VALDNÉA CASAGRANDE DALVI

CONTRIBUIÇÕES DA ANATOMIA PARA A TAXONOMIA E FILOGENIA DE GENTIANACEAE JUSS.

Tese apresentada a 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: 21 de fevereiro de 2014.

Profª Renata Maria Strozi Alves Meira
(Coorientadora)

Profª Maria Fernanda Aguiar Calió
(Coorientadora)

Prof. Élder Antônio Sousa Paiva

Profª Dayana Maria Teodoro Francino

Profª Aristéa Alves Azevedo
(Orientadora)

À minha família

Dedico.

A resposta certa, não importa nada: o essencial é que as perguntas estejam certas

(Mário Quintana)

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BIOGRAFIA

Valdnéa Casagrande Dalvi, filha de José Aguilar Dalvi e Maria Lídia Casagrande Dalvi, nasceu em Castelo, ES, em 14 de julho de 1981.

Em agosto de 2003 iniciou o curso de Ciências Biológicas (Licenciatura) na Universidade Federal de Ouro Preto (UFOP) concluindo em outubro de 2007.

Na UFOP foi bolsista de Iniciação Científica durante dois anos, trabalhando com anatomia ecológica, no período de 2005 a 2007 e bolsista de Extensão, trabalhando com Educação Ambiental, durante o ano de 2005. Desenvolveu atividades de monitoria voluntária nas disciplinas Biologia de Criptógramas e Biologia de Espermatófitas.

Em 2008 iniciou o mestrado, pelo Programa de Pós-Graduação em Botânica, da Universidade Federal de Viçosa-UFV, Minas Gerais; obtendo o título de mestre em Botânica em fevereiro de 2010, com a dissertação intitulada: "Anatomia dos órgãos vegetativos de espécies neotropicais de Gentianaceae Jussieu: contribuições à taxonomia e filogenia".

Em 2010 iniciou o doutorado pelo Programa de Pós-Graduação em Botânica, da Universidade Federal de Viçosa-UFV, Minas Gerais; submetendo-se a defesa e aprovação da tese no dia 21 de fevereiro de 2014.

CONTEÚDO

RESUMO

DALVI, Valdnéa Casagrande, D.Sc., Universidade Federal de Viçosa, fevereiro de 2014. **Contribuições da anatomia para a taxonomia e filogenia de Gentianaceae Juss..** Orientadora: Aristéa Alves Azevedo. Coorientadoras: Renata Maria Strozi Alves Meira e Maria Fernanda Aguiar Calió.

A família Gentianaceae está incluída na ordem Gentianales e compreende sete tribos monofiléticas (Chironieae, Exaceae, Gentianeae, Helieae, Potalieae, Saccifolieae e Voyrieae) 91 gêneros e cerca de 1700 espécies. Helieae representa um grupo de taxonomia complicada e controversa. Embora os dados moleculares tenham contribuído substancialmente para o conhecimento das relações filogenéticas em Gentianaceae, a circunscrição de alguns gêneros e a distinção das espécies permanecem problemáticas, especialmente pela insuficiência de dados morfológicos e/ou anatômicos. Os objetivos do trabalho foram: 1- investigar a origem, anatomia e histoquímica dos coléteres foliares em *Macrocarpaea obtusifolia*; 2- avaliar a presença, tipo e padrão de distribuição de nectários extraflorais (NEFs) foliares em espécies neotropicais; 3- averiguar a presença de NEFs em caules; 4- correlacionar os NEFs com o padrão de distribuição geográfico dos táxons de Gentianaceae, investigando sobre a evolução destas estruturas; e 5- selecionar caracteres anatômicos úteis para a taxonomia de Helieae. Procedimentos usuais em anatomia para coleta e processamento das amostras foram conduzidos. Para investigar a evolução dos nectários, uma filogenia para a família foi elaborada a partir de dados disponíveis no genBank e a evolução dos caracteres foi reconstruída. Para os estudos anatômicos em Helieae análises de similaridade utilizando o Índice de Sorensen`s foram conduzidas. Os resultados obtidos permitiram comprovar que as estruturas secretoras presentes na base das folhas de *Macrocarpaea obtusifolia* são coléteres. Estes possuem origem protodérmica e diferem dos tipos relatados na literatura para as demais espécies de Gentianaceae. Dados sobre a presença, o tipo e o padrão de distribuição de NEFs foliares em 27 espécies neotropicais de Gentianaceae demonstraram, pela primeira vez, que estas estruturas são comuns nas espécies neotropicais evidenciando a importância taxonômica dos NEFs. A constatação de NEFs nos caules de aproximadamente 50% das espécies (17 de 38) evindencia que estes são também comuns em caules. A diversidade e a evolução de NEFs em folhas de Gentianaceae foram avaliadas em um contexto filogenético. A presença/ausência, o tipo e a disposição de NEFs em linhagens particulares de Gentianaceae auxiliam a

delimitação e caracterização de grupos. A presença de NEFs está relacionada ao padrão de distribuição geográfico das espécies. Os resultados de anatomia foliar de 60 espécies de Helieae, distribuídas em 21 gêneros, juntamente com as análises de similaridade permitiram identificar caracteres úteis para a delimitação e reconhecimento de espécies e da maioria dos gêneros da tribo. O acúmulo de informações obtidas nas excursões de campo possibilitou elaborar um guia para identificação das espécies de Gentianaceae da Cadeia do Espinhaço.

ABSTRACT

DALVI, Valdnéa Casagrande, D.Sc., Universidade Federal de Viçosa, February, 2014. **Anatomic contributions for taxonomy and phylogeny of Gentianaceae Juss..** Adviser: Aristéa Alves Azevedo. Co-advisers: Renata Maria Strozi Alves Meira and Maria Fernanda Aguiar Calió.

The family Gentianaceae is included in the order Gentianales and is made up of seven monophyletic tribes (Chironieae, Exaceae, Gentianeae, Helieae, Potalieae, Saccifolieae and Voyrieae), 91 genera and about 1700 species. Helieae comprises a complicated and controversial taxonomy group. Although molecular data has contributed substantially to the understanding of phylogenetic relationships in Gentianaceae, the circumscription of some genera and species distinction remains problematic, especially the lack of morphological and/or anatomical data. The objectives of this study were: 1 - to investigate the origin, anatomy and histochemistry of foliar colleters in *Macrocarpaea obtusifolia*, 2 - assess the presence, type and distribution pattern of foliar extrafloral nectaries (EFNs) in neotropical species, 3 - to determine the presence of EFNs in stems, 4 – correlate the EFNs with the geographical distribution pattern of Gentianaceae taxa, investigating the evolution of these structures, and 5 - select useful anatomical characters for the taxonomy of Helieae. Common anatomic procedures for collecting and processing the samples were conducted. To investigate the evolution of nectaries, a phylogeny for the family was constructed from data available in the GenBank and the evolution of the characters was reconstructed. For anatomical analyzes in Helieae, similarity analyses were performed using the Sorensen's Index. The results obtained allowed for establishing that the secretory structures at the base of the leaves of *Macrocarpaea obtusifolia* are colleters. These are of protodermic origin and differ from the types reported in literature for other species of Gentianaceae. Data on the presence, type and distribution pattern of foliar EFNs in 27 neotropical Gentianaceae species demonstrated, for the first time, that these structures are common in neotropical species, indicating the taxonomic significance of EFNs. The finding of EFNs on the stems of approximately 50% of species (17 of 38) shows that they are also common on stems. Diversity and evolution of EFNs on leaves of Gentianaceae were evaluated in a phylogenetic context. The presence/absence, type and arrangement of EFNs in specific lineages of Gentianaceae aid in the delineation and characterization of groups. The presence of EFNs is related to the geographical distribution pattern of the species. The

results of foliar anatomy of 60 Helieae species, distributed in 21 genera, together with the similarity analysis, allowed for identification of useful characters for distinguishing and recognizing species and most of the genera of the tribe. The accumulation of information obtained in field excursions permitted preparation of a guide for identifying species of Gentianaceae from the Espinhaço mountains.

INTRODUÇÃO GERAL

Gentianaceae está incluída na ordem Gentianales juntamente com Apocynaceae, Loganiaceae, Gelsemiaceae e Rubiaceae (APG, 1998; 2003; 2009). Embora tenha distribuição cosmopolita (exceto no continente Antártico) com a maioria das espécies ocorrendo de forma concentrada nas regiões temperadas (Sousa & Lorenzi, 2008) a maior diversidade genérica encontra-se nas Américas Central e do Sul (Albert & Struwe, 2002). Gentianaceae tem representantes em diferentes tipos vegetacionais ocorrendo em formações florestais (florestas temperadas e pluviais) e formações savânicas (campos, restingas e caatinga) (Barroso *et al.*, 1991; Guimarães, 2002; Struwe *et al*., 2002).

Em relação ao hábito, a maioria das espécies de Gentianaceae é herbácea ocorrendo também representantes arbóreos, arbustivos e trepadeiras. Destaca-se, ainda, a presença de espécies saprófitas representadas pelos gêneros *Voyria* Aubl., *Voyriella* (Miq.) Miq. e *Cotylanthera* Bl. (Struwe *et al.,* 2002). Além da variedade de habitats e hábitos, Gentianaceae apresenta uma grande diversidade morfológica (Albert & Struwe, 2002). As plantas apresentam: folhas simples, geralmente glabras, com filotaxia oposta; padrão de venação geralmente acródromo, podendo ocorrer o tipo broquidódromo; estípulas ausentes; látex ausente; coléteres na base das folhas e sépalas; caule de seção transversal circular ou retangular com aletas; inflorescências terminais ou axilares, cimosas, podendo ocorrer flores solitárias; flores bissexuais, actinomorfas, raramente zigomorfas; corola com prefloração contorta; estames epipétalos; ovário súpero, bicarpelar, com glândulas ou disco nectarífero na base e placentação parietal; ausência de estigma subdividido; frutos frequentemente secos, podendo ocorrer frutos coriáceos ou carnosos; presença de seco-iridoides e xantonas e ausência de alcaloides (Struwe *et al*., 2002; Calió, 2009; Judd *et al*., 2009).

Embora ocorra grande diversidade morfológica, a única característica exclusiva da família dentro da ordem Gentianales é a placentação do tipo parietal (Albert & Struwe, 2002). Na prática, a presença ou a ausência de características observadas em outras famílias da ordem Gentianales e de Asteridae são utilizadas para distinguir as Gentianaceae, o que dificulta sua caracterização morfológica e, consequentemente impõe empecilhos para o estabelecimento da circunscrição da família.

Desde a primeira proposta de classificação de Gentianaceae elaborada por Jussieu (1789), a circunscrição da família foi alterada pela exclusão de Menyanthoideae (Wagenitz, 1964) e posteriormente pela inclusão de Potalieae, que estava inclusa em Loganiaceae (Leewenberg & Leenhouts, 1980), e Saccifoliaceae, anteriormente tratada como uma família monotípica (Maguire & Pires, 1978; Struwe *et al*., 1998; Thiv *et al*., 1999).

No final do século XX, com o avanço dos estudos moleculares, trabalhos com enfoque filogenético (De Laet & Smets, 1996; Backlund *et al.*, 2000; Struwe *et al.,* 2002; APG, 2003; 2009) permitiram uma nova proposta de classificação, sendo estabelecidas seis tribos monofiléticas (Chironieae, Exaceae, Gentianeae, Helieae, Potalieae e Saccifolieae), além de um gênero, *Voyria*, com posição taxonômica incerta (Struwe *et al*., 2002). Recentemente, Merck *et al*. (2013) restabeleceram o gênero *Voyria* como uma tribo Voyrieae. Atualmente a família Gentianaceae compreende sete tribos monofiléticas: Chironieae, Exaceae, Gentianeae, Helieae, Potalieae, Saccifolieae and Voyrieae (Struwe *et al*., 2002, Merckx *et al*. 2013), com 91 gêneros e cerca de 1700 espécies (Gentian Research Network, 2014).

Destas, Helieae, com distribuição exclusivamente neotropical, destaca-se como um grupo, constituído por 23 gêneros e cerca de 220 espécies, de taxonomia complexa e controversa com difícil delimitação e identificação das espécies e dos gêneros (Weaver, 1972; Struwe *et al*., 2002). A maioria dos gêneros da tribo possui poucas espécies, com distribuição geográfica restrita e carece de estudos de revisão taxonômica. Dados moleculares acrescidos de dados morfológicos, micromorfológicos e palinológicos sustentam a monofilia da tribo e de diversos gêneros e apontam para a necessidade de reclassificação de alguns gêneros parafiléticos como *Chelonanthus* Gilg e *Irlbachia* Mart. (Struwe *et al*., 2009). Nestes estudos, *Celiantha* Maguire, *Prepusa* Mart. e *Senaea* Taub. aparecem como grupo-irmão do restante dos gêneros de Helieae que foram agrupados em dois subclados ("subclado *Symbolanthus*" e "subclado *Macrocarpaea*"). Tais resultados indicam a necessidade de aporte de dados advindos de novas fontes com o intuito de contribuir para a resolução das relações filogenéticas entre os gêneros da tribo e delimitação adequada destes gêneros polifiléticos.

Embora os dados moleculares tenham contribuído substancialmente para o conhecimento das relações filogenéticas do grupo, ainda há dificuldades quanto à circunscrição dos gêneros e a distinção das espécies (Albert & Struwe, 2002). A insuficiência de dados sobre a morfologia e, em especial, sobre a anatomia das espécies

(Mészaros *et al*., 2002) constitui uma lacuna do conhecimento e obstáculo à identificação de possíveis sinapomorfias para a família, tribos e gêneros.

Pesquisas têm demonstrado a importância dos caracteres anatômicos como fonte promissora de dados para a taxonomia em diferentes níveis hierárquicos (Solereder, 1908; Metcalfe & Chalk, 1979; Dickison, 2000, Reis *et al.,* 2004; Gomes *et al*., 2005, Rio *et al*., 2005, Cardoso & Sajo, 2006, Teixeira & Gabrielli, 2006, Calvente *et al.*, 2008, Silva & Potiguara, 2008, Gomes *et al.,* 2009, Pelegrin *et al.,* 2009, Araújo *et al*., 2010, Francino, 2010, Melo *et al.,* 2010; Le Roux *et al.,* 2011, Stpiczynska *et al.,* 2011; Chauveau *et al*., 2012; Blanco *et al.,* 2013; Coutinho *et al*., 2013).

No entanto, em Gentianaceae, estudos anatômicos são escassos e geralmente abordam os órgãos reprodutivos, destacando-se trabalhos sobre palinologia (Nilsson, 2002), morfologia, anatomia e micromorfologia de sementes (Bouman *et al*., 2002) e anatomia floral (Gopal & Puri, 1962). Estudos anatômicos mais restritos fornecem dados sobre a micromorfologia de papilas em flores de *Gentianella* sect. *Gentianella* (Greimler *et al*., 2004); a anatomia e histoquímica de tricomas secretores em *Gentiana* L. (Renobales *et al*., 2001) e a anatomia da madeira em espécies de Potalieae (Jansen & Smets, 1998) e Helieae (Carlquist & Grant, 2005).

Recentemente, estudos anatômicos com órgãos vegetativos revelaram caracteres promissores para a taxonomia de espécies neotropicais de Coutoubeinae-Chironieae (Delgado, 2008; Delgado *et al.,* 2009), Helieae (Delgado, 2008; Dalvi, 2010; Delgado *et al.*, 2011*a*,b) e Saccifolieae (Dalvi *et al*., 2013). O padrão de venação, o tipo de medula no caule, o tipo de mesofilo, o tipo e a distribuição dos estômatos, os tecidos associados aos feixes vasculares e a presença, o tipo e a distribuição das estruturas secretoras foram úteis para a taxonomia nestes grupos.

Dentre as estruturas secretoras, embora os coléteres representem uma da sinapomorfias da ordem Gentianales (Judd *et al*., 2009) poucos são os estudos sobre anatomia destas estruturas e a natureza química do secretado, restringindo-se a espécies dos gêneros *Gentiana* L., *Gentianella* Moench, *Gentianopsis* Ma, *Comastoma* (Wettst.) Toyok., *Swertia* L. (Renobales *et al*., 2001) e *Calolisianthus* Gilg (Delgado *et al*., 2011*a*,*b*). Não foram encontrados dados sobre o desenvolvimento dos coléteres na família.

Em relação aos nectários, estruturas não-usuais denominados nectaríolos foram descritos por Vogel (1998) para espécies de *Irlbachia* Mart. (Helieae) e representantes de mais seis famílias não correlacionadas, podendo ocorrer isolados ou agrupados em

nectários conspícuos. Estruturas semelhantes anatomicamente foram descritas para folhas de espécies de *Calolisianthus* (*Delgado et al*., 2011*a*,*b*), C*urtia* Cham. & Schltdl.*, Hockinia* Gardner (Dalvi *et al*. 2014), *Deianira* Cham. & Schltdl. (Dalvi, 2010) e *Macrocarpaea* (Griseb.) Gilg (Cardinelli *et al*., 2010). O registro da ocorrência de nectários foliares em diferentes gêneros é recente e comprova que tais estruturas têm sido negligenciadas nos estudos com Gentianaceae. Tal fato pode ser atribuído à escassez de estudos anatômicos foliares e ao diminuto tamanho destas estruturas secretoras, que exige a utilização de técnicas adequadas, como a diafanização, para constatação da sua presença e caracterização, ao microscópio.

Levando em consideração os problemas taxonômicos encontrados em Gentianaceae, especialmente dentro da tribo Helieae, e a carência de informações acerca das estruturas secretoras na família, estudos anatômicos foram conduzidos na tentativa de esclarecer os seguintes questionamentos:

1- As estruturas secretoras comumente encontradas na base das folhas de espécies de Gentianaceae realmente correspondem a coléteres? Como são caracterizadas anatomicamente estas estruturas? Qual a natureza química do produto secretado? Qual a origem ontogenética destas estruturas?

2- Nectários foliares são comuns em espécies de Gentianaceae neotropicais? Como são caracterizadas anatomicamente estas estruturas? Existem diferentes padrões de distribuição dos nectários ao longo do limbo foliar? Estes padrões são constantes dentro de determinados grupos, podendo ser importantes ferramentas taxonômicas?

3- Nectários também podem ocorrer em caules de espécies herbáceas? São anatomicamente semelhantes aos encontrados nas folhas? Qual sua relevância taxonômica e ecológica?

4- A presença de nectários foliares é característica peculiar das Gentianaceae tropicais ou ocorrem em grupos restritos às regiões temperadas? Como se deu a evolução destas estruturas dentro da família? Existe correlação da distribuição geográfica da família com a presença de nectário foliar? Poderiam os nectários foliares representar uma sinapomorfia para a família?

5- Como a anatomia foliar pode contribuir para a resolução dos problemas taxonômicos dentro da tribo Helieae? Os caracteres anatômicos são relevantes em nível de espécies ou de gêneros? Quais os caracteres anatômicos relevantes dentro da tribo?

Com o intuito de responder a estes questionamentos, o trabalho foi organizado em capítulos redigidos na forma de artigos científicos incluindo:

CAPÍTULO I: "Foliar colleters in *Macrocarpaea obtusifolia* (Gentianaceae): anatomy, ontogeny and secretion" - publicado no periódico *Botany*

CAPÍTULO II: "Extrafloral nectaries in neotropical Gentianaceae: occurrence, distribution patterns, and anatomical characterization" - publicado no periódico *American Journal of Botany*

CAPÍTULO III: "Are nectaries common structures on the stem of Gentianaceae species?" - submetido ao periódico *Plant Systematics and Evolution*

CAPÍTULO IV: "Diversity and evolution of extrafloral nectaries in Gentianaceae" - submetido ao periódico *Annals of Botany*

CAPÍTULO V: "Anatomia foliar de Helieae (Gentianaceae): considerações taxonômicas" - será submetido ao periódico *Plant Systematics and Evolution.*

Também foi possível elaborar um guia de campo com fotos ilustrativas para reconhecimento das espécies de Gentianaceae ocorrentes ao longo da Cadeia do Espinhaço. Este guia segue como:

CAPÍTULO VI: "Gentianaceae of the Espinhaço Mountain Range, Brazil. Guia de campo" - publicado no *The Field Museum*, Chicago, USA.

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CAPÍTULO 1

FOLIAR COLLETERS IN *MACROCARPAEA OBTUSIFOLIA* (GENTIANACEAE): ANATOMY, ONTOGENY AND SECRETION

Valdnéa Casagrande Dalvi, Lucas Siqueira Cardinelli, Renata Maria Strozi Alves Meira, and Aristéa Alves Azevedo

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ARTICLE

Foliar colleters in *Macrocarpaea obtusifolia* (Gentianaceae): anatomy, ontogeny, and secretion

Valdnéa Casagrande Dalvi, Lucas Siqueira Cardinelli, Renata Maria Strozi Alves Meira, and Aristéa Alves Azevedo

Abstract: Colleters are secretory structures located in reproductive and (or) vegetative organs of many eudicots. In Gentianaceae Juss., the presence of foliar colleters has been neglected, and anatomical and histochemical studies are scarce. The objectives of this study were to investigate the anatomy, ontogeny, and chemical nature of the secretion found in *Macrocarpaea obtusifolia* (Griseb.) Gilg colleters to establish a relationship between their structure and function and check whether these structures are similar to those described for other genera of the Gentianaceae and other families of the Gentianales. Samples of leaves at different developmental stages were collected and processed for anatomical and histochemical analysis using light microscopy and scanning electron microscopy. Colleters in *M. obtusifolia* have a protodermal origin, are of standard type, and are not vascularized. Young colleters are translucent and produce an abundant amount of sticky secretion. Later, they turn yellowish with a blackened region at the apex of the head, and the secretion, composed of polysaccharides and proteins, becomes less abundant and brownish. During senescence, the process begins with complete degradation and cell collapse of the secretory portion. The colleters of the standard type in *M. obtusifolia* have been observed for the first time in the Gentianaceae and represent additional evidence that reinforces how common this type of colleter is in the Gentianales. Such results provide new information on the anatomy, ontogeny, histochemistry, and colleter types of Gentianaceae.

Key words: micromorphology, mucilage, proteins, secretory structures.

Résumé : Les collerettes constituent des structures sécrétrices localisées dans les organes reproducteurs ou végétatifs de plusieurs eudicotylées. Chez les Gentianaceae, on a négligé la présence de collerettes foliaires; en effet, rares sont les études anatomiques et histochimiques. L'objectif des auteurs était d'examiner l'anatomie, l'ontogénie, et la nature chimique de la sécrétion se retrouvant dans les collerettes du *Macrocarpaea obtusifolia* (Griseb.) Gilg, afin d'établir la relation entre leur structure et leur fonction et de vérifier si ces structures sont semblables à celles décrites chez d'autres genres de Gentianaceae et d'autres familles des Gentianales. Ils ont récolté des échantillons de feuilles à divers stades de développement et effectué des analyses anatomiques et histochimiques à l'aide de la microscopie photonique et à électronique par balayage. Chez le *M. obtusifolia*, les collerettes ont une origine protodermique, sont de type standard et non vascularisées. Les jeunes collerettes translucides produisent une bonne quantité de sécrétion collante. Plus tard, elles deviennent jaunes avec une région à col noir à l'apex de la tête et la sécrétion, composée de polysaccharides et de protéines, devient moins abondante et brunit. Pendant la sénescence, le processus débute avec une dégradation complète avec un affaissement des cellules de la portion sécrétrice. On a observé pour la première fois les collerettes de type standard chez le *M. obtusifolia*, ce qui représente une preuve additionnelle supportant jusqu'à quel point ce type de collerette est commun chez les Gentianales. De tels résultats fournissent une nouvelle information sur l'anatomie, l'ontogénie, l'histochimie, et les types de collerettes des Gentianaceae. [Traduit par la Rédaction]

Mots-clés : micromorphologie, mucilage, protéines, structures sécrétrices.

Introduction

Colleters are secretory structures located on the adaxial surface of reproductive and (or) vegetative organs found in approximately 60 families of eudicots (Thomas 1991), whereas in monocotyledons, they occur only in the Orchidaceae (Leitão and Cortelazzo 2008; Mayer et al. 2011). The secretion of colleters is mostly composed of mucilage (Fahn 1979; Thomas 1991; da Silva et al. 2012) or a mixture of mucilage and terpenes and (or) resin (Fahn 1979; Subramanian et al. 1989; Mangalan et al. 1990; Barreiro and Machado 2007). However, other chemical compounds have been identified in the exudate as proteins in Apocynaceae (Dave et al. 1987; Thomas et al. 1989), Aquifoliaceae (González and Tarragó 2009), Orchidaceae (Mayer et al. 2011), and Rubiaceae (Thomas and Dave 1990; Klein et al. 2004; de Castro Miguel et al. 2006); phenolic compounds in Apocynaceae (Martins 2012), and lipids in Apocynaceae (Appezzato-da-Glória and Estelita 2000), Fabaceae (Paiva 2009), Orchidaceae (Mayer et al. 2011), and Rubiaceae (Machado et al. 2012). Although the presence of various metabolites, in addition to mucilage, has been demonstrated in the colleter exudate of several species, no correlation between different chemical constitutions and taxonomic groups has been observed.

The mucilaginous secretion produced by the colleters is deposited on the meristems and developing tissues and organs, which ensures lubrication and protection against dehydration (Fahn 1979; Thomas et al. 1989; Thomas and Dave 1989*a*, 1989*b*; Thomas 1991; Evert 2006; Paiva 2009), as demonstrated in colleters of coffee flowers by Mayer et al. (2013). An additional role attributed to mucilage is to act as a substrate for bacterial growth, thus facilitating

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symbiotic interactions (Horner and Lersten 1968; Lersten 1974*a*, 1974*b*; Lersten 1975; Lersten and Horner 1976). Klein et al. (2004) suggested that the proteins in the colleter secretions of *Simira* (Rubiaceae) species are also associated with a defense function against microorganisms, and de Castro Miguel et al. (2006) confirmed the antifungal activity of these proteins in *Bathysa nicholsonii* K. Schum. (Rubiaceae).

To correctly identify a secretory structure as a colleter, criteria such as location, morphology, and chemical nature of the secretions have been used by different researchers (Leitão and Cortelazzo 2008). Paiva (2009) added the need for detailed anatomical and histochemical studies.

The presence and type of colleters have proven to be useful characters for taxonomy and phylogeny (Lersten 1975; Thomas 1991; González 1998; Simões et al. 2004; González and Tarragó 2009; da Silva et al. 2012). The presence of colleters is one of the synapomorphic characteristics of the order Gentianales, which includes the families Gentianaceae, Apocynaceae, Gelsemiaceae, Loganiaceae, and Rubiaceae (Struwe and Albert 2002). Many studies on types of colleters were performed with representatives from the Gentianales, especially from the Apocynaceae (Dave et al. 1988; Thomas and Dave 1989*b*; Simões et al. 2004; Martins et al. 2010, Martins 2012) and Rubiaceae (Lersten 1975; Thomas 1991; Barreiro and Machado 2007; Machado et al. 2012). Calycine colleters have been reported in different genera of Gentianaceae (Renobales et al. 2001; Mészaros et al. 2002; Struwe et al. 2002); however, reports of the presence of foliar colleters and studies of the anatomy and chemical nature of the secretion are scarce and restricted to species of the genera *Gentiana* L., *Gentianella* Moench, *Gentianopsis* Ma, *Comastoma* Toyok., *Swertia* L. (Renobales et al. 2001), *Calolisianthus* Gilg (Delgado et al. 2011), and *Curtia* Cham. & Schltdl. and *Hockinia* Gardner (Dalvi et al. 2013). No data were found on the development of colleters in the family Gentianaceae.

Macrocarpaea (Griseb.) Gilg is the fourth largest genus of Gentianaceae and the largest of the tribe Helieae (Struwe and Albert 2002), with most species only having been described recently (Grant and Struwe 2001, 2003; Grant 2003, 2004, 2005, 2007, 2008; Grant and Weaver 2003). The genus has a Neotropical distribution (Struwe and Albert 2002), with eight species in Brazil that are endemic to the Amazon, the Atlantic forest, and the Cerrado (Grant and Trunz 2011; Guimarães et al. 2012). *Macrocarpaea obtusifolia* (Griseb.) Gilg is found in Atlantic forests and high-altitude fields in southeastern Brazil (Grant and Trunz 2011), and during the collections, an abundant sticky secretion was observed at the base of leaves. Given this observation, it has been hypothesized that this secretion is produced by colleters because these structures are one of the synapomorphies of Gentianales. The objectives of this study were to investigate the anatomy, ontogenetic development, and chemical nature of the product of the secretory structures found on the leaf base of *M. obtusifolia*, verify whether these structures are similar to those described for other genera of the Gentianaceae and other families of the Gentianales, and establish a relationship between their structure and function.

Material and methods

Basal regions of leaves and apical meristems of *M. obtusifolia* were collected in high-altitude fields in the Parque Estadual da Serra do Brigadeiro, Minas Gerais, Brazil. The fertile material was pressed and deposited in the collections of the VIC Herbarium of the Federal University of Viçosa (UFV), Viçosa, Minas Gerais, Brazil, under the numbers VIC 32.543 (Valente et al. 1620), VIC 37.278 (Dalvi et al. 85), and VIC 37.279 (Dalvi et al. 86).

The basal regions of leaves at different stages of development (i.e., from leaf primordium in apical meristem to senescent leaves in the most basal nodes of the stem) were sampled (Fig. 1A). Four leaf-growth stages (I, II, III, and IV) were selected to describe the ontogeny of secretory structures: I corresponds to the region of apical meristem and young leaves until the fourth node; II includes leaves from the fifth and sixth nodes; III includes leaves from the seventh and eighth nodes; and IV corresponds with senescent leaves from the most basal nodes.

Table 1. Histochemical tests used to characterize the colleters of *Macrocarpaea obtusifolia* (Gentianaceae).

Metabolic group	Histochemical test (reference)
Polysaccharides	
Total polysaccharides	Periodic acid – Schiff (McManus 1948)
Acid mucilage and (or) pectins	Ruthenium red (Gregory and Baas 1989)
Acid	Alcian Blue (Pearse 1980)
Mucopolysaccharides	
Starch	Lugol (Johansen 1940)
Phenolic compounds	
General phenolic	10% ferric chloride (Johansen 1940)
	Potassium dichromate (Gabe 1968)
Tannins	Vanillin hydrochloric acid (Mace and Howell 1974)
Lignins	Phloroglucinol (Johansen 1940)
Flavonoids	Aluminium chloride* (Charrière-Ladriex 1976)
Flavonoids	Neutral lead acetate* (Charrière-Ladriex 1976)
Lipids	
Total lipid	Sudan IV (Pearse 1980)
Total lipid	Sudan black B (Pearse 1980)
Acid and neutral lipids	Nile blue sulphate (Cain 1947)
	Neutral red* (Kirk 1970)
Alkaloids	Wagner's reagent (Furr and Mahlberg 1981)
	Dragendorff reagent (Svendsen and Verpoorte 1983)
Proteins	
Total proteins	Coomassie Brilliant Blue (Fisher 1968)
Total proteins	Xylidine Ponceau (O'Brien and McCully 1981)

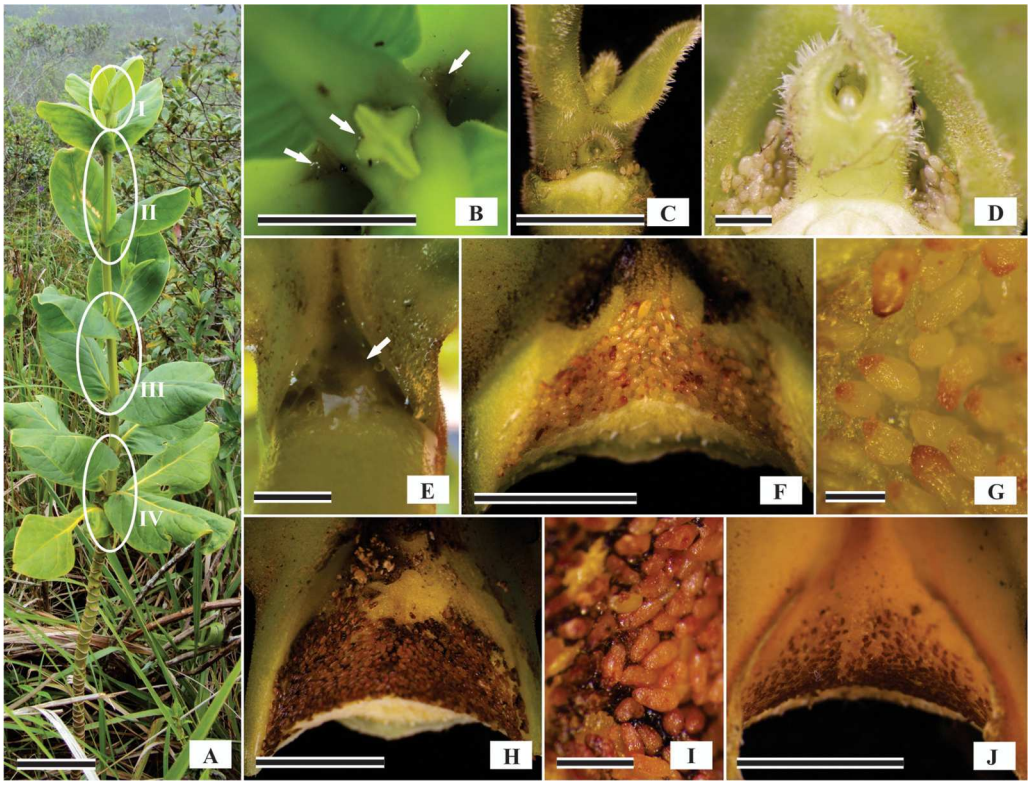
*Fluorescence microscopy (UV light).

For ontogeny and structural characterization, samples were fixed in FAA (formaldehyde, acetic acid, and 50% ethyl alcohol, 1:1:18 *v*/*v*/*v*) for 48 h under a vacuum and stored in 70% ethanol (Johansen 1940). Subsequently, the material was dehydrated with tertiary butyl alcohol and embedded in histological paraffin with dimethyl sulfoxide (DMSO) (Histosec Merck, Darmstadt, Germany) or dehydrated in an ethanol series and embedded in a methacrylate resin (Historesin Leica; Leica Microsystems, Heidelberg, Germany). Transverse and longitudinal sections, 5–8 μm thick, were obtained using an automatic advance rotary microtome (model RM2155; Leica Microsystems, Deerfield, Illinois, USA). The material was then deparaffinized and stained with 1% aqueous safranin and astra blue (48 h) and aqueous astra blue 1% (2 min) (Gerlach 1969, modified). The material included in the historesin was stained with Toluidine Blue O at pH 4.7 (O'Brien et al. 1964). The slides were mounted in resin (Permount; Fisher Scientific, New Jersey, USA).

Different fixatives were used for histochemical tests: FAA (Johansen 1940) to identify polysaccharides, proteins, and water-soluble phenolic localization; neutral-buffered formaldehyde solution (phosphate buffer, formalin, 9:1 *v*/*v*) (Lillie 1965) to identify lipids and lipid-soluble phenolic compounds, and a formalin–ferrous sulphate solution (SFF, formalin, 9:1 *v*/*v*) (Johansen 1940) to identify general phenolic compounds. The histochemical tests are summarized in Table 1.

For histochemical tests, some samples were sectioned on a table microtome (LPC model; Rolemberg and Bhering Trade and Import LTDA, Belo Horizonte, Brazil) and some were dehydrated in an ethanol series, embedded in histological paraffin with DMSO, and

Fig. 1. Developmental stages of the colleters of *Macrocarpaea obtusifolia*. (A) Four developmental stages analyzed. (B–D) Stage I. (B) Translucent secretion. (C and D) Apical meristem showing the position and colour of the structures. (E–G) Stage II. (E) Accumulation of secretion. (F) General view. (G) Detail of the secretory portion with blackish apex. (H and I) Stage III. (H) Structures blackened in the process of senescence. (I) Detail of reduced and blackened secretion. (J) Stage IV, reduction in the number of colleters, indicating the process of abscission. Scale bars: (A) 10 cm; (B, C) 1 cm; (D, G, I) 0.1 cm; (E, F, H, J) 0.5 cm.



sectioned to a thickness of 7 μm using a rotary microtome. The respective controls were processed concurrently with the tests according to protocol. Samples not subjected to the reagent (blank) were also observed using light microscopy and fluorescence microscopy under UV light.

Sections were analysed in the Laboratory of Plant Anatomy of UFV using a light microscope (Olympus model AX70TRF; Olympus Optical, Tokyo, Japan) equipped with a U-Photo System and digital camera (AxioCam HRc; Zeiss, Göttingen, Germany), and an epifluorescence microscope (HBO 50-W; Olympus Optical, Tokyo, Japan) with a mercury lamp and filter block (exciter filter BP 340–380, dichroic mirror 459, barrier filter LP-430; Ushio-USH 102D, Japan).

For analysis with a scanning electron microscope, samples fixed in Karnovsky solution (Karnovsky 1965) were dehydrated in an ethanol series and subjected to critical-point drying using CO_2 (CPD 020; Bal-Tec, Balzers, Liechtenstein). The samples were then fixed on supports and sputter coated with gold (FDU 010; Bal-Tec, Balzers, Liechtenstein). The analysis was performed and data documented at the Center for Microscopy and Microanalysis at UFV using a scanning electron microscope (Leo 1430 VP model; Zeiss, Cambridge, England).

Results

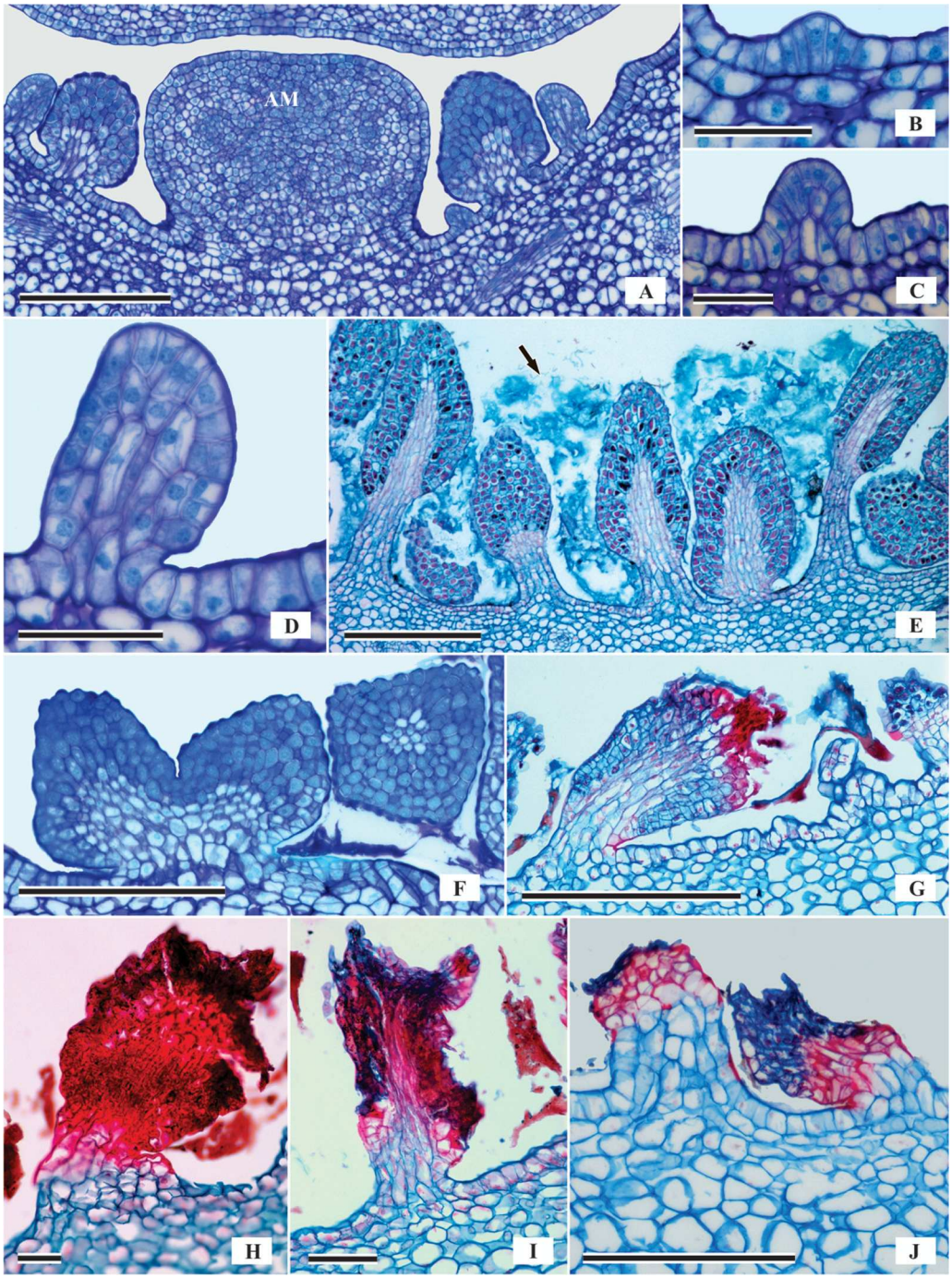
Localization and macroscopic characterization of colleters and secretions

The colleters of *M. obtusifolia* are clustered on the adaxial side of the leaf, near to its insertion into the stem. In stage I, the colleters are whitish with an abundant sticky translucent secretion (Figs. 1B–1D). In stage II, they are yellowish with a darkened apical end but still with abundant translucent secretion (Figs. 1E–1G). In stage III, colleters are brownish and the secretion is much less evident and is blackish (Figs. 1H and 1I). In stage IV, the senescent leaf, only a part of these structures remains and the secretion is reduced (Fig. 1J).

Ontogenetic development and anatomical characterization

The colleters of *M. obtusifolia* have asynchronous development and are formed early during the leaf primordial stage I (Fig. 2A). Early in development, some cells of the protoderm are noticeably larger than others (Fig. 2B). These cells undergo successive periclinal and anticlinal divisions, culminating in the formation of a projection (Fig. 2C). Ulterior growth is a result of successive divisions in several planes (Fig. 2D) and cellular expansion, giving rise to the mature, fully developed structure (Figs. 2E and 2F). The mature colleters are of different sizes and can be sessile (Figs. 2E and 2F) or present a peduncle of varying heights (Fig. 2F). Generally, they are digitiform (Fig. 2E), although they can also be bifurcated (Fig. 2F). They have a secretory portion comprising a central axis of elongated, thin-walled, and vacuolated nonsecretory cells (Figs. 2E and 2F) surrounded by several layers of isodiametric secretory cells that are thin-walled with large nuclei and dense cytoplasm (Figs. 2E and 2F). The peduncle contains isodiametric cells with denser cytoplasm than cells of the central axis (Fig. 2E). The colleters are nonvascularized and covered with a thin cuticle (Fig. 2E). In the postsecretion phase, cells degrade and collapse from the apex towards the base of the secretory portion (Fig. 2F). These changes can occur in stage II but are intensified in stages III (Fig. 2G) and IV (Fig. 2H), usually causing abscission of the secretory portion (Fig. 2I). In the region where the peduncle is in contact with the secretory portion (Fig. 2I), there is a formation of cell layers similar to wound tissue. Moreover, asynchrony in development is also observed, with some structures in early stages of

Fig. 2. Ontogeny of the colleters of *Macrocarpaea obtusifolia* (transverse sections). (A–D) Stage I. (A) Apical meristem (AM), asynchronous development of structures. (B) Enlargement of the protoderm cells. (C) Anticlinal and periclinal divisions. (D) Early differentiation of cells of the central axis and secretory cells. (E and F) Stage II. Mature structures at the stage of secretion. (E) General view showing colleters with different sizes and abundant secretion (arrow). Secretory portion formed by a central axis of vacuolated cells and secretory cells with dense cytoplasm and large nuclei. Peduncles of different sizes are observed, as well as sessile structures. (F) Sessile structure, bifurcated, with observable cuticle. (G) Stage III. Marked necrosis at the apical part of the secretory portion and early necrosis at the base of the peduncle showing the onset of senescence of the colleters. (H–J) Stage IV. (H and I) Collapse of the cells of the secretory portion. (J) Formation of a healing layer in the abscission region of the colleters. Scale bars: (A, E–G, J) 200 μm; (B–D, H, I) 50 μm.

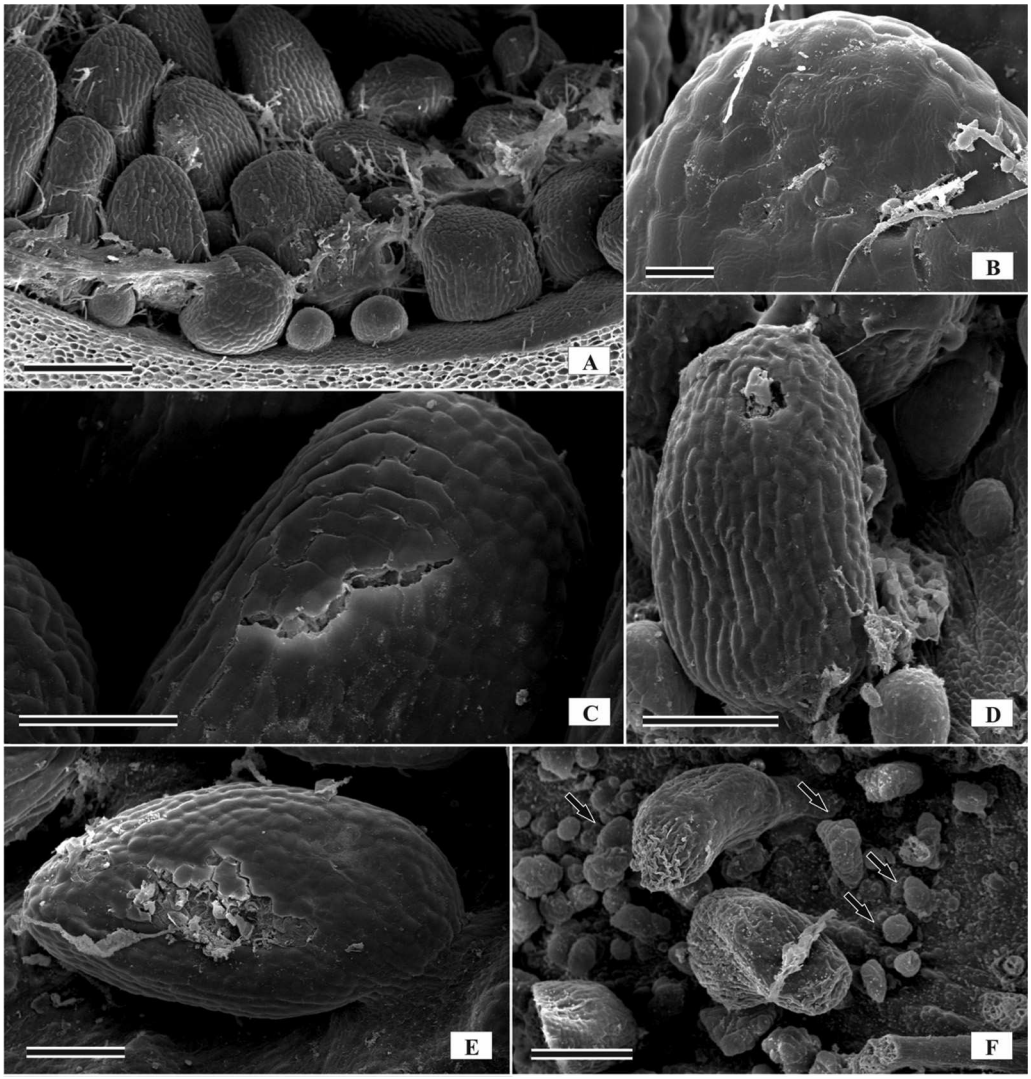


senescence and others with complete abscission of the secretory portion.

Micromorphological characterization

Micromorphological analyses confirmed asynchrony in the development of colleters (Fig. 3A) and copious secretion covering the meristems and young leaves (stage I; Fig. 3A). In some regions of the secretory portion, the cuticle presents a smoother and dilated surface, making it impossible to detect the boundary between cells, although small fissures are observed (Fig. 3B). At a later stage, the cuticle is disrupted (Fig. 3C) and the secretion is extravasated (Fig. 3D). In the more advanced stages (III and IV), the

Fig. 3. Scanning electron micrographs showing micromorphology of colleters of *Macrocarpaea obtusifolia*. (A and B) Stage I. (A) Asynchrony in the development and presence of secretion. (B) Dilated surface the cuticule. (C) Stage II showing fissure at the apex of the secretory portion. (D and E) Stage III showing regions of extravasation of the secretion and detachment of the cuticle, respectively. (F) Stage IV. Base of the peduncles (arrows) after abscission of the secretory cells of the colleters. Scale bars: (A, F) 200 μm; (B–E) 100 μm.



cuticle is broken up and detached from the entire region of the secretory portion (Fig. 3E). After abscission of the secretory portion, protrusions corresponding to the peduncles remain (Fig. 3F).

Histochemical characterization

A variation in colour of the secretion was noticeable in fresh and fixed samples not subjected to histochemical tests (control). In stages I and II, the secretion is translucent, whereas in stages III and IV (Fig. 4A), it displays a blackish colour.

General polysaccharides were detected using the periodic acid-Schiff (PAS) test in all developmental stages of colleters (Fig. 4B) in both the cytoplasm of the secretory cells and the exudate that accumulates outside the structure. The tests with Alcian Blue 8GX (Fig. 4C) and ruthenium red revealed, respectively, acidic mucopolysaccharides and acidic mucilages and (or) pectin only in the secretion.

Proteins were detected in the secretion and cytoplasm of secretory cells with Coomassie Brilliant Blue G-250 and Xylidine Ponceau (XP). The reaction with Coomassie Brilliant Blue G-250 was most intense in the secretion (Fig. 4D), whereas the XP test showed stronger reaction in the cytoplasm (Fig. 4E).

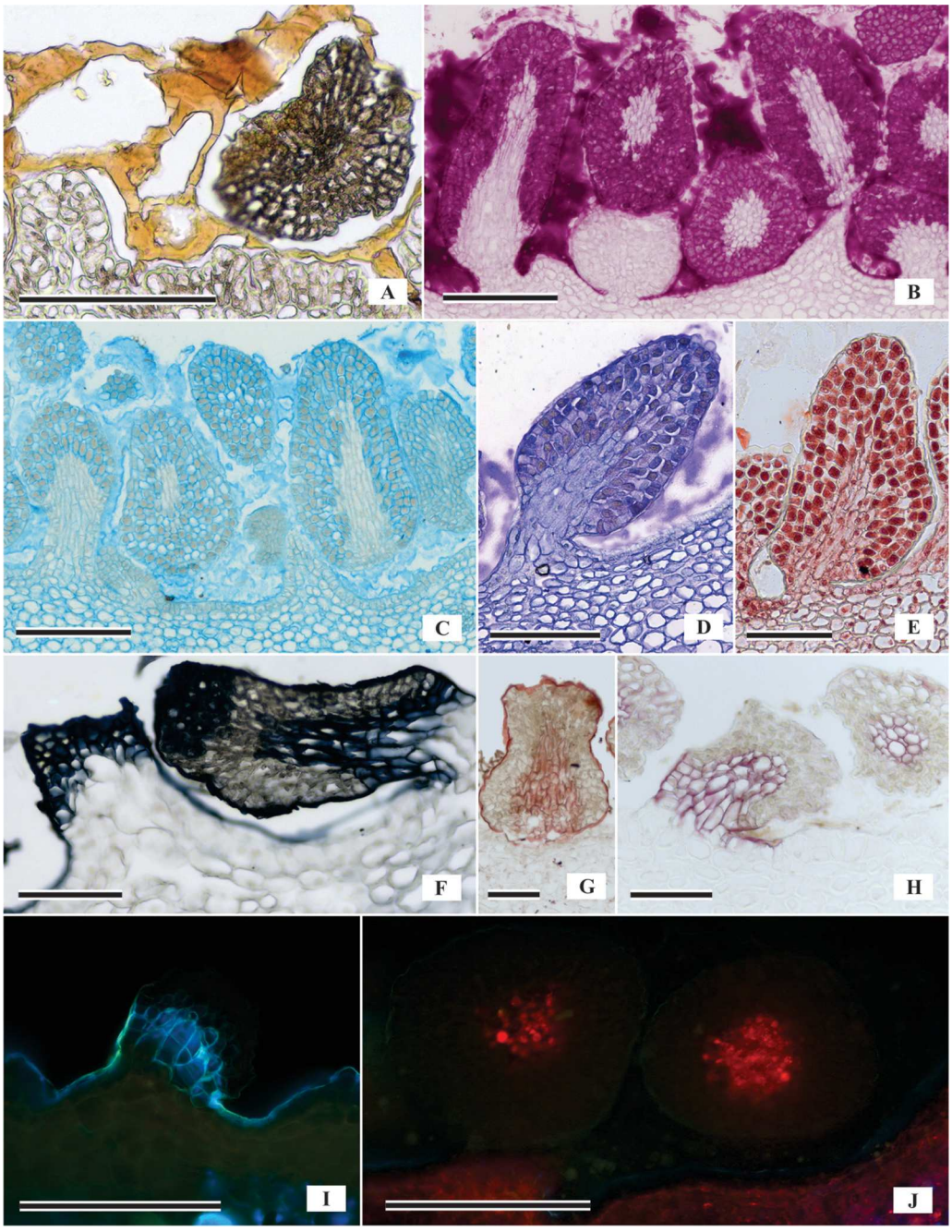
All tests used for detection of lipid compounds were negative in the secretion, which revealed only structural lipids such as those of the cuticle (Figs. 4F and 4G). Interestingly, a positive reaction was observed in the cell walls of the central axis and in the region where the peduncle was in contact with the secretory portion (Figs. 4F and 4G) in the advanced stages of development (III and IV).

The results were negative for phenolic compounds in the secretory portion and in secretion at all stages of development. Lignification of cell walls in the central axis and in the contact region between the peduncle and the secretory portion in stages III and IV was observed, as detected by phloroglucinol (Fig. 4H) and neutral lead acetate (Fig. 4I). Red autofluorescence under UV light (Fig. 4J) identified chloroplasts in cells of the central axis in the initial stages of development; this fluorescence was no longer detected in the senescent stage of the secretory structures. None of the other tests showed a positive reaction. It was thus determined that the secretion is composed of polysaccharides and proteins.

Discussion

The morphoanatomy of secretory structures present at the base of leaves of *M. obtusifolia* and the chemical nature of the secretion

Fig. 4. Histochemistry of the colleters of *Macrocarpaea obtusifolia*. (A) Stage IV. Material fixed only in formaldehyde – acetic acid solution (FAA), secretion with a brownish colour. (B–E) Stage II. (B and C) Testing for polysaccharides. (B) Polysaccharides in the secretion and secretory cells (periodic acid – Schiff – PAS). (C) Acid mucilages in the secretion evidenced by Alcian Blue 8GX. (D and E) Positive reaction for proteins in secretion and secretory cells. (D) Coomassie Brilliant Blue G-250. (E) Xylidine Ponceau. (F–I) Stage IV. (F and G) Tests for lipids, indicating a positive reaction in cells of the central axis and the cuticle. (F) Sudan Black B. (G) Sudan IV. (H) Test for lignins, with phloroglucinol, indicating a positive reaction in cells of the central axis. (I) Neutral lead acetate revealing flavonoids in the abscission region of the colleter. (J) Stage II. Autofluorescence under UV light showing chloroplasts in the region of the central axis. Scale bars: (A–C, I, J) 200 μm; (D–H) 100 μm.



are consistent with those described for colleters (Fahn 1979; Thomas et al. 1989; Thomas and Dave 1989*a*, 1989*b*, 1990; Thomas 1991; Evert 2006).

Regarding their morphology, the colleters of *M. obtusifolia* can be classified as standard (Lersten 1974*a*, 1974*b*) because they comprise a central axis that is covered by secretory cells, which, however, do not exhibit the typical palisade aspect. They are anatomically distinct from colleters so far described for other species of Gentianaceae and exhibit higher structural complexity compared with colleters in *Calolisianthus* (Delgado et al. 2011), *Curtia* and *Hockinia* (Dalvi et al. 2013), *Gentiana*, *Gentianella*, *Gentianopsis*, *Comastoma*, and *Swertia* (Renobales et al. 2001).

These colleters are formed by a group of secretory cells, either sessile or short pedunculated, and do not exhibit a central axis or any bifurcation. Given the differences observed between *M. obtusifolia* and species from other genera of the Gentianaceae, such results may indicate the existence of patterns that might reflect the evolution of these secretory structures in the Genti-

anaceae, as reported in the Rubiaceae (Lersten 1974*a*, 1974*b*) and Apocynaceae (Simões et al. 2004), both families from order Gentianales. Studies on the diversity of foliar colleters in the Gentianaceae, covering species from different genera and tribes, combined with molecular data, will be performed. This will allow inferences on the evolution of this character within the group.

The absence of vascularization observed in the colleters of *M. obtusifolia* has also been reported for other species of Gentianaceae (Renobales et al. 2001; Delgado et al. 2011; Dalvi et al. 2013) and for other families of angiosperms (Appezzato-da-Glória and Estelita 2000; Paiva and Machado 2006; De-Paula and Oliveira 2007; Vitarelli and Santos 2009). Although Carlquist (1969) reported that vascularization is directly related to the size of the structure and not the developmental stage, this statement does not seem valid for the Gentianaceae, because although the colleters of *Macrocarpaea* are larger than the foliar colleters of other species of the same family, these are not vascularized.

The colleters of *M. obtusifolia* can be considered as glandular trichomes (Foster 1949; Fahn 1974), owing to their exclusively protodermal origin. Renobales et al. (2001) also considered the colleters of Gentianaceae as trichomes, although no ontogenetic studies were conducted. However, Decraene et al. (1998) emphasized that colleters differ from ordinary multicellular trichomes in having a specific position and function, e.g., as protecting the developing meristem by producing a viscous secretion.

On the one hand, the mucilaginous secretion of the colleters provides protection and lubrication of the buds in the initial phase of development of the leaf primordia (Fahn 1979). Mucilages are of a mixed nature, consisting primarily of acid and (or) neutral heteropolysaccharides, proteins, and phenolic substances that form colloidal solutions that become viscous when in contact with water (Priolo De Lufrano and Caffini 1981; Gregory and Baas 1989; Roshchina and Roshchina 1993). Furthermore, the presence of pectin helps to maintain the viscous appearance of the secretion because it is a hygroscopic polysaccharide with high water absorption capacity (Nobel et al. 1992). Thus, the characteristics of the secretion observed in the colleters of *M. obtusifolia*, together with the position of the colleters and their early differentiation, confirm the function previously described, which is assigned to colleters in general, as demonstrated in colleters of coffee flowers by Mayer et al. (2013).

On the other hand, mucilage can attract opportunistic organisms such as fungi and bacteria, causing damage to the meristems (Martins 2012); however, the proteins observed in the secretion of the colleters of *M. obtusifolia* could have antifungal activity, as pointed out by de Castro Miguel et al. (2006) in *B. nicholsonii* (Rubiaceae), and be associated with defense mechanisms against microorganisms (Klein et al. 2004). Future studies will be performed aiming to clarify the importance of the presence of proteins in the colleter secretion of Gentianaceae species from functional and phylogenetic perspectives. The proteins observed in the colleter secretion of *M. obtusifolia* may represent a character shared by families of the Gentianales, because they also occur in the colleter secretions of Apocynaceae (Dave et al. 1987; Thomas et al. 1989) and Rubiaceae (Thomas and Dave 1990; Klein et al. 2004; de Castro Miguel et al. 2006). However, absence of protein in the secretion is a common feature in colleters of the Myrtaceae, as reported by da Silva et al. (2012) for 52 species belonging to 17 genera of the Myrtoideae.

Chloroplasts found in the cells of the central axis during the early stages of colleter development in *M. obtusifolia* might be related to the production of the precursors of the secreted compounds, given that these structures decrease substantially after the secretory phase of the colleter. Chloroplasts were also visualized by Klein et al. (2004), although their function has not been clarified; therefore, studies are needed to clarify the ultrastructure of these chloroplasts and their function in the colleters.

The senescent process of *M. obtusifolia* colleters, which begins at the apex and extends to the base of the secretory portion, has been reported as common in other species of Aquifoliaceae (González and Tarragó 2009), Apocynaceae (Dave et al. 1987; Thomas et al. 1989; Thomas and Dave 1989*a*, 1989*b*; Appezzato-da-Glória and Estelita 2000), and Rubiaceae (Dave et al. 1988). In some species, the colour change in colleters has been associated with the initiation of senescence (Thomas and Dave 1990; Thomas 1991; Appezzatto-da-Glória and Estelita 2000; Barreiro and Machado 2007) and with the presence of phenolic compounds in the secretion (Thomas et al. 1989; Martins 2012). However, in *M. obtusifolia*, although colour change occurs during the colleter senescent process, phenolic compounds in the secretion were not detected in any stage of development.

During the senescence of *M. obtusifolia*, lignification and deposition of fatty compounds occur in the walls of the central axis cells in the contact area between the secretory portion and the peduncle. This finding indicates that lignification, and most likely suberization, are involved in colleter abscission in *M. obtusifolia*, similar to what happens during leaf abscission (Dickison 2000; Evert 2006) and in colleters of other species (Thomas and Dave 1989*a*, 1989*b*). This process is most probably related to protection by preventing the action of pathogens and leaf tissue damage after colleter senescence. In *Cariniana estrellensis* (Raddi) Kuntze (Lecythidaceae), the accumulation of phenolic compounds was observed in the colleter abscission zone (Paiva 2012).

In *M. obtusifolia*, colleters remain intact after the secretory phase, which suggests that the secretion is eliminated by cuticle pores (Vitarelli and Santos 2009). Furthermore, ultrastructural studies are necessary for clarifying the mechanism of elimination of colleter secretion in *M. obtusifolia*.

The results presented here reveal the presence of foliar colleters in *Macrocarpaea*, thus extending the reports of occurrence of these secretory structures to yet another genus of the Gentianaceae. Furthermore, the standard type of colleters is being reported for the first time in the Gentianaceae, although such type is a common characteristic of the Gentianales. The proteins observed in the secretion of the colleters of *M. obtusifolia* should be given attention in further studies that would aim to verify whether their presence is a common characteristic of the Gentianaceae, as verified in other families of Gentianales such as the Rubiaceae and Apocynaceae. These data also provide new information on the anatomy, ontogeny, and chemical nature of colleter secretions in the Gentianaceae, which can be useful for future work focussing on the ecological, taxonomic, and phylogenetic characteristics of this family.

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CAPÍTULO 2

EXTRAFLORAL NECTARIES IN NEOTROPICAL GENTIANACEAE: OCCURRENCE, DISTRIBUTION PATTERNS, AND ANATOMICAL CHARACTERIZATION

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Extrafloral nectaries in neotropical Gentianaceae: Occurrence, distribution patterns, and anatomical characterization[1]

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- *Premise of the study:* Extrafloral nectaries (EFNs) are structures that secrete nectar and protect plants against herbivores and pathogens. In Gentianaceae, these structures have been described in species of *Calolisianthus*, *Fagraea*, and *Anthocleista* and are important morphological markers for taxonomic and phylogenetic studies. To establish a foundation for further studies, we investigated the occurrence, distribution patterns, and anatomy of EFNs on leaves of 27 species belonging to 13 genera and three tribes of neotropical Gentianaceae.
- *Methods:* Leaf samples were diaphanized, stained with basic fuchsin, and mounted in glycerinated gelatin. Cross sections were obtained from material embedded in methacrylate or paraffin, stained, and mounted in Permount. Polysaccharides were histochemically stained with periodic acid–Schiff stain. Samples were also examined with scanning electron microscopy.
- *Key results:* Unusual EFNs, visible only with light microscopy, were formed of modified epidermal cells. Each EFN consisted of 2–5 secretory cells encircling a central cell. The EFNs varied in size and in the shape and arrangement of the adjacent cells surrounding the secretory cells. EFNs occurred in all analyzed species as isolated units distributed throughout the leaf blade or as aggregates; aggregates were generally visible to the naked eye. Based on their occurrence as aggregates or isolated units and on their location on the leaf blade, six distribution patterns were identified.
- *Conclusions:* This is the first comprehensive study of EFNs on the leaves of neotropical Gentianaceae. The data suggested that NEFs evolved from isolated units for NEFs in aggregates. The results represent a new source of data for future ecological, systematic, and phylogenetic studies in Gentianaceae.

Key words: Chironieae; Coutoubeinae; epidermal nectaries; foliar anatomy; Helieae; nectarioles; Saccifolieae; secretory structures.

Extrafloral nectaries (EFNs) are structures that secrete nectar, a solution of mono- and disaccharides composed mainly of fructose, glucose, and sucrose (Fahn, 1979; Lanza, 1988; Koptur, 1992; Heil, 2008) and are located on vegetative organs (Schmid, 1988). They play an important ecological role by protecting plants against herbivores and pathogens (Elias, 1972, 1980; Fahn, 1979; Pacini and Nicolson, 2007).

Highly diverse in morphology, EFNs include glandular structures that differ considerably in location, size, and form (Weber and Keeler, 2013). They range from single-celled nectar-secretion hairs to "formless" glandular tissue, complex raised cups, and shallow bowl-like depressions and can be highly vascularized or nonvascularized (Elias, 1983).

Extrafloral nectaries have been considered important for understanding taxonomic and phylogenetic relationships and for enhancing the adaptive success of various plant groups (Solereder, 1908; Fahn, 1979; Francino et al., 2006; Marazzi et al., 2006; Thadeo et al., 2008; Conceição et al., 2009; Coutinho et al., 2010; Weber and Keeler, 2013). They appear to have evolved numerous times independently; they occur in approximately 108 families of vascular plants and in 33 of 65 angiosperm orders (Weber and Keeler, 2013) and are more abundant in tropical than temperate communities (Morellato and Oliveira, 1991; Koptur, 1992; Oliveira et al., 1999).

Although EFNs have been reported to be absent in Gentianaceae (Elias, 1983), these structures have been observed in species of *Calolisianthus* (Calió, 2009), *Fagraea*, and *Anthocleista* (Struwe et al., 1994; Mészaros et al., 2002). Additionally, EFNs have been anatomically described in the leaves of *Calolisianthus pendulus*, *C. speciosus*, and *C. amplissimus* (Delgado et al., 2011a, b).

Gentianaceae is a monophyletic family within the order Gentianales, as demonstrated by molecular phylogenetic studies (Bremer and Struwe, 1992; Struwe et al., 1994; De Laet and Smets, 1996; Backlund et al., 2000; Albert and Struwe, 2002; APG III, 2003). This family contains approximately 1700 species and 91 genera distributed in six tribes (Chironieae, Exaceae, Gentianeae, Helieae, Potalieae, and Saccifolieae), in addition to the genus *Voyria*, whose position remains uncertain

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(Albert and Struwe, 2002; Struwe et al., 2002; Gentian Research Network, 2012). Although Gentianaceae is cosmopolitan, two-thirds of its genera are tropical (Mészaros et al., 1996). The tribes Helieae and Saccifolieae and the subtribe Coutoubeinae (tribe Chironieae) are exclusively neotropical (Albert and Struwe, 2002; Struwe et al., 2002).

Given the importance of EFNs as morphological markers in taxonomic and phylogenetic studies, we investigated the occurrence, distribution patterns, and anatomical characteristics of extrafloral nectaries in the leaves of species belonging to the tribes Chironieae, Helieae, and Saccifolieae (Gentianaceae).

MATERIALS AND METHODS

Material was collected during field expeditions from high-altitude, rocky vegetation complexes in the states of Minas Gerais and Bahia, Brazil, from March 2008 to July 2011 and deposited in the VIC Herbarium of the Universidade Federal de Viçosa (UFV). Additional herbarium material was acquired from national and international herbaria. Leaves from 27 species and 13 genera belonging to three tribes (Saccifolieae, Chironieae, and Helieae) of Gentianaceae were analyzed (Appendix 1).

Field-collected material was fixed in FAA (formaldehyde, acetic acid, 50% ethanol, 1 : 1 : 18 v/v) under vacuum for approximately 48 h, then dehydrated and stored in 70% ethanol (Johansen, 1940). Herbarium materials were rehydrated (Smith and Smith, 1942) and stored in 70% ethanol.

Whole, completely expanded leaves were processed by diaphanization with 10% w/v sodium hydroxide, bleached with 10% v/v sodium hypochlorite, stained with 0.001% w/v basic alcoholic fuchsin (Foster, 1950, modified), and mounted in glycerinated gelatin. Slides were sealed with colorless nail polish. The occurrence, distribution, and structure of nectaries were examined in frontal view.

Fragments from the median region of the leaf blade and fragments containing nectaries were selected to prepare cross sections and longitudinal sections. Some of these samples were dehydrated in an ethanol series and then embedded in methacrylate (Historesin, Leica Instruments, Heidelberg, Germany), and some were dehydrated in a tert-butanol series (Johansen, 1940) and then embedded in histological paraffin with dimethyl sulfoxide (DMSO; Histosec-Merck). Sections were made using a rotary microtome (model RM2155, Leica Microsystems, Deerfield, USA) with a thickness of 4 μm (for samples embedded in resin) or 8 μm (samples embedded in paraffin). Sections of samples embedded in resin were stained with toluidine blue, pH 4.7 (O'Brien et al., 1964), while those from samples embedded in paraffin were stained with astra blue and safranin (Gerlach, 1969). Permanent slides were mounted in synthetic resin (Permount; Fisher Scientific, New Jersey, USA). Sections obtained from fixed samples were stained with periodic acid–Schiff (PAS) to verify the presence of polysaccharides (Maia, 1979).

Photographs of the material were taken using a light microscope (Olympus AX70TRF; Olympus Optical, Tokyo, Japan) equipped with a U-photo system and a digital camera. Schematic drawings of the nectary distribution patterns were made using a light microscope equipped with a camera lucida (Olympus CBA, Olympus Optical, Tokyo, Japan).

For further micromorphological analysis, fragments of field-collected samples were dehydrated using an ethanol series and critical-point dried (model CPD 030, Bal-Tec, Balzers, Liechtenstein) using CO_2. Materials obtained from herbaria were not dehydrated. After drying, the samples were coated with a thin layer of gold (metalizer FDU010, Bal-Tec, Balzers, Liechtenstein) and analyzed at the Microscopy and Microanalysis Nucleus at UFV using a scanning electron microscope equipped with an image-capture system (model LEO 1430 VP, Zeiss, Cambridge, UK).

RESULTS

***Occurrence and distribution*—**All analyzed species bore EFNs that varied in their patterns of occurrence, distribution along the leaf blade, and anatomical structure.

The EFNs occurred in isolated secretory units that were visible only by microscopy and dispersed along the leaf blade and/or in aggregates forming nectaries that were generally visible to the naked eye (Table 1). In all species, EFNs in aggregates occurred only on the abaxial surfaces of leaf blades.

Six distribution patterns were identified based on the locations of EFNs on the leaf blade (Table 1): (I) EFNs isolated and dispersed on the abaxial surface only; (II) EFNs isolated and dispersed on both surfaces; (III) EFNs in aggregates at the base of the leaf blade only; (IV) EFNs in aggregates at the apex only and isolated EFNs dispersed on both surfaces; (V) EFNs in aggregates at both the base and the apex and isolated EFNs dispersed on both surfaces; and (VI) EFNs in aggregates at the base only and isolated EFNs dispersed on both surfaces.

All species of Saccifolieae (Table 1) had isolated EFNs dispersed over the leaf blade, interspersed among the stomata, and lacked EFNs in aggregates (types I and II). Type I was observed in *Hockinia montana* (Fig. 1A), *Curtia diffusa* (Fig. 1B), *C. verticillaris* (Fig. 1C), and *C. conferta.* Type II was observed in *Tapeinostemon spenneroides*, *T. longiflorum* var. *longiflorum*, *Voyriella parviflora*, and *C. tenella* (Fig. 1D, E), which seldom presented EFNs.

Species of Chironieae (Table 1) exhibited isolated, dispersed EFNs (type I) or EFNs in aggregates (type III). Type I was observed in *Coutoubea ramosa* (Fig. 2A), *C. spicata* (Fig. 2B), *Schultesia australis*, *S. brachyptera*, and *Symphyllophyton caprifolioides*. The EFNs of *S. caprifolioides* were scarce and difficult to detect even by microscopy. *Deianira damazioi* exhibited type III (Fig. 2C, D); the nectaries in aggregates were easily observed in the field because they were visible to the naked eye as a set of small points (Fig. 4A).

Species of Helieae (Table 1) possessed both isolated, dispersed EFNs and EFNs in aggregates in three distribution patterns (types IV, V, and VI). Type IV was observed in *Chelonanthus purpurascens* (Fig. 3A, B), with isolated EFNs dispersed abundantly over the abaxial surface and sparsely on the adaxial surface and EFNs in aggregates easily observed in diaphanized samples. However, the EFNs in aggregates could hardly be detected in the field. *Calolisianthus pedunculatus* (Fig. 3C, D), *Chelonanthus alatus*, *C. acutangulus*, *C. angustifolius*, *C. grandiflorus*, *C. viridiflorus*, *Helia brevifolia*, and *H. oblongifolia* showed type V. In *Chelonanthus viridiflorus* (Fig. 4B) and *Calolisianthus pedunculatus* (Fig. 4D), two yellow-green EFN aggregates at the base of the leaf blade were easily observed in the field. Otherwise, the apical EFN aggregates were difficult to observe in the field despite being observable in diaphanized material. *Macrocarpaea obtusifolia* (Fig. 3E, F), *M. glaziovii*, and *M. rubra* exhibited type VI. In these species, although the two EFN aggregates at the leaf base were inconspicuous, they were easily observed in the field on the oldest leaves because they appeared as yellowish points, as observed in *M. obtusifolia* (Fig. 4C).

***Anatomical characterization and histochemistry*—**In all analyzed species, the EFNs (Figs. 5, 6) were nonvascularized and made up of a single epidermal central secretory cell (CSC) surrounded by concentrically arranged secretory cells (SC). The SCs were voluminous, with dense cytoplasm and a conspicuous nucleus, while the central cell was reduced in size (Fig. 5). In the contact region between the SCs and the CSC, all stains tested showed intense coloration. For example, this intense staining can be observed for toluidine blue in *Curtia diffusa* (Fig. 5A) and *Hockinia montana* (Fig. 5B), astra blue and safranin in *Coutoubea ramosa* (Fig. 5C) and *Calolisianthus*

TABLE 1. Occurrence, distribution, and anatomy of leaf extrafloral nectaries (EFNs) in neotropical species of Gentianaceae.

Species	Distribution of EFNs on leaf		Anatomical structure of nectaries		
	Occurrence	Type	No. of secretory cells (Rarely)	Shape of adjacent cells relative to other epidermal cells	Size relative to stomata
Tribe Saccifolieae					
Curtia conferta (Mart.) Knobl.	Isolated, dispersed	I	2 (3)	Similar	Similar
Curtia diffusa (Mart.) Cham.	Isolated, dispersed	I	2 (3)	Similar	Similar
Curtia tenella (Mart.) Cham.	Isolated, dispersed	II	2	Similar	Similar
Curtia verticillaris (Sprengel) Knobl.	Isolated, dispersed	I	2 (3)	Similar	Similar
Hockinia montana Gardner	Isolated, dispersed	I	3 (4)	Similar	Similar
Tapeinostemon longiflorum var. *longiflorum* Maguire & Steyerm.	Isolated, dispersed	II	2 (3)	Similar	Similar
Tapeinostemon spenneroides Benth.	Isolated, dispersed	II	2 (3)	Similar	Similar
Voyriella parviflora (Miq.) Miq.	Isolated, dispersed	II	3	Similar	Similar
Tribe Chironieae (Coutoubeinae)					
Coutoubea ramosa Aubl.	Isolated, dispersed	I	4 (3)	In concentric series	Larger than the stomata
Coutoubea spicata Aubl.	Isolated, dispersed	I	4 (3 or 5)	In concentric series	Larger than the stomata
Deianira damazioi E.F.Guim.	In aggregates	III	4-5	Similar	Larger than the stomata
Schultesia australis Griseb.	Isolated, dispersed	I	3 (4)	Similar	Similar
Schultesia brachyptera Cham.	Isolated, dispersed	I	4 (3)	Similar	Similar
Symphyllophyton caprifolioides Gilg	Isolated, dispersed	I	4-5	Similar	Similar
Tribe Helieae					
Calolisianthus pedunculatus (Cham. & Schltdl.) Gilg	In aggregates and isolated, dispersed	V	3 (4 or 5)	Similar	Similar
Chelonanthus alatus (Aubl.) Pulle	In aggregates and isolated, dispersed	V	3 (4)	Similar	Similar
Chelonanthus acutangulus (Ruiz & Pav.) Gilg	In aggregates and isolated, dispersed	V	3 (4)	Similar	Similar
Chelonanthus angustifolius (Kunth) Gilg	In aggregates and isolated, dispersed	V	3 (4)	Similar	Similar
Chelonanthus grandiflorus (Aubl.) Chodat & Hassl.	In aggregates and isolated, dispersed	V	3 (4)	Similar	Similar
Chelonanthus purpurascens (Aubl.) Struwe & V. A. Albert	In aggregates and isolated, dispersed	IV	3 (4)	Similar	Similar
Chelonanthus viridiflorus (Mart.) Gilg	In aggregates and isolated, dispersed	V	3 (4)	Similar	Similar
Helia brevifolia Cham.	In aggregates and isolated, dispersed	V	4 (3 or 5)	Similar	Similar
Helia oblongifolia Mart.	In aggregates and isolated, dispersed	V	3 (4)	Similar	Similar
Macrocarpaea glaziovii Gilg	In aggregates and isolated, dispersed	VI	3 (4)	Similar	Similar
Macrocarpaea obtusifolia (Griseb.) Gilg	In aggregates and isolated, dispersed	VI	3 (4 or 5)	Similar	Similar
Macrocarpaea rubra Malme	In aggregates and isolated, dispersed	VI	3 (4)	Similar	Similar
Tetrapollinia caerulescens (Aubl.) Maguire & B. M. Boom	In aggregates and isolated, dispersed	VI	3 (4)	Similar	Similar

Notes: Type I, EFNs isolated and dispersed on the abaxial surface only; type II, EFNs isolated and dispersed on both surfaces; type III, EFNs in aggregates at the base of the leaf blade only; type IV, EFNs in aggregates at the apex only and isolated EFNs dispersed on both surfaces; type V, EFNs in aggregates at both the base and the apex and isolated EFNs dispersed on both surfaces; and type VI, EFNs in aggregates at the base only and isolated EFNs dispersed on both surfaces.

pedunculatus (Fig. 5D), and PAS in *Curtia verticillaris* (Fig. 5E). In *Deianira damazioi* (Fig. 5F), the CSC showed intense staining when treated with PAS.

The EFNs varied in the number of secretory cells (SC) and in the size and arrangement of the adjacent epidermal cells (Table 1).

In most of the analyzed species, the EFNs were similar in size to the stomata (Fig. 6A–C), and the adjacent cells surrounding the EFNs were similar in shape and size to the remaining epidermal cells (Fig. 6B, C). In two *Coutoubea* species, however, the EFNs were clearly larger than the stomata, and the adjacent epidermal cells exhibited a distinctive shape, occurring in a concentric series, as in *Coutoubea ramosa* (Fig. 6D).

In species of Saccifolieae, the EFNs were made up of two or three SCs. The EFNs of *Curtia* and *Tapeinostemon* had two SCs (Fig. 6A, B), while those of *Hockinia montana* (Fig. 6C) and *Voyriella parviflora* predominantly possessed three SCs. In Coutoubeinae-Chironieae, three to five SCs were observed. In all Helieae species, the EFNs predominantly contained three SCs (Fig. 6E, F), but variants with four (or rarely five) SCs also occurred (Fig. 6G). In this tribe, no striking differences were noted in the size and shape of the adjacent epidermal cells (Fig. 6E–G, I), but the SCs in the aggregated EFNs of *Calolisianthus pedunculatus* displayed densely stained cytoplasm (Fig. 6H).

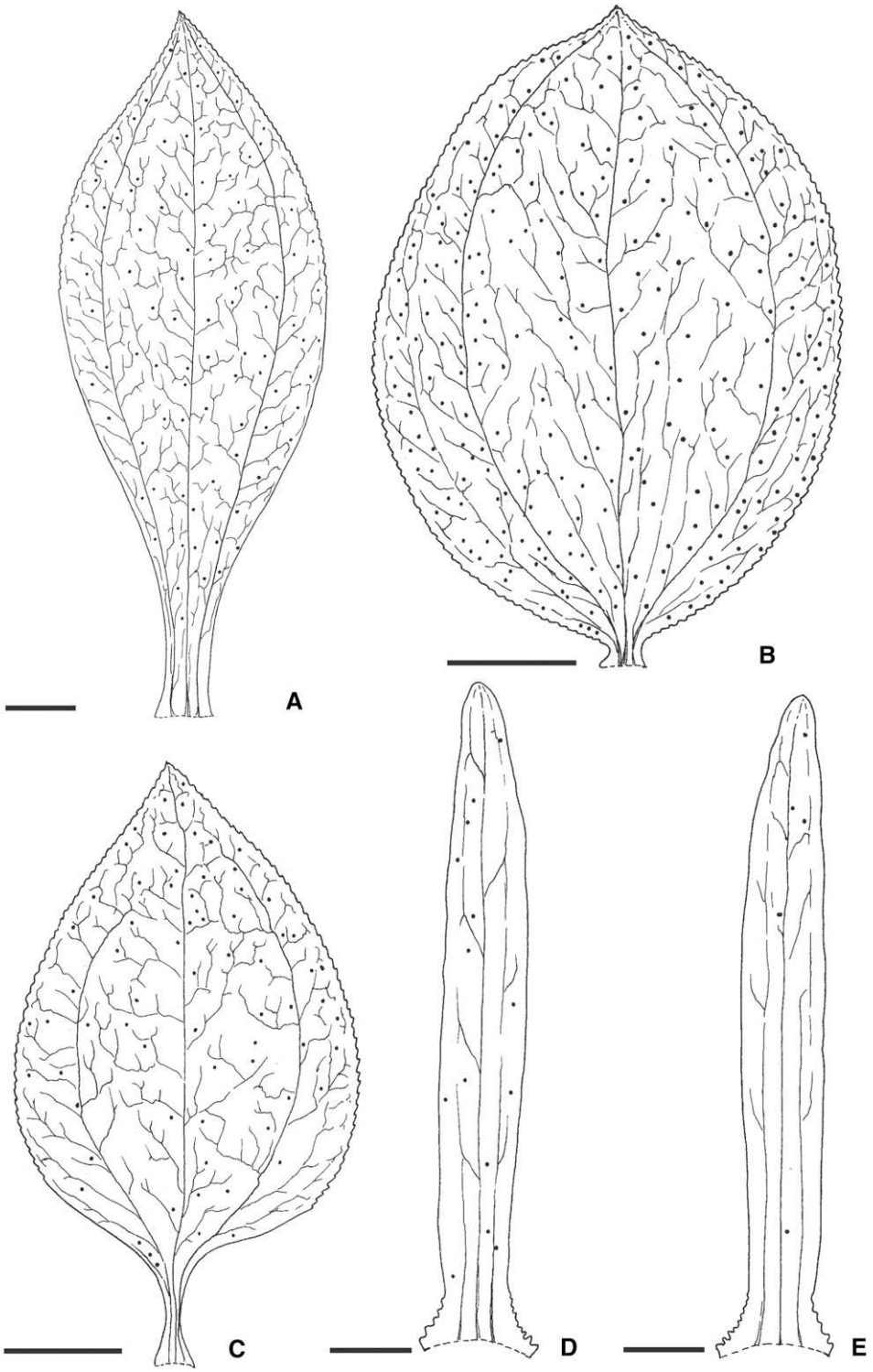

Fig. 1. Diagrams of distribution patterns of extrafloral nectaries on the leaves of Saccifolieae species (Gentianaceae). (A–D) Abaxial surface, (E) adaxial surface. (A) *Hockinia montana*. (B) *Curtia diffusa*. (C) *Curtia verticillaris*. (D, E) *Curtia tenella*. Scale bars = 2 mm.

DISCUSSION

The general structure of the EFNs examined in the leaves of these 27 species of neotropical Gentianaceae corresponds to the unusual, minute nectariferous structures termed nectarioles by Vogel (1998) and observed on the sepals of *Irlbachia* (Vogel, 1998) and on the leaves of *Calolisianthus* species (Delgado et al., 2011a, b). Vogel (1998) and Delgado et al. (2011b) interpreted the central region of these structures as a canal or pore rather than a central cell, as clearly observed in the present study. The coloration present in the contact region between the secretory cells and the central cell corresponds to the labyrinthine walls

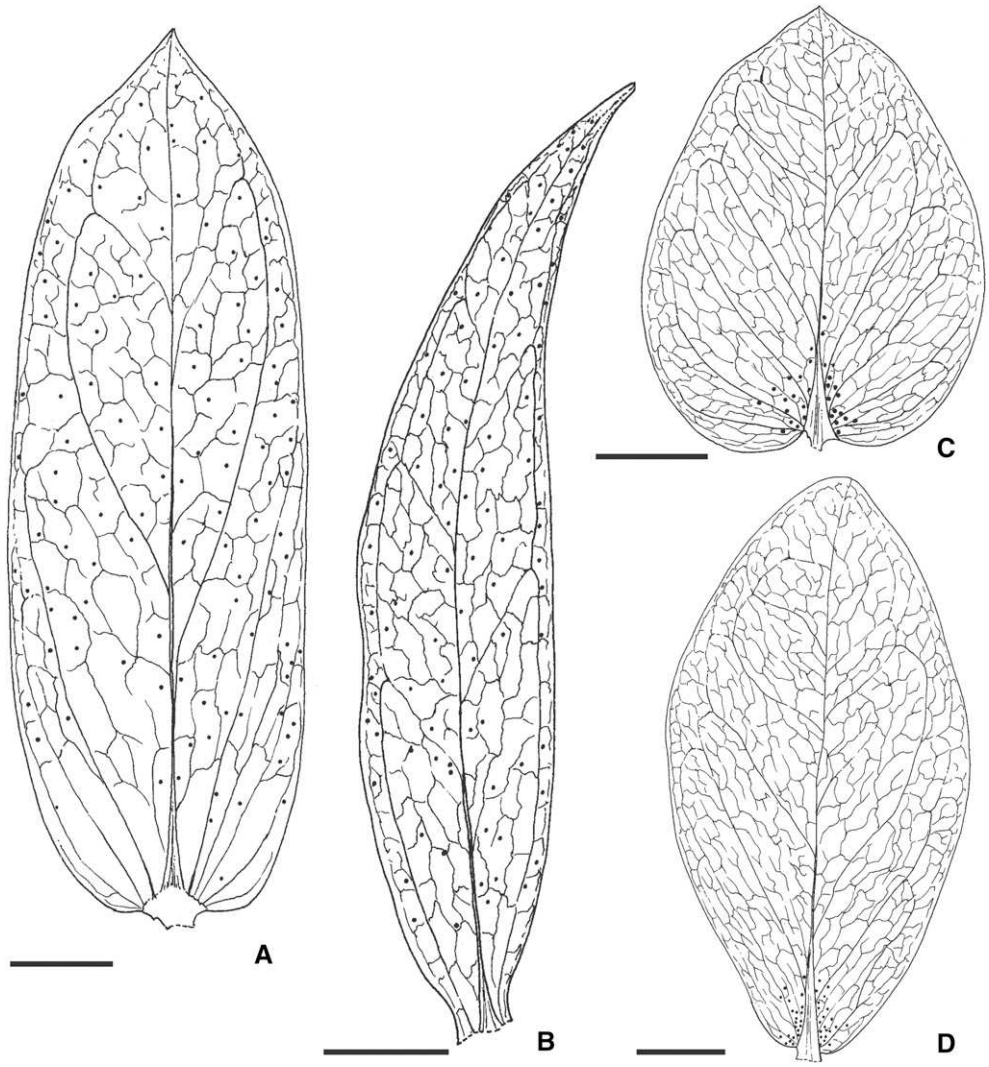

Fig. 2. Diagrams of distribution patterns of extrafloral nectaries on the abaxial surfaces of the leaves of Chironieae species (Gentianaceae). (A) *Coutoubea ramosa*. (B) *Coutoubea spicata*. (C, D) *Deianira damazioi*, apical and basal leaves, respectively. Scale bars = 5 mm.

described by those authors. Further ontogenetic and ultrastructural studies are underway to clarify whether the nectar produced by the secretory cells (SC) is transferred to the central cell (CSC), where it accumulates and is subsequently eliminated.

In the traditional sense, nectarioles include anatomically heterogeneous secretory structures formed by one or a few cells and distributed singly or in clusters composing macroscopic nectaries (Vogel, 1998). However, this term is broad and confusing because it can include glands, idioblasts, clusters of mesenchymatic cells, and secretory trichomes.

Bernardello (2007) analyzed the basic nectary types described by Vogel (1977) as mesenchymatous, epithelial, and trichomatous and by Vogel (1998) as nectarioles. This analysis verified some overlap among nectarioles, trichomatous nectaries, and epithelial nectaries. Therefore, Bernardello (2007) suggested the term "epidermal nectaries" to denote the wide variety of few-celled epidermal nectariferous structures. We do not use the term nectarioles for the nectar-secreting structures found on the leaves of certain species of Gentianaceae; rather, we prefer the terminology proposed by Bernardello (2007).

The observation of foliar EFNs in all species examined indicates that such structures are common in Gentianaceae. The use of this character in taxonomy is promising because EFNs have been considered rare or infrequent in Gentianales, previously observed only in a few species of Loganiaceae and Apocynaceae (Elias, 1983).

The lack of information about nectaries in Gentianaceae (Struwe et al., 1994; Mészaros et al., 2002; Calió, 2009; Delgado et al., 2011a, b) may be due to their small size and the difficulty of observing these structures without magnification in the field and in herbarium samples. Therefore, it is essential to use appropriate techniques, such as diaphanization, to confirm the presence of isolated EFNs and to evaluate their potential for taxonomic and phylogenetic studies in Gentianaceae.

The different EFN distribution patterns have diagnostic value and may be important for distinguishing species within genera or genera within tribes. Six EFN distribution patterns occur in the studied species and appear to be taxonomically useful at the tribe level. Either type I or type II occurs in all studied species of Saccifolieae; these patterns may represent a unifying character because the studied species represent four of the five

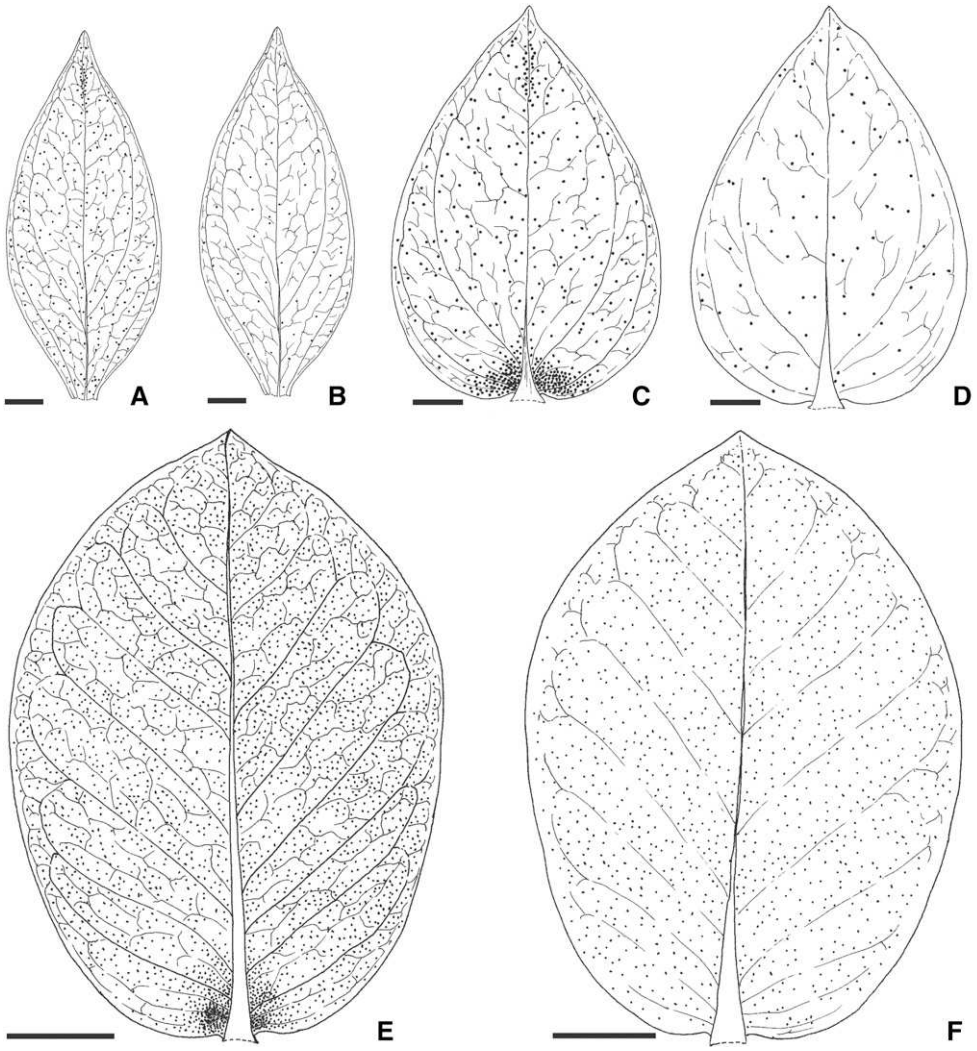

Fig. 3. Diagrams of distribution patterns of extrafloral nectaries on the leaves of Helieae species (Gentianaceae). (A, C, E) Abaxial surface. (B, D, F) Adaxial surface. (A, B) *Chelonanthus purpurascens*. (C, D) *Calolisianthus pedunculatus*. (E, F) *Macrocarpaea obtusifolia*. Scale bars: (A–D) = 5 mm; (E, F) = 20 mm.

genera and 50% of the species assigned to the tribe (Albert and Struwe, 2002).

EFNs are present in all analyzed species of Coutoubeinae (subtribe Chironieae). However, no EFNs were identified in earlier anatomical studies of *Schultesia gracilis*, *Deianira nervosa*, *D. pallescens*, and *D. erubescens* (Delgado et al., 2009). Thus, although the presnt study includes four of the five genera belonging to the subtribe (Albert and Struwe, 2002), the taxonomic value of this character requires further evaluation using a larger sample of *Schultesia* and *Deianira* species. This research is especially important because the position of *Deianira* in Coutoubeinae is controversial (Mészaros et al., 2002; Struwe et al., 2002).

All studied species of Helieae exhibit EFNs (types IV, V, or VI). Those of *Calolisianthus pedunculatus* show a type V distribution, as described for *Calolisianthus pendulus* and *C. speciosus* by Delgado et al. (2011a). However, *Calolisianthus amplissimus* (Delgado et al., 2011a) exhibits type IV, which also occurs in *Chelonanthus purpurascens*, the only species of this genus with purple flowers. All other studied species of *Chelonanthus* show type V. These data reinforce the assignment of *C. amplissimus* and *C. purpurascens* to the genus *Chelonanthus*, as suggested by Calió (2009) based on molecular phylogenetic data. In *Macrocarpaea bubops* and *M. noctiluca,* the two areas of orange coloration described by Grant and Struwe (2003) exhibit morphology similar to the EFNs (type VI) observed in the three *Macrocarpaea* species studied here.

The data obtained in this study on the occurrence of nectaries as isolated, dispersed units or in aggregates permit inferences about the evolutionary history of this character in Gentianaceae. Saccifolieae is considered the most basal lineage in the family, followed by Chironieae (Coutoubeinae) and then by Helieae, which occupies the most derived position among these taxa (Struwe et al., 2002). Only isolated, dispersed nectaries occur in Saccifolieae; both isolated, dispersed nectaries and nectaries in aggregates at the leaf base occur in Coutoubeinae (Chironieae); and both nectaries in aggregates (forming basal pairs and/or a single unit at the apex) and isolated, dispersed nectaries on both surfaces occur in all examined representatives of Helieae. Thus, the EFNs of Gentianaceae most likely arose as isolated, dispersed nectaries on the abaxial surface of the leaf blade, followed by isolated, dispersed

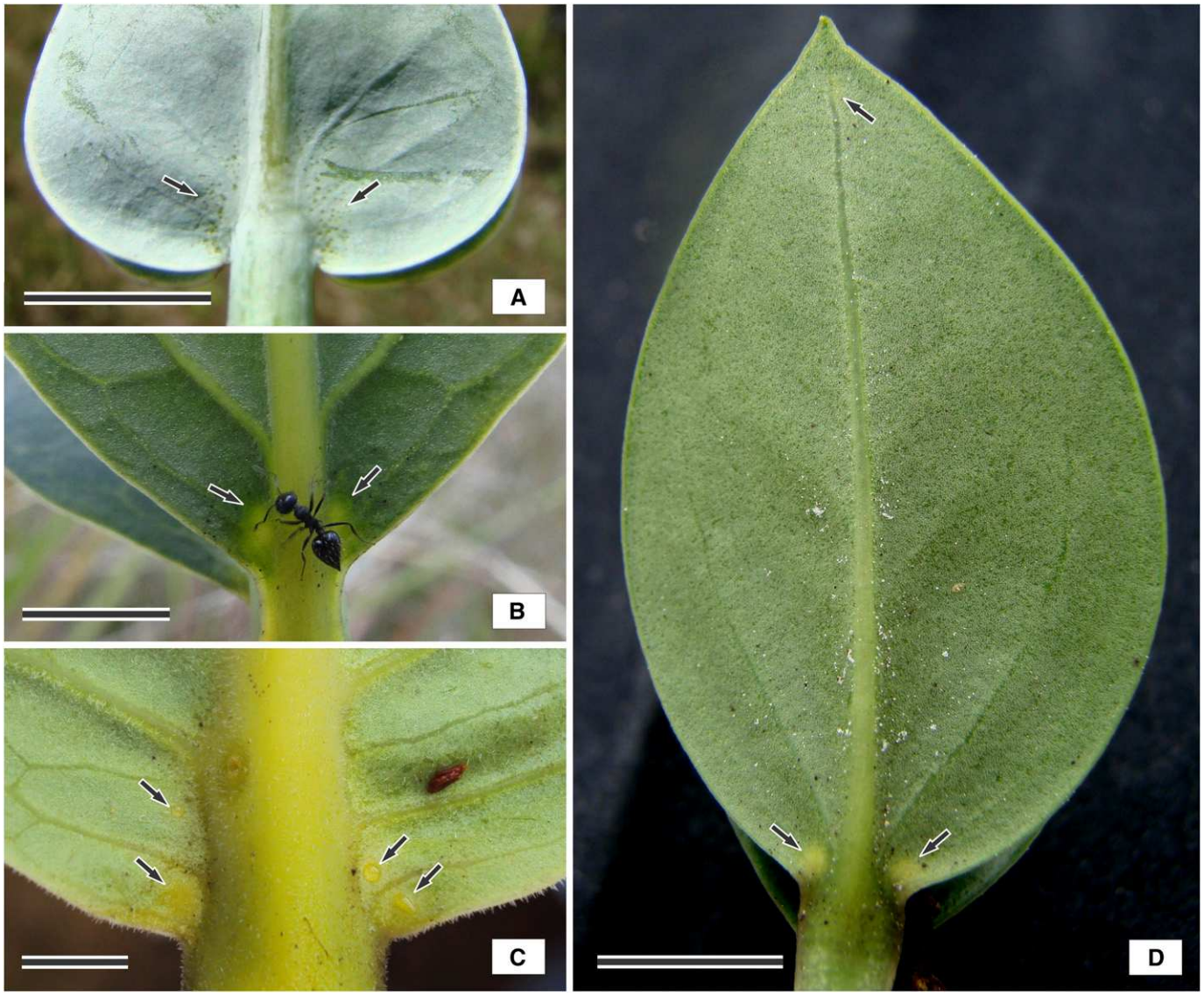

Fig. 4. Extrafloral nectaries of Gentianaceae observed without magnification in the field (abaxial surface). (A) *Deianira damazioi*. (B) *Chelonanthus viridiflorus*. (C) *Macrocarpaea obtusifolia*. (D) *Calolisianthus pedunculatus*. Arrows indicate the position of nectaries. Scale bars = 1 cm.

nectaries over both leaf surfaces. Later, aggregated nectaries appeared on the leaf bases, giving rise to paired EFNs. The most derived condition is the universal presence of nectaries in aggregates (at the base or apex of the leaf) together with isolated, dispersed nectaries. In intermediate phases (subtribe Coutoubeinae), the loss of isolated nectaries apparently occurred concomitantly with the rise of aggregated nectaries. This hypothesis should be tested in future studies using combined morphological and molecular data from representatives of all tribes.

EFNs are generally more abundant in plants from tropical environments than in plants from temperate regions (Morellato and Oliveira, 1991; Koptur, 1992; Oliveira et al., 1999). The presence of EFNs on the leaves of all studied species of neotropical Gentianaceae reinforces this pattern. To verify the correlation between the presence of EFNs and the geographic distributions of Gentianaceae species, however, additional studies incorporating representatives of all tribes, including those from temperate climates, are needed.

The occurrence of EFNs predominantly or exclusively on the abaxial surface of the leaf blade in species of Gentianaceae may represent a strategy to reduce nectar loss due to evapotranspiration, thus increasing the availability of this resource to ants compared to other plants (Keeler and Kaul, 1979; Leitão et al., 2005; Paiva and Machado, 2006). The presence of dispersed nectaries with minute secretion rates and of aggregates with visible secretions may favor ants patrolling in search of nectar and function as a defense mechanism, as observed by previous authors (Elias and Gelband, 1976; Morellato and Oliveira, 1994). In the field, we have seen ants visiting EFNs only in *Chelonanthus viridiflorus* (Fig. 4B), but they have also been reported to visit those of *Calolisianthus speciosus* (Delgado et al., 2011b). Of the three species studied by Delgado et al. (2011b), they reported the presence of ants at EFNs of *Crematogaster*, *Camponotus*, *Brachymyrmex*, and *Linepithema* and only at the base of leaves of *C. speciosus* during the rainy period. The lack of information about the importance of these

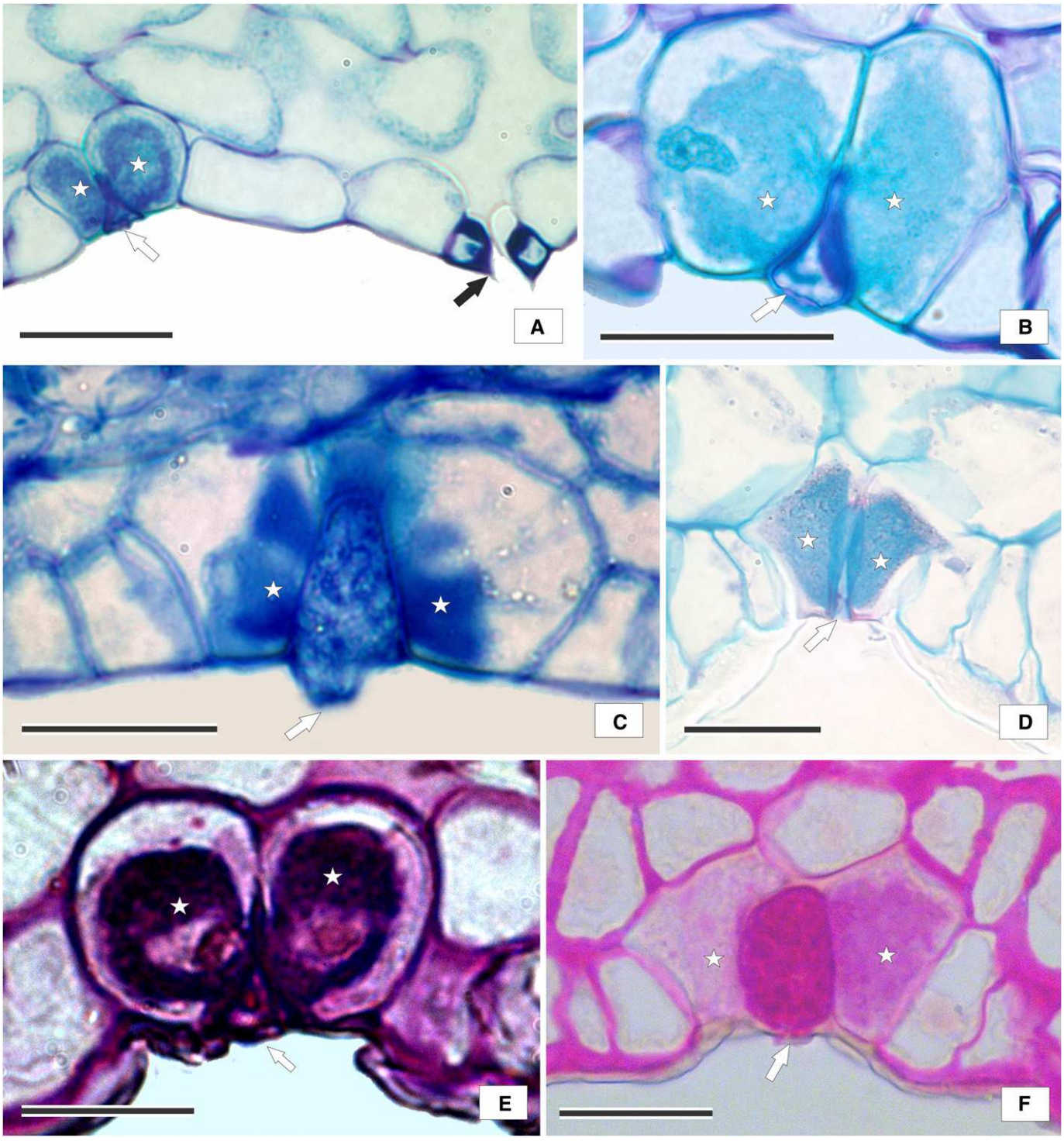

Fig. 5. Extrafloral nectaries of species of Gentianaceae in transverse section. (A) *Curtia diffusa*. (B) *Hockinia montana*. (C) *Coutoubea ramosa*. (D) *Calolisianthus pedunculatus*. (E, F) PAS-stained sections. (E) *Curtia verticillaris*. (F) *Deianira damazioi*. Black arrows indicate stomata, white arrows indicate central cells, and open stars indicate the labyrinthine nectary walls. Scale bars = 30 µm.

secretory structures for the adaptive success of Gentianaceae emphasizes the need for ecological studies.

This study presents the first record of EFNs on the leaves of species of Gentianaceae and clearly shows that EFNs are common secretory structures in neotropical Gentianaceae. The distribution patterns described here can be used to make inferences about the evolutionary history of these structures. These data represent a new source of information for future ecological, systematic, and phylogenetic research in Gentianaceae.

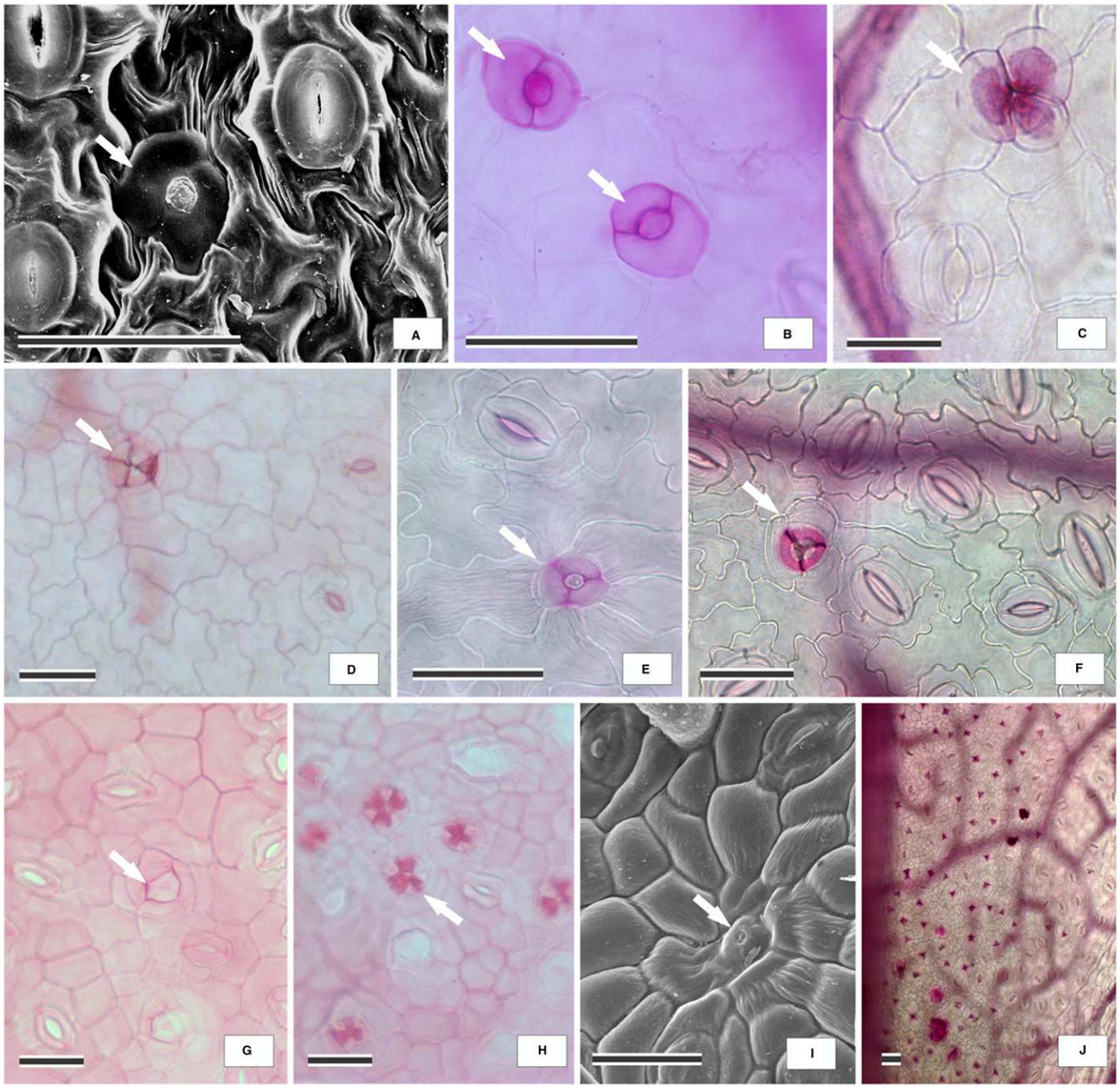

Fig. 6. Extrafloral nectaries of species of Gentianaceae in frontal view. (A–G, I) Isolated nectaries dispersed over the leaf blade. (H, J) Nectaries in aggregates at the base of the leaf. (A) *Curtia diffusa*. (B) *Curtia verticillaris*. (C) *Hockinia montana*. (D) *Coutoubea ramosa*. (E) *Chelonanthus purpurascens*. (F) *Chelonanthus alatus*. (G, H) *Calolisianthus pedunculatus*. (I) *Macrocarpaea obtusifolia*. (J) *Chelonanthus grandiflorus*. Scale bars = 50 µm.

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APPENDIX 1. Gentianaceae species studied and voucher information.

Tribe—*Taxon*: *Voucher* (Herbarium code). The herbaria acronyms: INPA = Instituto Nacional de Pesquisas da Amazônia; MBM = Museu Botânico Municipal; NYBG = New York Botanical Garden; RB = Jardim Botânico do Rio de Janeiro; SP = Instituto de Botânica; SPF = Universidade de São Paulo; VIC = Universidade Federal de Viçosa.

Helieae—*Calolisianthus pedunculatus*: *Valente et al. 2466* (VIC), *Valente et al. 2468* (VIC), *Dalvi et al. s/n* (VIC); *Chelonanthus acutangulus*: *Benson 8302* (INPA); *Nee 48250* (NYBG), *Killip and Smith 22537* (NYBG); *Chelonanthus alatus*: *Zarucchi et al. 3028* (RB), *Brocki 14* (INPA), *Groppo et al. 956* (INPA); *Chelonanthus angustifolius*: *Prance et al. s/n* (SP), *Cárdenas et al. 4010* (INPA), *Silva et al. 405* (INPA), *Gangi and Silvano 4011* (INPA); *Chelonanthus grandiflorus*: *Stevenson et al. 952* (INPA), *Todzia et al. 2307* (INPA), *Silva et al. 618* (INPA), *Albuquerque et al. 695* (INPA); *Chelonanthus purpurascens*: *Mantone 1007* (RB), *Dalvi et al. 30* (VIC), *Dalvi et al. 48* (VIC), *Dalvi et al. 62* (VIC), *Pirani et al. 5441* (SPF); *Chelonanthus viridiflorus*: *Dalvi and Francino 12* (VIC), *Dalvi and Francino 13* (VIC), *Lombardi et al. 3879* (RB), Alves et al. 2250 (SP), *Romaniuc Neto et al. 615* (SP); *Helia brevifolia*: *Serafim 27* (RB), *Costa et al. 5762* (SPF), *Silva et al. 724* (SP); *Helia oblongifolia*: *Harley et al. s/n* (SP), *Seccantini 180* (SPF); *Macrocarpaea glaziovii*: *Nadruz et al. 1992* (RB), *Rossini and Bausen 539* (SPF), *Pessoa et al. 249* (RB); *Macrocarpaea obtusifolia*: *Valente et al. 1620* (VIC), *Dalvi et al. 85* (VIC), *Dalvi et al. 86* (VIC); *Macrocarpaea rubra*: *Falkenberg 3846* (MBM), *Bovini et al. 2733* (RB), *Oliveira 686* (MBM), *Cervi et al. 6353* (UPCB); and *Tetrapollinia caerulescens*: *Dalvi and Francino 22* (VIC), *Dalvi and Francino 23* (VIC), *Hopkins et al. 1766* (INPA).

Saccifolieae—*Curtia conferta*: *Brade s/*n (RB); *Curtia diffusa*: *Dalvi et al. s/n* (VIC); *Curtia tenella*: *Dalvi et al. 01* (VIC), *Dalvi et al. 04* (VIC); *Curtia verticillaris*: *Dalvi et al. 44* (VIC), *Dalvi et al. 45* (VIC), *Dalvi et al. 72* (VIC), *Dalvi and Francino 15* (VIC); *Hockinia montana*: *Dalvi et al. 99* (VIC), *Dalvi et al. 100* (VIC), *Valente and Meira 1284* (VIC), *Valente et al. 620* (VIC); *Tapeinostemon longiflorum* var. *longiflorum*: *Maguire et al. s/n* (NYBG), *Maguire et al. s/n* (NYBG), *Maguire et al. s/n* (NYBG); *Tapeinostemon spenneroides*: *Steyermarch s/n* (NYBG), *Steyermarch et al. s/n* (NYBG), *Tillet and Tillet s/n* (NYBG); and *Voyriella parviflora*: *Pires and Silva 1641* (NYBG), *Cristenson and George 1796* (NYBG), *Maguire et al. s/n* (NYBG).

Chironieae—*Coutoubea ramosa*: *Cid Ferreira 9584* (RB), *Romaniuc Neto et al. 493* (RB), *Silva et al. 3804* (RB), *Marquete et al. s/n* (RB); *Coutoubea spicata*: *Heiden et al. 960* (RB), *Hatschbach et al. 75269* (MBM), *Ratter et al. s/n* (INPA); *Deianira damazioi*: *Valente et al. 2458* (VIC), *Valente et al. 2459* (VIC), *Dalvi and Pereira 07* (VIC), *Dalvi and Pereira 08* (VIC); *Schultesia brachyptera*: *Barroso and Elsie 92* (RB), *Romaniuc Neto et al. 431* (SP), *Faria et al. s/n* (SP); *Schultesia australis*: *Hatschbach s/n* (RB), *Hatschbach et al. 79187* (SPF), *Hatschbach and Zardini 41012* (MBM); and *Symphyllophyton caprifolioides*: *Fonseca et al. 6076* (RB), *Gottesberg and Gottesberg s/n* (SP), *Ratter et al. s/n (*MBM*)*.

CAPÍTULO 3

ARE NECTARIES COMMON STRUCTURES ON THE STEM OF GENTIANACEAE SPECIES?

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Are nectaries common structures on the stem of Gentianaceae species?

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Abstract: Extrafloral nectaries (EFNs) are structures that secrete nectar, being reported on leaves, cotyledons and seldom on stems. In the Gentianaceae, EFNs were described on leaves of Neotropical species and on the stem only of *Curtia* species and *Hockinia.* The aim of this study was to verify if stem EFNs have general occurrence in the Gentianaceae, and investigate if there is a correlation between such occurrence and the geographical distribution of species, subtribes and/or tribes. Samples were obtained from internodal regions of materials collected in the field and from herbarium specimens of 38 species and 26 genera comprising representatives of five tribes. Cross sections were obtained from material embedded in methacrylate, stained and mounted in Permount. Data on geographical distribution pattern were extracted from herbarium specimens and literature. EFNs are non-vascularized and composed by modified epidermal cells. They could be observed in 17 Neotropical species, all of which belong to tribes Helieae, Saccifolieae, Potalieae and Coutoubeinae. *Cicendia quadrangularis* and *Zygostigma australe* (Chironieae-Chironiinae) occur in the Neotropics but do not possess EFNs. These structures were not found in species of others regions. These results show how common are EFNs on the stem of the Gentianaceae, particularly in neotropical species. The data reported herein on the spread occurrence and geographic distribution pattern are original and may be useful for future works with multidisciplinary approaches, such as functional anatomy, ecology, taxonomy and phylogeny.

Key words: Chironiieae, Gentianeae, Helieae, nectary, Potalieae

Introduction

Nectaries are structures that secrete nectar, a solution composed of monosaccharides and disaccharides, amino acids, proteins and trace amounts of others compounds (Elias 1983). According to their occurrence along the plant body, nectaries have been referred to as floral nectaries (FNs), when located on floral parts, and extrafloral nectaries (EFNs), when situated on vegetative organs (Schmid 1988). EFNs are usually reported on leaves, specifically on the adaxial surface of the petiole or rachis, between pairs of leaflets, rarely along the midvein (Elias 1980, 1983), and on cotyledons (Zimmerman 1932). On the other hand, the occurrence of EFNs on the stem is limited to reports on nodal regions (Elias 1983, Oliveira and Oliveira-Filho 1991, Machado et al. 2008). EFNs play an important ecological role by protecting plants against herbivores and pathogens (Elias 1972, 1980, Fahn 1979, Pacini and Nicolson 2007). However, such role has not yet already been investigated on stem EFNs.

EFNs occur in approximately 108 families of vascular plants and in 33 of the 65 angiosperm orders (Weber and Keeler 2013). Although plants bearing EFNs are widely distributed around the world, Bentley (1977) highlights that these structures are more abundant in species from tropical communities, rather than in species from temperate ones. In the Gentianaceae, EFNs were described on leaves of different neotropical genera, such as *Calolisianthus* (Delgado et al. 2011a, b, Dalvi et al. 2013), *Curtia*, *Coutoubea, Chelonanthus*, *Deianira*, *Helia, Hockinia*, *Macrocarpaea*, *Schultesia, Symphyllophyton, Tapeinostemon, Tetrapollinia* and *Voyriella* (Dalvi et al. 2013, 2014), and on the stem of *Curtia* species and *Hockinia montana* (Dalvi et al. 2014). Dalvi et al. (2013) verified the presence of EFNs in 27 neotropical species (13 genera) of the Gentianaceae, demonstrating the existence of a correlation between the presence of leaf EFNs and the pattern of geographic distribution.

The Gentianaceae contains 1700 species and 91 genera distributed along seven tribes: Chironieae, Exaceae, Gentianeae, Helieae, Potalieae, Saccifolieae and Voyrieae (Struwe et al. 2002, Merckx et al. 2013, Gentian Research Network, 2014). The family is cosmopolitan (except for the Antarctic continent), most of the species being concentrated in temperate regions (Sousa and Lorenzi 2008). However, the greatest genera diversity lies in South and Central America (Struwe and Albert 2002).

Taking into account the diversity of the Gentianaceae and its wide distribution, the aims of this work were to verify how common is the occurrence of EFNs on the stem of its species, and to investigate if the same correlation verified in leaves, between occurrence and distribution of species and subtribes or tribes, exists for stem EFNs.

Materials and methods

Field expeditions were performed in states of Minas Gerais and Bahia, Brazil, for sample collection and fixation for anatomical studies. Samples of the third to fifth internodes were fixed in FAA (formaldehyde, acetic acid, 50% ethanol, 1:1:18 v/v) and stored in 70% ethanol (Johansen 1940). In the case of *Potalia resinifera*, a tree species, fragments of branches were sampled from the herbarium specimen. Collected materials were deposited at the VIC Herbarium of Universidade Federal de Viçosa (UFV), Minas Gerais, Brazil. Additional herbarium materials were acquired from national and international herbaria, and were rehydrated (Smith and Smith 1942) and stored in 70% ethanol.

Samples were dehydrated in ethanol series, embedded in methacrylate (Historesin, Leica Instruments, Heidelberg, Germany) (Johansen 1940) and then sectioned in a rotary microtome (model RM2155, Leica Microsystems Inc., Deerfield, USA). Transverse and paradermal sections 7 µm thick were stained with toluidine blue pH 4,7

(O' Brien et al. 1964) and permanent slides were mounted with synthetic resin (Permount- Fisher Scientific, New Jersey, USA).

Material analysis and image capture were performed under a light microscope (Olympus AX70TRF; Olympus Optical, Tokyo, Japan) equipped with an U-photo system and a digital camera, at Laboratory of Plant Anatomy, UFV.

For the study of geographical distribution of species and tribes or subtribes, information was obtained from herbarium data and compiled from literature. Analyzed species, organized by tribes and followed by their respective collectors, herbaria, collection sites and geographic distribution patterns, are listed in Table 1.

Results

We analyzed 38 species (26 genera), comprising representatives of tribes Chironieae, Gentianeae, Helieae, Potalieae and Saccifolieae (Table 1).

Neither in materials collected in the field nor in those from herbaria, are stem EFNs visible to the naked eye. No secretion or other macro-morphological evidence related with the presence of EFNs were detected. Under light microscopy, however, EFNs were observed on internodes of the stems in about 50% of the species (17 out of the 38 analyzed species) and in 12 out of the 26 analyzed genera (Table 1).

The EFNs are non-vascularized and have a single central epidermal cell surrounded by concentrically arranged secretory cells, as observed in *Tetrapollinia caerulescens* (Fig. 1a). Secretory cells have evident nucleus and the region of contact with the central cell stained intensely when treated with toluidine blue (Figs. 1a-f). EFNs are scattered distributed and interspersed among stomata (except in *Potalia resinifera,* where stomata could not be detected).

EFNs were visualized in all 11 analyzed species in six genera of tribe Helieae (Table 1), as demonstrated with *Tetrapollinia caerulescens* (Fig. 1a), *Calolisianthus pedunculatus* (Fig. 1b) and *Chelonanthus grandiflorus* (Fig. 1c). In Saccifolieae, a single species, *Voyriella parviflora*, was analyzed (Table 1), and it presented EFNs. The presence of EFNs was constant in Potalieae, as observed in *Neurotheca loeselioides* (Fig. 1d), subtribe Faroinae; and in *Potalia resinifera*, subtribe Potaliinae. On the other hand, EFNs were absent in all seven analyzed taxa of tribe Gentianeae.

In Chironieae, EFNs were present in three species from different genera and absent in the other species. In subtribes Coutoubeinae, Canscorinae and Chironiinae the pattern of presence or absence of EFNs remained constant. All three species of Coutoubeinae presented EFNs, as found in *Symphyllophytton caprifolioides* (Fig. 1e) and *Schultesia pachyphylla* (Fig. 1f), while in Canscorinae (*Canscora diffusa*) and in Chironiinae EFNs were not found.

During the field expeditions, the presence of ants patrolling stems, leaves, buds and flowers was recorded only on individuals of *Calolisianthus pedunculatus*, *Calolisianthus speciosus* and *Chelonanthus viridiflorus,* all of which are species with leaf EFNs visible to the naked eye.

Chironieae presents different distribution patterns among its three tribes. Canscorinae occurs in the Paleotropics, *Canscora difusa* being registered on Asia, Africa and Australia. Chironiinae presents pantropical to tempered distribution, and occurrence in the Mediterranean is registered for *Blackstonia perfoliata* subs. *grandiflora*, *Centautium erythraea, Centautium maritimum, Centautium pulchellum*, *Cicendia filiformis*, *Cicendia quadrangulares* and *Schenkia spicata*. *Cicendia filiformis* also occurs in Europe, *Cicendia quadrangula*res in North and South America and *Schenkia spicata* in Europe, Asia and Africa. *Chironia baccifera* is endemic to Africa;

Gyrandra tenuiflora and *Zeltera stricta* occur in Mexico; the two species of *Sabatia* occur in North America, and *Zygostigma australe* occurs only in the Neotropics, specifically in Brazil, Argentina and Uruguay. Coutoubeinae is restricted to the Neotropics: *Schultesia pachyphylla* and *Symphyllophytton caprifolioides* are endemic to Brazil and *Xestea lisianthoides* occurs in South and Central America (Table 1).

The Gentianeae are restricted to temperate-alpine regions, with representatives in the Asian, Australian, African, American and European continents (Table 1).

Tribe Helieae has a distribution restricted to the Neotropics, with species endemic to Brazil, such as *Calolisianthus pedunculatus*, *Calolisianthus speciosus*, *Irlbachia nemorosa*; to South America (*Adenolisianthus arboreus*, *Chelonanthus alatus*, *Chelonanthus purpurascens, Chelonanthus viridiflorus, Helia oblongifolia, Tetrapollinia caerulescens*); and to Latin America (South and Central America), such as *Chelonanthus alatus* and *Chelonanthus grandiflorus* (Table 1).

The Potalieae has pantropical distribution. *Neurotheca loeselioides* occurs in Africa and South America, while *Potalia resinifera* has a distribution restricted to the Neotropics (Amazon basin and Andes) (Table 1).

The Saccifolieae is also restricted to the Neotropics, *Voyriella parviflora* occurring in South America and Panama (Table 1).

Regarding the correlation between presence/absence of EFNs and distribution pattern of the species, tribes or subtribes, the presence of stem EFNs was constant in species with distribution restricted to the Neotropics, including all representatives of tribes Helieae, Saccifolieae, subtribe Coutoubeinae (Chironieae), and *Potalia resinifera* (Potalieae - Potaliinae). *Neurotheca loeselioides* (Potalieae-Faroinae), besides occurring in the Neotropics, is also found in Africa and possesses EFNs. Exceptions could be seen in Chironieae, in which *Cicendia quadrangularis* and *Zygostigma australe* (Chironieae -

Chironiinae), both occurring in South America, showed no EFNs. In contrast, these structures are absent in all species with distribution only in temperate-alpine regions (Gentianinae), as well as in species restricted to the Paleotropics (Chironieae - Canscorinae) and in those with occurrence on pantropical to temperate regions (Chironieae - Chironiinae) (Table 1).

Discussion

Recent studies indicate that EFNs are common in leaves of Gentianaceae species (Mészaros et al. 2002, Calió 2009, Delgado et al. 2011a, Dalvi et al. 2013, 2014). Although the presence of stem EFNs is considered uncommon in most eudicotyledonous families (Elias 1983, Machado et al. 2008), data obtained on the present study demonstrate that stem EFNs have wide occurrence along different tribes of the Gentianaceae.

EFNs of the Gentianaceae were described by Vogel (1998) as unusual nectaries on the sepals of *Irlbachia* species and were termed nectarioles by the author. The anatomical structure of stem EFNs is similar to that observed in leaf EFNs (Delgado et al. 2011 a, b, Dalvi et al. 2013, 2014). Although it was not possible to observe secretion on stem EFNs of the studied species, the presence of voluminous nucleus and dense cytoplasm indicates that these nectaries are probably active. The coloration in the contact region between the secretory cells and the central cell, as reported in leaf EFNs, corresponds to the labyrinthine walls, as demonstrated by ultrastructural studies made by Delgado et al. (2011b).

Stem EFNs, as well as foliar nectaries, have probably been neglected due to the need for microscopic analyses, in most cases, for their identification, as stated by Dalvi et al. (2013). Generally, stem anatomical studies in the Gentianaceae are even scarcer

than those conducted with leaves and are restricted to a few species of *Deianira*, *Schultesia* (Delgado et al. 2009), *Curtia* and *Hockinia* (Dalvi et al. 2014). In these genera, EFNs were reported on stem of only seven *Curtia* species and *Hockinia montana*.

Comparing the data obtained here with those of Dalvi et al. (2013), we observed that the presence of EFNs on leaves is directly related to the presence of EFNs in stems, and all neotropical studied species of the Gentianaceae have EFNs on leaves and/or stems. However, the expansion of data on the occurrence of EFNs on leaves of species with distribution outside the Neotropics is essential to support this relationship.

The presence of ants in *Calolisianthus pedunculatus*, *Calolisianthus speciosus* and *Chelonanthus viridiflorus* is probably related to the presence of conspicuous macroscopic EFNs at the base of leaves, with apparent secretion that attracts ants (Delgado et al. 2011b, Dalvi et al. 2013). In other Gentianaceae species, which present nectaries isolated and dispersed along the leaf blade, the presence of ants has not been recorded and apparent secretion was not detected (Delgado et al. 2011b, Dalvi et al. 2013, 2014), the same as for species with stem EFNs. Therefore, it is necessary to clarify the role played by both leaf and stem EFNs on the defense against herbivores and pathogens, as well as the process of nectar secretion. Correlations between the presence of stem EFNs and geographic distribution of species, subtribes or tribes could be observed. EFNs are present in all species with distribution restricted to the Neotropics, which includes representatives of the Helieae, Saccifoliae and Coutoubeinae (Chironieae). An exception was found only in *Zygostigma australe* (Chironiinae-Chironieae), a species restricted to South America and that does not present EFNs. The other species with EFNs also have representatives in the Neotropics, such as two species of the Potalieae, a pantropical tribe: *Potalia resinifera* (Potaliinae),

restricted to the Neotropics, and *Neurotheca loeselioides,* recorded for South America and Africa. *Cicendia quadrangularis* (Chironiinae- Chironieae), which occurs in the Mediterranean and in disjoint areas of South America, also did not present EFNs. It is noteworthy that this species and other species of the genus are concentrated in the Mediterranean region, which could explain their absence of EFNs. The other species, despite of presenting a pantropical or pantropical to temperate distribution, do not occur in neotropical areas and do not have EFNs. Bentley (1977) highlights that EFNs are more abundant in species from the tropics when compared to species from temperate regions, a constant pattern in the Gentianaceae.

The neotropical region (South and Central America) possesses great importance on the evolutionary history of the Gentianaceae, as it was a center of diversification of the family, and comprises its most basal lineages, represented by Saccifolieae and Chironieae, except for a few genera of the latter, which occur in temperate regions (Albert and Struwe 2002). In such regions, 47 genera (54%) of the Gentianaceae can be found, 36 (77%) of which are endemic (Struwe and Albert 2002).

We observed the occurrence of EFNs in species of the most basal tribe (Saccifolieae), in neotropical species of Chironieae and Potalieae, and in species of Helieae, an exclusively neotropical tribe. At the same time, we could observe the non-occurrence of EFNs in species of neither temperate nor paleotropical regions. In view of these evidences, the following questions can be raised: is the presence of EFNs a plesiomorphic character in the family? Did the evolution of this structure take place along the diversification of the family? The answers to these questions are being investigated by our group.

The occurrence of stem EFNs recorded in this work for different species of the Gentianaceae and its relation with their pattern of geographical distribution, along with

recent data on leaf EFNs, opens perspectives for future works with multidisciplinary approaches, such as functional anatomy, ecology, taxonomy and phylogeny.

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Figures

Fig 1. Stem extrafloral nectaries (EFNs) of Gentianaceae species in transverse section. **a** *Tetrapollinia caerulescens*. **b** *Calolisianthus pedunculatus*. **c** *Chelonanthus grandiflorus*. **d** *Neurotheca loeselioides.* **e** *Symphyllophytton caprifolioides*. **f** *Schultesia pachyphylla*. Black arrows indicate EFNs, white arrows indicate central cells. Scale bars: 50 μm

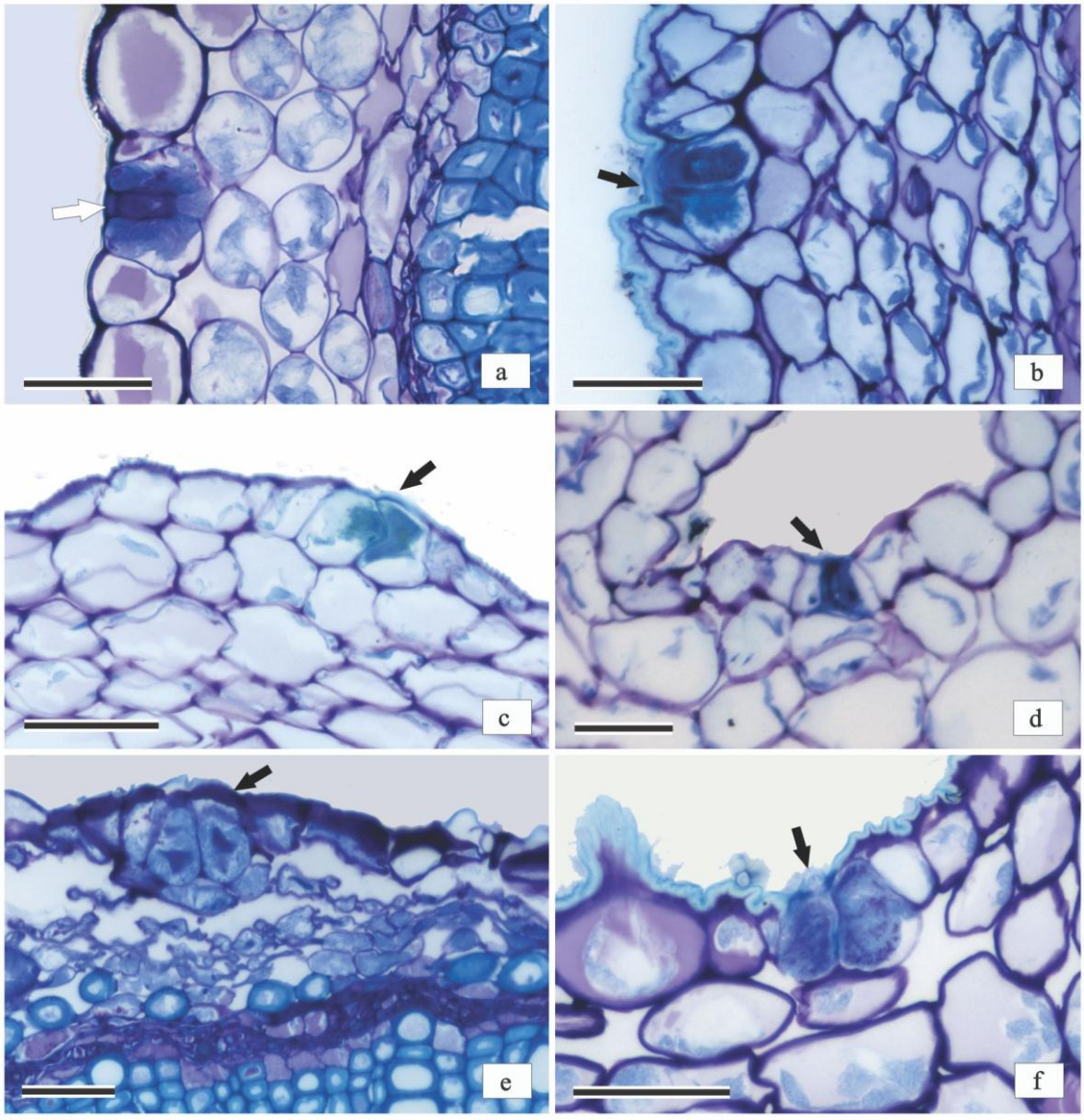


Table 1: Sampled Gentianaceae species, organized by tribes, with their collectors (herbaria), collection sites and geographical distribution of tribes, subtribes and species (literature)

Tribe/subtribe/species	Collector and herbarium	Local collection	Geographical distribution (TRIBE and species)
CHIRONIEAE - Subtribe Canscorinae			PALEOTROPICAL (Struwe et al. 2002)
Canscora diffusa (Vahl) R. Br. ex Roem. & Schult.	D.A.Nangoma and K.Kaunda 201 (NYBG) NR (NYBG) Koyama et al. s.n. (NYBG)	Malawi, Africa Cameroon, Africa Doi Inthanon, Thailand	Asia, India, Africa, Madagascar and Australia (Thiv and Kadeiret 2002)
CHIRONIEAE - Subtribe Chironiinae			PANTROPICAL TO TEMPERATE (Struwe et al., 2002)
Blackstonia perfoliata (L.) Huds. subsp grandiflora	J.Lewalle 8763 (NYBG)	Tétouan, Maroc	Mediterranean (Mansion and Struwe 2004)
Centaurium erythraea Rafn	I.Cordeiro et al. 2993 (SP) H.Luedrewaldt 51 (SP) R.Kral 75992 (SP)	Rio de Janeiro, Brazil Rio de Janeiro, Brazil São Paulo, Brazil	Mediterranean (Mansion and Struwe 2004)
Centaurium maritimum (L.) Fritsch	Pajarón 53 (NYBG) Pajarón 53 (MBM)	Los Barrios, Spain Los Barrios, Spain	Mediterranean (Mansion and Struwe 2004)
Centaurium pulchellum (Sw.) Druce	A.W.Cusick 1133 (NYBG) A.W.Cusick 34481 (NYBG) W.D.Longbotton 13784 (NYBG)	Ohio, USA Ohio, USA Maryland, USA	Mediterranean (Mansion and Struwe 2004)
Chironia baccifera L.	H.J.T.Venter 10676 (NYBG) R.Brand et al. 175 (NYBG) s.c. (NYBG)	South Africa, Africa South Africa, Africa South Africa, Africa	Southern Africa (Mansion and Struwe 2004); Africa and Madagascar (Gentian Research Network 2013)
Cicendia filiformis Delarbre	J.Stefani s.n. (US) P.Aellen s.n. (US) J.Stefani s.n. (SP)	NR Solenzara, France NR	Mediterranean and Western Europe (Struwe et al. 2002)
Cicendia quadrangularis (Lam.) Griseb.	H.W.Camp 3516 (NYBG) B.Ertter et al. s.n. (NYBG) s.c. (NYBG)	Chimborazo, Ecuador California, USA California, USA	Mediterranean (Mansion and Struwe 2004); disjunct distribution in Southern and Western North America; and South America, from Ecuador to Argentina (Struwe et al. 2002)
Gyrandra tenuiflora (Martens & Galeotti) Mansion	A.Neil and B.R.Harriman s.n. (NYBG) T.Gviniashvili et al. 464 (NYBG)	Ozaukee, USA Georgia, USA	Mountains of Western Mexico (Mansion 2004)
Sabatia angularis (L.) Pursh	J.B.Nelson and A.Aurich 16714 (NYBG)	South Carolina, USA	USA (Gentian Research Network 2013)

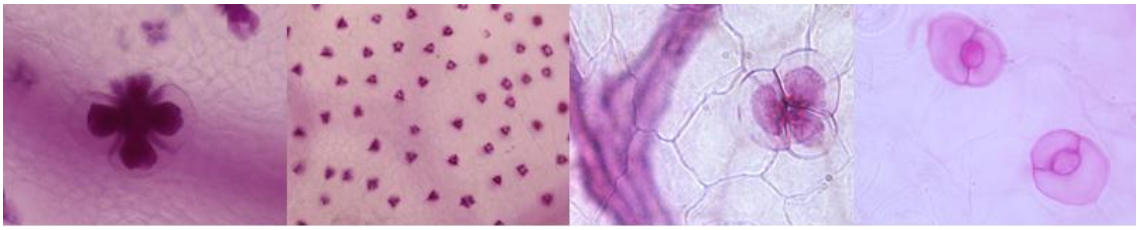
	F.W.H. 10462 (NYBG)	North Carolina, USA	
	R.Dale Thomas 124348 (NYBG)	Louisiana, USA	
Sabatia campestris Nutt.	G.L.Webster and R.L.Wilbur 3261 (NYBG)	Louisiana, USA	USA (Gentian Research Network 2013)
	R.Dale Thomas 100115 (NYBG)	Louisiana, USA	
	R.Dale Thomas and C. Amazon (NYBG)	Louisiana, USA	
Schenkia spicata (L.) Mansion	J.Risler and R.A.Kerrigan 403 (NYBG)	Alroy Downs, Australia	Mediterranean (Mansion and Struwe 2004);
	D.E.Symon 15270 (NYBG)	South Australia, Australia	Western Europe to Eastern Asia and North
	H.P. Vonow 911 (NYBG)	South Australia, Australia	Africa; introduced in North America
			(Mansion 2004)
Zeltnera stricta (Schiede) Mansion	C.R.Broome and R.M. Lloyd 634 (NYBG)	Mexico	Endemic to South and Central Mexico
	C.R.Broome 746 (NYBG)	Mexico	(Mansion 2004)
	C.R.Broome and R.M. Lloyd 620 (NYBG)	Mexico	
Zygostigma australe (Cham. & Schltdl.) Griseb.	G.Hatschbach et al. 71812 (MBM)	Paraná, Brazil	Brazil, Argentina and Uruguay (Struwe et al.
	A.Usteri s.n. (SP)	São Paulo, Brazil	2002)
	F.C.Hoehne s.n. (SP)	São Paulo, Brazil	
CHIRONIEAE - Subtribe Coutoubeinae			NEOTROPICAL (Struwe et al. 2002)
Schultesia pachyphylla Griseb.	J.R.Pirani et al. 5386 (SPF)	Bahia, Brazil	Brazil (Guimarães 2002)
	V.C.Dalvi et al. 51 (VIC)	Bahia, Brazil	
	V.C.Dalvi et al. 74 (VIC)	Bahia, Brazil	
Symphyllophyton caprifolioides Gilg	J.A.Ratter et al. 6742 (INPA)	Maranhão, Brazil	South America (Mansion and Struwe 2004);
	I.Gottesberg and G.Gottesberg s.n. (SP)	Goiás, Brazil	and Brazil (Guimarães and Saavedra 2013)
	J.A.Ratter et al. s.n. (MBM)	Maranhão, Brazil	
Xestaea lisianthoides Griseb.	P.H.Gentle 9052 (US)	Cayo, Honduras	Central and South America (Struwe et al.
	P.C.Standley 30379 (US)	France Field, Panama	2002)
	E.P.Killip 3362 (US)	Panama	
GENTIANEAE - Subtribe Gentianinae			TEMPERATE-ALPINE (Struwe et al. 2002)
Gentiana sedifolia H. B. K.	L.B.Holm-Nielsen and J. Jamarilho s.n. (US)	Tungurahua, Ecuador	*Asia, Europe, North and South America,
	P. Acevedo-Rodzg s.n. (US)	Cochabamba, Bolivia	Northwest Africa, and East Australia
	J.L. Clark 719 (US)	Tungurahua, Ecuador	(Struwe et al. 2002)
GENTIANEAE - Subtribe Swertiinae			TEMPERATE-ALPINE (Struwe et al. 2002)
Bartonia paniculata (Michx.) Muhl.	L.K. Magrath 17310 (NYBG)	Oklahoma, USA	*North America, from Texas and Florida to
	H.E. Ahles 36920 (NYBG)	North Carolina, USA	Newfoundland (Struwe et al. 2002)
Bartonia virginica (L.) Britton, Sterns & Poggenb	M.L. Fernald s.n. (NYBG)	Nova Scotia, Canada	*North America, from Texas and Florida to Newfoundland (Struwe et al. 2002)
	R.C.Bean and D. White s.n. (NYBG)	Nova Scotia, Canada	
	M.L. Fernald and B. Long s.n. (NYBG)	Nova Scotia, Canada	
Gentianella amarella (L.) Börner	C.G. Alm s.n. (NYBG)	Torneträsk, Sweden	Europe and North America (Gentian
	N.Jacobsen and J. Suedsen s.n. (NYBG)	NR	Research Network 2013)

	Harry Smith s.n. (NYBG)	Sweden	
Halenia corniculata (L.) Cornaz	H.H.Iltis et al. 636 (NYBG)	Siberia	Asia (von Hagen 2003)
	N.Naruhashi s.n. (NYBG)	Hokkaido, Japan	
	H.H.Iltis et al. 873 (NYBG)	Lake Baikal, Siberia	
Halenia palmeri A. Gray	Rogers McVaugh 21741 (NYBG)	Durango, Mexico	Mexico (von Hagen 2003)
	N/H. Holmgren and T. K. Lowrey 8073 (NYBG)	Durango, Mexico	
	F.W.Pennell s.n. (NYBG)	Durango, Mexico	
Lomatogonium carinthiacum (Wulf.) Rchb.	V.Zuer (NYBG)	Altai, Russia	*North America, temperate Asia, and Europe (Struwe et al. 2002)
	Otar Abdalazed et al. 686 (NYBG)	Kazbegi, Georgia	
	G.Nakahutsrishvili and O.Abdalazed 160 (NYBG)	Kazbegi, Georgia	
HELIEAE			NEOTROPICAL (Struwe et al. 2002)
Adenolisianthus arboreus Gilg	J.J.Wurdack and L.S.Adderley s.n. (NYBG)	Amazonas, Brazil	South America (Lepis 2009)
	B.G.S.Ribeiro 1060 (RB)	Amazonas, Brazil	
Calolisianthus pedunculatus (Cham. & Schltdl.) Gilg	V.C.Dalvi et al. 98 (VIC)	Minas Gerais, Brazil	Brazil (Calió 2009)
	V.C.Dalvi et al. 102 (VIC)	Minas Gerais, Brazil	
	V.C.Dalvi et al. 109 (VIC)	Minas Gerais, Brazil	
Calolisianthus speciosus Gilg	G.Valente et al. 1969(VIC)	Minas Gerais, Brazil	Brazil (Calió 2009)
	G.Valente et al. 1941 (VIC)	Minas Gerais, Brazil	
	G.Valente et al. 2237 (VIC)	Minas Gerais, Brazil	
Chelonanthus alatus (Aubl.) Pulle	C.Todzia et al. 2213(INPA)	Amazonas, Brazil	Central and South America (Lepis 2009)
	E.Brocki 14 (INPA)	Amazonas, Brazil	
	M.Groppo Jr. et al. 956 (INPA)	Amazonas, Brazil	
Chelonanthus albus (Spruce ex Progel) Badillo	F.E.Miranda and M.C.C. Miranda 829 (INPA)	Amazonas, Brazil	Central and South America (Lepis 2009)
	J.Chagas s.n. (INPA)	Amazonas, Brazil	
	W.Rodrigues and J. Chagas 4503 (INPA)	Amazonas, Brazil	
	P.J.Maas et al s.n. (NYBG)	Amazonas, Brazil	
Chelonanthus grandiflorus (Aubl.) Chodat & E. Hassl.	M.F.Silva et al. 618 (INPA)	Amazonas, Brazil	Central and South America (Lepis,2009)
	L.A.Maia et al 403 (INPA)	Amazonas, Brazil	
	D.W.Stevenson et al. s./n. (INPA)	Amazonas, Brazil	
Chelonanthus purpurascens (Aubl.) Struwe, S. Nilsson & V.A. Albert	V.C.Dalvi et al. 34 (VIC)	Bahia, Brazil	South America (Lepis 2009)
	V.C.Dalvi et al. 52 (VIC)	Bahia, Brazil	
	V.C.Dalvi et al. 61 (VIC)	Bahia, Brazil	

Chelonanthus viridiflorus (Mart.) Gilg	J. A. Ratter et al. s.n. (INPA)	Roraima, Brazil	South America (Lepis 2009)
	V.CDalvi and D,M,T,F.Francino 03 (VIC)	Minas Gerais, Brazil	
	V.C.Dalvi and D.M.T.F.Francino 12 (VIC)	Minas Gerais, Brazil	
Helia oblongifolia Mart.	M.M.K. Carra and P. J .M. Maas (SP)	NR	Brazil and Paraguay (Calió 2009)
	M.F. Calió 205 et al. (SPF)	Minas Gerais, Brazil	
	A.C.Brade s.n. (SP)	São Paulo, Brazil	
Irlbachia nemorosa (Willd. ex Roem. & Schult.) Merr.	W.Montovani and D.M.S.Rocha s.n. (SPF)	Amazonas, Brazil	Brazil (Guimarães and Saavedra 2013)
	Fábio de Barros 947 (SP)	Amazonas, Brazil	
Tetrapollinia caerulescens (Aubl.) Maguire & B.M. Boom	R.M.Harley et al 25990 (SP)	Bahia, Brazil	Central and South America (Gentian Research Network 2013)
	M.F.Calió et al 154 (SPF)	Minas Gerais, Brazil	
	Fábio de Barros 862 (SP)	Mato Grosso do Sul, Brazil	
POTALIEAE - Subtribe Faroinae			PANTROPICAL (Struwe et al. 2002)
Neurotheca loeselioides (Spruce ex Progel) Baill.	M.N.Silva et al. 182 (INPA)	Pará, Brazil	Northern South America, tropical Africa, and Western Madagascar (Struwe et al. 2002)
	B.W.Nelson et al. 1483 (INPA)	Amazonas, Brazil	
	O.P.Monteiro and J.F.Ramos 832 (INPA)	Amazonas, Brazil	
	A.Janssen 131 (RB)	Amazonas, Brazil	
POTALIEAE - Subtribe Potaliinae			PANTROPICAL (Struwe et al., 2002)
Potalia resinifera Mart.	N.A.Rosa et al. 5487 (SPF)	Amazonas, Brazil	Brazil (Amazonas) and Andes (Struwe and Albert 2004)
	M.Goppo et al. 882 (SPF)	Amazonas, Brazil	
	A.Henderson and F.G.Padilha 2034 (NYBG)	Loreto, Peru	
SACCIFOLIEAE			NEOTROPICAL (Struwe et al. 2002)
Voyriella parviflora Miq.	M.J.Pires and N.T.Silva 1641 (NYBG)	Pará, Brazil	South America and Panama (Struwe et al. 2002)
	E.A.Chritenson and S.R.George 1796 (NYBG)	French Guiana	
	Bassett Maguire et al. s.n. (NYBG)	Suriname	

(*) Distribution pattern of the genus. S.c.= Collector not reported. Acronyms of herbaria according to Index Herbariorum

CAPÍTULO 4



DIVERSITY AND EVOLUTION OF EXTRAFLORAL NECTARIES IN GENTIANACEAE

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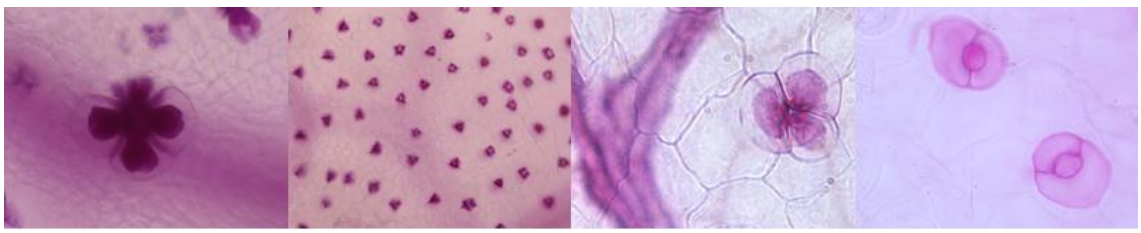

ORIGINAL ARTICLE

Diversity and evolution of extrafloral nectaries in Gentianaceae

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Abbreviated title: Extrafloral nectaries in Gentianaceae

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• *Background and Objectives* Extrafloral nectaries (EFN) have been recently reported for neotropical Gentianaceae and exhibit unusual anatomical structure. We aimed to investigate if EFNs are common in leaves of Gentianaceae species, determine if the presence/absence and topographic and anatomical diversity of EFNs are useful for taxonomy, study the evolution of EFNs in a phylogenetic context, and examine the relationship between the geographical distribution of species and the presence of EFNs.

• *Methods* The anatomical characteristics of foliar EFNs were obtained by using standard anatomy techniques. Data on the geographic distribution pattern were obtained from literature and herbarium specimens. Phylogenetic reconstruction was elaborated based on data from the Genbank. Phylogenetic analyses and divergence time estimates were conducted. For reconstructions of the ancestral state, parsimony (Mesquite), BBM and S-DIVA (RASP) analyses were used.

• *Key Results* Foliar EFNs occurred in 92 of 179 species and 34 of 69 genera. They are epidermal, avascularized structures, occurring scattered throughout the leaf blade and/or in aggregates. They are present in Saccifolieae, Helieae and Voyrieae, partially present in Chironieae and Potalieae, and absent in Gentianeae and Exaceae. The divergence time analysis places the most recent common ancestor of the Gentianaceae in the Late Cretaceous, about 70.4 Mya. The presence of EFNs is reconstructed as the ancestral condition for Gentianaceae. Losses of EFNs occurred in three independent lineages (Exaceae, Chironieae and Gentianeae). The Neotropics were reconstructed as the ancestral area for Gentianaceae, Saccifolieae, Chironieae, Potalieae, Helieae, and Voyrieae.

• *Conclusions* The diversity and evolution of EFNs in Gentianaceae, addressed for the first time in a phylogenetic context, demonstrates that the presence of EFNs is a ancestral condition in the family. Characteristics of EFNs in particular lineages assist

the delineation and characterization of groups. The geographical distribution pattern of the species was correlated with the EFNs and the occurrence of ants. The importance of EFNs in the evolution and diversification of Gentianaceae should be considered.

Keywords: Chironieae, Exaceae, extrafloral nectaries, foliar anatomy, Gentianeae, Gentianaceae, Helieae, nectarioles, Potalieae, Saccifolieae, secretory structures, Voyrieae

INTRODUCTION

Extrafloral nectaries (EFNs) are nectar glands with extremely diverse morphology, anatomy, and location in the plant body (Zimmerman, 1932; Elias, 1983; Schmid 1988; Marazzi *et al*., 2013*a, b*). EFNs have been observed in 3,941 species belonging to 745 genera and 108 families of vascular plants, and are registered in at least 457 different phylogenetic lineages (Weber and Keller, 2013). This data indicates that EFNs represent a fundamental innovation for the diversification of certain plant groups (Marazzi *et al*., 2006; Marazzi and Sanderson, 2010). The high phenotypic diversity of EFNs and their occurrence in different groups has intrigued scientists for centuries and they have been interpreted by some authors as a result of convergent or parallel evolution selected in response to similar ecological factors (Rico-Gray and Oliveira, 2007).

The extrafloral nectar is rich in sugars, amino acids, vitamins, water, and other organic compounds (Elias, 1983), making up a valuable food source for many animals (Ruhren and Handel, 1999), especially ants (Mackay and Whalen, 1998). Thus, the EFNs are responsible for establishing ecological ant-plant mutualism relationships and play a role in defense against herbivores (Koptur *et al*., 1998; Vesprini *et al.,* 2003; Heil *et al*., 2004; Oliveira and Freitas, 2004; Nascimento and Del-Claro, 2010; Marazzi *et al.*, 2013*a*). According to Nogueira *et al*. (2012*a*), evolution of EFNs and the ant-guarding system may be significantly affected by intrinsic factors such as the emergence of new morphological structures in the same organ, and extrinsic factors such as biotic and abiotic changes associated with geographical distribution of the plants.

Regarding the geographical distribution, although EFNs are reported for plants in temperate regions, they are more common in species of tropical and subtropical regions (Bentley, 1977; Koptur, 1992; Sttot and Pemberton, 1998; Oliveira *et al.,* 1999; Pacini and Nicolson, 2007). Correlations between the presence of EFNs and geographic

distribution of species within the same taxonomic group are scarce (Gómes-Acevedo *et al.,* 2010; Dalvi *et al*., 2013). On the other hand, numerous studies have been performed considering taxa with EFNs in different habitats (Bentley, 1976; Keeler 1979*a*, *b*; Keeler 1980; Oliveira and Leitão-Filho, 1987; Pemberton, 1988; Pemberton, 1990; Oliveira and Oliveira-Filho, 1991; Koptur, 1992; Morellato and Oliveira, 1991; Sugiura *et al*., 2010) or when analyzing the diversity of EFNs in plants of the same taxonomic group (Melo *et al*., 2010).

Morphological studies conducted in the 1980's reported nectaries as rare structures in Loganiaceae and Apocynaceae, and absent in Gentianaceae (Elias, 1980). However, foliar EFNs were described more recently for 33 species of tropical Gentianaceae, representing 13 genera and three tribes (Delgado *et al*., 2011*a*, *b*; Dalvi *et al*., 2013, 2014). Nectaries were also observed in sepals of the species *Irlbachia* (Gentianaceae) and denominated nectarioles (Vogel, 1998), where this term was considered inadequate and the term nectary was maintained (Dalvi *et al*., 2013). Nectaries of Gentianaceae exhibit an unusual anatomical structure, are avascular and formed by a small number of modified secretory epidermal cells (Vogel, 1998; Delgado *et al*., 2011*a;* Dalvi *et al*., 2013, 2014) with a central cell by where the secretion is eliminated (Dalvi *et al*., 2013). The EFNs occur in isolated and scattered units along the leaf blade and/or in aggregated/concentrated units (Delgado *et al*., 2011*a*; Dalvi *et al*., 2013, 2014).

The Gentianaceae family consists of seven monophyletic tribes: Chironieae, Exaceae, Gentianeae, Helieae, Potalieae, Saccifolieae, and Voyrieae (Struwe *et al*., 2002, Merckx *et al*. 2013), with 91 genera and nearly 1700 species (Gentian Research Network, 2014).

Although of cosmopolitan distribution, most species of Gentianaceae (over 50%) are concentrated in temperate regions with representatives from four of the five major genera: *Gentiana*, *Gentianella*, *Halenia* and *Swertia* (Albert and Struwe, 2002). On the other hand, those of the Neotropics comprise greater phylogenetic and taxonomic diversity, including 47 genera corresponding to more than half the genera of the family (54%). Merckx *et al*. (2013) highlighted the importance of the Neotropical species for the diversification of Gentianaceae, indicating that the family has a tropical origin, and temperate northern lineages are the result of secondary radiations during evolution of the group.

In the Neotropics 77% of genera are endemic, but tropical Africa (with 52%) and Asia (with 44%) are also considered generic endemism centers (Albert and Struwe, 2002). This high endemism may explain the wide variation in habit, morphology and anatomy of the Gentianaceae species (Struwe *et al*. 2002).

Morphologically, the only unique characteristic of Gentianaceae among the Gentianales is the parietal placentation (Albert and Struwe, 2002). The other characteristics, although useful for recognition of the family, are shared with related groups at different hierarchical levels (Struwe *et al*., 2002; Calió, 2009; Judd *et al.*, 2009). Some of the tribes and/or subtribes did not show morphological synapomorphies, but only molecular synapomorphies, and the monophyly of the genera and limits of the species still need to be elucidated (Albert and Struwe, 2002).

Given the wide geographic distribution of Gentianaceae and new records of the presence of EFNs on leaves of Neotropical representatives, this study aims to: (1) investigate if the EFNs are common structures on leaves of Gentianaceae species, (2) evaluate if the presence/absence and topographic and anatomical diversity of EFNs are useful for the taxonomy of Gentianaceae, (3) study the evolution of EFNs within the

family in a phylogenetic context, and (4) examine the relationship between geographical distribution of the Gentianaceae species and the presence of foliar EFNs.

MATERIAL AND METHODS

Taxon sampling and plant material. Several DNA markers have been used for phylogenetic reconstruction within Gentianaceae. The most comprehensive phylogenetic study on the family level is that of Struwe *et al*. (2002), in which molecular data from *trnL* intron and *matK* were used. Another region that has been extensively employed is the internal transcribed spacer region (ITS) of nuclear ribosomal DNA (e.g., Frasier *et al*., 2008, Mansion and Struwe, 2004; Struwe *et al*., 2009; Yuan and Küpfer, 1995). Therefore, we delineated our sampling for the phylogenetic analyses based on the availability in the Genbank of sequences for these three regions. In most cases, we included species with sequences for at least two of these regions, but species with sequence for only one of the regions were also included. Three species of Apocynaceae, Loganiaceae and Rubiaceae (all members of Gentianales) were used as outgroup taxa. In the end, the molecular dataset comprised 124 taxa, with 121 belonging to Gentianaceae; these encompass 59 genera, and represent all seven tribes currently recognized in the family. Genbank accession numbers are listed in the Supplementary Information Appendix 1.

To verify the occurrence and anatomical characteristics of foliar EFNs in Gentianaceae, material collected in the field and samples from herbarium specimens of national and international herbaria were used. Field expeditions were performed in the states of Amazonas, Minas Gerais and Bahia (Brazil) and leaf samples collected in the field were fixed for anatomical analyzes. Specimens were dried and pressed, and

deposited in the VIC Herbarium at the Universidade Federal de Viçosa (UFV), Minas Gerais, Brazil (Supplementary Information Appendix 2).

Data available in literature on the presence foliar EFNs in Gentianaceae (Dalvi *et al*. 2013, 2014) was used. Altogether, the presence of foliar EFNs was investigated in 179 species, comprising 121 species used in phylogenetic reconstruction and 58 others. For most species an average of three individuals were analyzed, totaling 679 specimens. This sample included 69 genera, eight subtribes and seven tribes, as well as three species used as the outgroup (Supplementary Information Appendix 1).

Sequence alignment. Sequences were pre-aligned with the Muscle alignment tool (Edgar, 2004) implemented in Geneious Pro v.6.1.6 (Biomatters) using default parameters, and subsequently the alignment was manually adjusted according to Simmons (2004). A few short regions of the *trnL* spacer and ITS could not be unambiguously aligned, therefore they were excluded from the final matrices.

Phylogenetic analyses and divergence time estimation. jModeltest v.0.1.1 (Posada, 2008) was used to select the substitution model for each data matrix using the Akaike Information Criterion. The best-fitting model for *trnL* and *matK* datasets was GTR+G, and for ITS it was GTR+I+G. Bayesian phylogenetic reconstructions were performed in MrBayes v.3.2.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) for the individual and for the combined molecular datasets. Bayesian runs consisted of ten million generations for two independent runs with four chains of Markov chain Monte Carlo (MCMC) each, sampling trees at every 1000th generation. Chain convergence (Effective Sample Size – ESS values >200) was checked using the likelihood plots for each run using Tracer v.1.5 (Rambaut and Drummond, 2009). The Potential Scale Reduction Factor was also used to check chain convergence and burn-in; values close to one indicate good convergence between runs (Gelman and Rubin, 1992).

The first 2500 sampled trees of each analysis were regarded as 'burn in' and discarded. A 50% majority rule consensus tree was then calculated using the remaining 14000 trees.

Using all datasets, we performed a Bayesian divergence time estimation in BEAST v.1.7.5 (Drummond *et al*., 2012), constraining the topology to reflect tribes and subtribes as they are currently circumscribed (e.g., Struwe *et al*., 2002). Furthermore, we constrained Voyrieae to be the third diverging lineage, according to results obtained by Merckx *et al*. (2013). The molecular data was partitioned by region, each analyzed with its own evolution model (the same used in the Bayesian analyses). The analysis was run using an uncorrelated lognormal relaxed molecular clock model, a Birth-death speciation model (Gernhard, 2008) with random starting tree, and the default operator. The procedure presented by Merckx *et al*. (2013) was followed for setting up the calibration priors. The first calibration point is the age of the Gentianales crown group that was set to 79 ± 10 million years ago (Mya) based on the molecular dating analysis of Janssens *et al*. (2009); these were set as the root of the tree (i.e., the tree model root height). The second point reflects the *Lisianthius* fossil pollen data from the Late Eocene (Graham, 1984; Yuan *et al*., 2005; Favre *et al*., 2010); hence, a normal distribution of 37 with standard deviation of 1 was applied to the crown node of Potalieae. The third calibration point reflects the age estimated for the crown node of the subtribe Swertiinae; we set a normal distribution of 15 ± 1 Mya for this node. These three nodes used for calibration were also constrained to be monophyletic. The Markov chains were run for 50 million generations sampling every 1000th generation. After verifying that effective sample sizes for all parameters exceeded 200, and that chains had reached stationarity in Tracer v1.5, we used TreeAnnotator v.1.7.5 to remove 10% of the trees as burn-in, and to estimate median node height and the 95% highest

posterior density (HPD) using the remaining 45000 trees. All analyses were carried out on the CIPRES Science Gateway (Miller *et al.,* 2010).

***Anatomy*.** Fully expanded leaves, whole or subdivided, were collected in the field and fixed in FAA (formaldehyde, acetic acid and 50% ethanol 1:1:18 v/v) for about 24 to 48 hours (Johansen, 1940). The herbarium samples obtained were submitted to the reverse herborization process (Smith and Smith, 1942). Thereafter, both materials were dehydrated in an ethanol series and stored in 70 % ethanol (Johansen, 1940).

To analyze the presence and distribution pattern of EFNs, leaves were subjected to the diafanization process using a 10% sodium hydroxide solution for about 2 hours, and 20% sodium hypochlorite solution for a variable time depending on the species (Johansen, 1940). The material was washed with distilled water, dehydrated in ethanol series, stained with 0.001% basic alcoholic fuchsin, hydrated and mounted in glycerine jelly. The slides were sealed with clear enamel.

After analysis of the clarified leaves, the species presenting EFNs were selected for inclusion of samples in historesin (Historesin, Leica Instruments, Heidelberg, Germany). Cross sections (middle region, base and apex of the leaf) were obtained using an automatic advance microtome (model RM 2155, Leica) equipped with glass knives. The sections were stained with toluidine blue pH 4.7 (O'Brien *et al.*, 1965) and mounted in synthetic resin (Permount-Fisher).

Analysis of the material and image capture were performed under an optical microscope (model Olympus AX70TRF, Olympus Optical, Tokyo, Japan) with U-Photo system and coupled digital camera (AxioCam HRc; Zeiss, Göttingen, Germany), in the Laboratory of Plant Anatomy, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil.

Character coding. We began the investigation on evolution of EFNs by assembling a matrix containing our findings of two characters: (1) absence/presence of EFNs, and (2) species geographic distribution (Table 1). Based on our observations, anatomical characters were coded as follows: absence/presence of EFNs: 0/A absent, 1/B present; occurrence of EFNs: 0/A EFNs absent, 1/B present in isolated and scattered units interspersed with the stomata, 2/C present in isolated and scattered units interspersed with the stomata and sometimes in aggregates located on the leaf base, sometimes on the apex or in both regions. For the purpose of optimizing this trait, in species where the occurrence of this structure was not constant in all specimens/leaf samples, but was present in at least one of the materials analyzed, the state was considered as present. Data regarding geographic distribution of species was mostly derived from literature, but also from herbaria material. The geographic distribution has been coded using major geographic units as follows: 0/A Neotropics, 1/B Neoartics, 2/C Paleartics, 3/D Tropical Africa, 4/E Orient, end 5/F Australian. Widespread species were coded as present in multiple regions, and only the natural distributions were taken into account (Table 1).

Ancestral character state reconstructions. To reconstruct anatomical ancestral states we used parsimony and Bayesian binary Markov chain Monte Carlo (MCMC) (BBM; Yu *et al.*, 2012). To reconstruct the ancestral geographic distribution we also performed statistical dispersal and vicariance analysis S-DIVA (Yu *et al.*, 2010). All analyses were performed using the maximum clade credibility tree and a random subset of 1000 post-burn-in trees from the combined BEAST runs. We used the program BayesRate v. 1.5 (Silvestro and Schnitzler, 2011, Silvestro *et al*., 2011) to subsample the trees and to trim the three outgroups before performing the reconstruction analyses.

Parsimony reconstructions were carried out in Mesquite v. 2·75 (Maddison and Maddison, 2011), under the 'unordered' model of character evolution. We optimized

character states on the 1000 trees randomly sampled from the combined BEAST runs, summarizing the ancestral states on the maximum clade credibility tree.

The resulting ancestral-state reconstructions were visually displayed by color-coding the branches of the maximum clade credibility tree. S-DIVA and BBM analyses were performed in RASP (Yu *et al*., 2012) using default parameters, except for the BBM runs that were set to 10 million generations, sampling at each 1000th generation, and with burn-in of 1000. For both analyses, we assumed a null distribution for the outgroup.

RESULTS

Phylogeny reconstruction and time estimation. The alignment lengths of the individual partitions were 695 (*trnL*), 743 (*matK*), and 728 (ITS) characters, resulting in a combined dataset measuring 2166 characters long. The Bayesian trees resulting from analysis of individual and combined datasets are shown in Supplementary Information Figs S1, S2, S3 and S4. The Bayesian Inference analysis using all three datasets combined resulted in a moderately well-resolved phylogeny. Due to the positioning of a few taxa, the family, some tribes and subtribes were not recovered as monophyletic groups, conflicting with previously established relationships (Struwe *et al.* 2002, 2009, Merckx *et al*. 2013). Specifically, the two species of Voyrieae included in this study were placed in a polytomy at the base of Gentianaceae (along with one of the outgroups), *Bisgoeppertia scandens* formed a clade with *Lisianthius jefensis* and *L. laxiflorus*, *Prepusa montana* emerged in a polytomy along with the clade corresponding to Potalieae and another clade with Gentianeae and Helieae. In most of these cases, relationships were not well-supported. Furthermore, the positioning of these particular taxa relied only on the information of a single dataset. Based on that we constrained

nodes for obtaining a topology congruent with previously established circumscriptions and relationships.

The divergence time analysis (Fig. 1; Table 2) places the most recent common ancestor (MRCA) of Gentianaceae in the Late Cretaceous, about 70.4 Mya. The estimated crown ages for the tribes Saccifolieae, Chironieae, and Potalieae vary between 36.4 and 36.2 Mya, suggesting that the origin of these groups would have occurred during the Late Eocene. An origin during the Early Oligocene was estimated for the MRCA of Gentianeae and Helieae (crown nodes of 30.7 and 29.4 Mya, respectively), whereas a more recent origin (Early Miocene) was estimated for Exaceae and Voyrieae (23.2 and 17.6 Mya, respectively).

Characterization of the foliar EFNs in Gentianaceae. In all species studied the EFNs are epidermal structures of diminutive size, in some cases resembling the stomata (Fig. 2). They are avascularized and generally formed by three radiated secretory cells (Fig. 2A-E) surrounding a center cell; however, variations of two (Fig. 2F), four or five (Fig. 2G) or approximately eight radiated cells (Fig. 2H) are also found. The radiated secretory cells are bulky, with pyriform shape, conspicuous nucleus and labyrinthine wall intensely stained by toluidine blue and/or basic fuchsin (Fig. 2I, J).

Occurrence and distribution pattern of foliar EFNs in different taxa of Gentianaceae. Nectaries were observed in 92 of the 179 species of Gentianaceae analyzed and in 34 of the 69 genera (Table 1). For all genera the presence/absence of EFNs was constant (Table 1) except for *Deianira* (present in *D. damazioi* and absent in *D. pallescens*).

EFNs are present on all species of the tribes Saccifolieae, Helieae and Voyrieae, and absent in all representatives of the tribes Gentianeae and Exaceae. In the tribes Chironieae and Potalieae, the presence/absence of EFNs was not constant (Table 1). In Chironieae EFNs were found only in representatives of the subtribe Coutoubeinae,

including species of the genera *Coutoubea*, *Deianira, Symphyllophytton*, *Schultesia* and *Xestea*, not being registered for *Deianira pallescens.* For *Schultesia guianensis* the pattern also did not remain constant since some specimens possessed nectaries while in others they were absent. In Potalieae, nectaries were observed in the three subtribes (Faroinae, Lisianthiinae and Potaliinae) (Table 1). All species of Lisianthiinae and Potaliinae presented EFNs as well as *Neurotheca loeselioides*, belonging to the subtribe Faroinae. In the other species of Faroinae included in this study the EFNs were absent (Table 1).

In relation to distribution along the leaf blade, the EFNs occur in two different forms/patterns: (I) isolated and scattered along the blade, interspersed with stomata, visible only in microscopy (Fig. 2) and (II) in aggregates/concentrated forming conspicuous or little conspicuous structures, generally visible to the naked eye and associated with the presence of isolated and scattered nectaries throughout the leaf surface. In the first case (I), EFNs can occur on both surfaces or restricted to the abaxial surface of the blade, never occurring on the adaxial surface. In the second case (II) they may be present in pairs on the leaf base (Fig. 3), or alone on the leaf apex (Fig. 4), or in both regions (at the base and apex), always restricted to the abaxial surface of the blade. In the field, nectaries in aggregates/concentrated can be observed in some species as areas of different colored materials, while in herbalized material this feature can hardly be detected. In most species, even when occurring in clusters, nectaries are only identified under a microscope.

The occurrence of EFNs in isolated and scattered units along the leaf blade or in aggregated/concentrated units remained constant within most groups.

In all species of the tribes Saccifoliaeae, Potalieae, Chironieae and Voyrieae the nectaries occur only as isolated and scattered along the leaf blade (Table 1). A single

exception was observed in the species *Deianira damazioi* (Chironieae), which showed no isolated EFNs, only aggregates/concentrated EFNs at the leaf base (Table 1). On the other hand almost all species of the tribe Helieae present aggregated/concentrated EFNs as well as isolated and scattered EFNs along the leaf blade (Table 1). The only exception was within the genre *Celiantha* in which three species showed no aggregated/concentrated EFNs.

Despite the occurrence of EFNs in aggregates/concentrated to be common for species of Helieae, this characteristic presented some variations within the tribe. In most species the nectaries consisted of two well-defined nectariferous areas on the leaf base visualized by microscopy, in clarified samples near the midrib, as observed in *Chelonanthus albus* (Fig. 3A, B), *Chelonanthus angustifolius* (Fig. 3C, E), *Chelonanthus viridiflorus* (Fig. 3D), *Chelonanthus grandiflorus* (Fig. 3E) and/or in the region near the leaf apex; also near the midrib a single gland area may be formed, as observed in *Neblinantha parviflora* (Fig. 4A), *Adenolisianthus arboreus* (Fig. 4B, C), *Chelonanthus angustifolius* (Fig. 4D, E), and *Irlbachia nemorosa* (Fig. 4F). In some cases, in these areas there is a large number of nectaries very close to each other and there are no interspersed stomata (Fig. 3A, B). On the other hand, in other species of the tribe the delimitation aggregated/concentrated EFNs are not so evident and/or the presence of this structure is not constant in all specimens or on all leaves of the same specimen examined, as occurs in *Aripuana cullmaniurum*, *Celiantha imthurniana*, *Chorisepalum ovatum, C. psychotrioides*, *C. sipapoanum*, *Irbachia pratensis*, *Lehmaniella splendens*, *Macrocarpaea angelliae*, *M. arborescens*, *M. dominguensis*, *M. valerii*, *Neblinantha neblinae*, *Prepusa alata, P. connata, P. hookeriana, P. montana*, *P. viridiflora*, *Roraimaea aurantiaca*, *Senaea caerulea*, *S. janeirensis*, *Tachia guianensis*, *T. loretensis*, *T. occidentalis*, *T. parviflora* and *Tetrapollinia caerulescens.* Another

variation observed was in relation to the aggregated/concentrated EFNs in the apical portion of the leaf. These, when present, are usually located near the midrib in the vertical direction of the leaf (Fig. 4), however, in *Macrocarpaea arborescens*, *M. dominguensis, Prepusa montana* and *Tachia parviflora* the EFNs are in the horizontally most distant region from the leaf apex.

Ancestral character state reconstructions. *Presence of foliar EFNs.* Ancestral character state reconstructions based on BBM and parsimony are depicted in Figs 5 and Supplementary Information Fig. S5. A summary of reconstructions at 21 nodes of particular interest is presented in Table 2. The ancestral state reconstructions of the parsimony and BBM analyses produced very similar results. The main difference between both analyses is that in the nodes the parsimony analysis resulted in an equivocal reconstruction, the BBM analysis reconstructed a single state with probability greater than 55% (except for node 3).

The presence of EFNs is reconstructed as the ancestral condition for Gentianaceae (Fig. 5 and Supplemetary Information Fig. S5, node 1), and also for all early diverging nodes (nodes 3, 5, 7, 12). Furthermore, the presence of EFNs is indicated as the most probable state for node 17 and the nodes that correspond to the tribes Saccifolieae, Potalieae and Helieae, the Chironieae subtribe Coutoubeinae, and Voyrieae (nodes 2, 13, 21, 10, 6, respectively). Losses of EFNs occurred in three independent lineages, corresponding to the tribes Exaceae, Chironieae and Gentianeae (nodes 4, 8 and 18).

Geographic distribution. Ancestral area reconstructions based on BBM, parsimony and S-DIVA are depicted in Figs 6 and Supplementary Information Figs S6 and S7. A summary of reconstructions at 21 nodes of particular interest is presented in Table 2. Results from the three methods are largely congruent for ancestral area reconstruction,

the exceptions being nodes with the same result for BBM and parsimony, but with larger, inclusive ancestral areas reconstructed by S-DIVA (nodes 1, 3, 9, 14, 17), and two nodes with different reconstructed patterns in each method (nodes 11 and 18).

Both BBM and parsimony reconstruct the Neotropics as the ancestral area for Gentianaceae (Figs. 5 and Supplementary Information Fig. S6, node 1), and also for all early diverging nodes (nodes 3, 5, 7, 12), whereas S-DIVA favors a broader ancestral area for the family and also for node 3 (Supplementary Information Fig. S7). The three methods indicate the Neotropics as the ancestral area for the nodes that correspond to the tribes Saccifolieae, Chironieae, Potalieae, Helieae and Voyrieae (nodes 2, 8, 13, 21, and 6, respectively). The BBM and parsimony methods reconstruct Tropical Africa as the ancestral area for Exaceae (Fig. 5 and Supplementary Information Fig. S6, node 4), while S-DIVA presents a broader ancestral area for this node (Supplementary Information Fig. S7). Lastly, the ancestral area of node 18, which corresponds to the tribe Gentianeae, is differently reconstructed in each method; the BBM favors the Neotropics as the most likely ancestral area (Fig. 5), the parsimony method results in an equivocal reconstruction (Supplementary Information Fig. S6), while the S-DIVA reconstructs three different areas in equal proportions that include the Neoartics, the Paleartics and the Orient region (Supplementary Information Fig. S7). Colonization of geographic areas other than the Neotropics took place after the Early Oligocene.

DISCUSSION

The present study included 75.8% of the genera and 100% of the tribes of Gentianaceae, accounting for 10.5% of the species of Gentianaceae and thus covering the major lineages of the family.

The presence of EFNs on the leaves of 92 species of Gentianaceae studied indicates that these structures occur not only in the family (Vogel, 1998; Delgado *et al*., 2011*a*,*b*; Dalvi *et al*., 2013, 2014), but are also relatively common and not absent as stated by Elias (1983). The importance of careful analyses that include anatomic studies for assessment of the presence or absence of EFNs in Gentianaceae is therefore evident, as highlighted by Dalvi *et al*. (2014).

Anatomically, the nectaries described for Gentianaceae are quite simple, consisting of a few secretory epidermal cells without vascularization. Avascularized nectaries formed by secretory epidermal cells have been recorded and described for other families such as Bignoniaceae (Elias and Gelband, 1976), Leguminosae (Elias, 1980; Paiva and Machado, 2006) and Convolvulaceae (Keeler and Kaul, 1979), but differ in structure from EFNs of Gentianaceae. These results indicate that this nectary is potentially unique to Gentianaceae.

The peculiar anatomy of EFNs of Gentianaceae supports the current circumscription of some groups. One example is the inclusion of the tribe Saccifolieae in Gentianaceae (Struwe *et al.,* 2002), previously considered the monotypic family Saccifoliaceae (Maguire and Pires, 1978; Struwe *et al*., 1998; 1999; Thiv *et al*., 1999). Besides *Saccifolium*, Struwe *et al.* (2002) included in this tribe the genera *Curtia, Hockinia*, *Tapeinostemom* and *Voyriella*. In the present study we verified the presence of EFNs in isolated and scattered units along the leaf blade in these four genera. Although *Saccifolium bandeirae* was not sampled in our work, the illustrations in the protologue of the species (Maguire and Pires, 1978) show nectaries similar to those of Saccifolieae species studied. Therefore, the presence and type/distribution of nectaries advocated the inclusion of Saccifoliaceae in Gentianaceae and delimitation of the tribe.

Regarding the tribe Potalieae, EFNs are present in representatives of the three subtribes, although not constant in all genera studied. EFNs of the species *Potalia* studied are slightly different from the other species studied of the same family and resemble the epidermal structures described as trichomes in the species *Anthocleista* (Wosu *et al*., 2012). The anatomical similarity of EFNs in *Anthocleista* and *Potalia* reinforces the proximity of these two genera both included in Potaliinae (Potalieae) and provides further evidence for the inclusion of Potalieae in Gentianaceae. The EFNs of the species Lisianthiinae, in turn, are similar to those found in *Neurotheca loeselioides* (Faroinae). However, the interpretation of the importance of EFNs in the delimitation and characterization of Faroinae (Potalieae) is limited since many genera of the subtribe Faroinae were not sampled in this study.

Voyria, a genus whose position was uncertain in the generic classification of Gentianaceae (Struwe *et al*., 2002), was recently reinstated as one of the tribes of the family, Voyrieae (Merck *et al*., 2013). The presence, type and distribution of EFNs in the species of Voyrieae make up new evidence backing the position of this tribe in Gentianaceae.

Due to low supporting values, Struwe *et al.* (2002) presented the subtribes Coutoubeinae and Canscorinae separately with caveats. The present study recovered the same uncertainties regarding the support of both subtribes, since the same molecular data was used to reconstruct the phylogeny of these groups. However, the presence of EFNs in practically all species of Coutoubeinae suggests that recognition of this lineage as a separate subtribe has merit from an anatomical point of view.

The presence of EFNs in all species of Helieae and Saccifolieae and the absence of species of Gentianaeae and Exaceae, for example, are anatomical attributes that aid in characterization of these groups. This data is relevant and can be included in

dichotomous keys that seek to distinguish the tribes/subtribes, similar to the key elaborated by Struwe *et al*. (2002). The addition of another morphological characteristic, in this case anatomical, to characterize and delineate major groups within Gentianaceae is of great value since there is scarcity of morphological and anatomical data for the family (Mészáros *et al*., 2002).

Our phylogenetic results generally recovered the relationships between groups already established and recognized in current classification systems of the family (Struwe *et al.,* 2002; 2009; Merckx *et al*., 2013, Gentian Research Network, 2014). Contrasting position with current classification of the tribes of Gentianaceae was obtained for the tribe Voyrieae, as well as *Bisgoesppertia scandens* and *Prepusa montana*; however, these results were based only on data from the ITS. The affinity of *Bisgoeppertia scandens* with species of *Lisianthius* and the uncertain affinity of *Prepusa montana* were also demonstrated in a recent study that employed data from the secondary structure of this molecular region in phylogenetic reconstruction (Molina and Struwe, 2009). However, because it does not include new data that might corroborate or refute the previously reconstructed hypotheses, we also chose to apply the current classification for the placement of these taxa. Regarding the tribe Voyrieae, the study of Merckx *et al*. (2013) was based on a larger sample for this particular genus, therefore their results are certainly more robust than those of the present study, which were based on data from only two species. Thus, we adopted the position of Voyrieae obtained in this other study.

Although our divergence time analysis of Gentianaceae was based on three sets of molecular data, sampling included less than 10% of all currently recognized species in the family. For this reason, and also due to the uncertainty of any divergence time estimate, dates obtained for clades should be considered with caution. The average

divergence ages obtained in our study for many nodes were very close to those presented by Merckx *et al*. (2013), but our 95% HPD intervals were generally higher. The main differences regarding ages obtained refer to the crown nodes of Gentianaceae, Saccifolieae and Helieae (approximately 10 Mya older in our estimates), as well as Exaceae and Voyrieae (10 or more Mya younger in our estimates). However, despite the limitations discussed above, we believe that our divergence time analysis of Gentianaceae provides a sufficiently robust framework for discussions regarding the evolution of EFNs in the family, since we sampled 64.8 % of the currently recognized genera and representatives of all tribes were included.

Reconstruction of the presence of EFNs in the common ancestor of Gentianaceae suggests that EFNs represent a likely synapomorphy of the group, whereas the absence of EFNs in Exaceae, Chironieae and Gentianeae corresponds to independent events of structure loss in these lineages. Regarding the biogeographical reconstructions, we found the Neotropics to be the ancestral area of the common ancestor of Gentianaceae (a result also obtained by Merckx *et al.,* 2013), as well as the majority of tribes of the family. Occupation of the extra-Neotropical areas results from members of the lineages corresponding to the tribes Exaceae, Gentianeae and Chironieae. Apparently, the occupation of these extra-Neotropical areas is related to convergent loss of EFNs; similarly, the presence of EFNs is associated with the distribution of species within the Neotropics.

Among the tribes whose species undoubtedly present EFNs, Helieae is that which presented greatest morphological and ecological diversity, comprising the second largest group in number of genera and species within the family (Albert and Struwe, 2002; Struwe *et al.,* 2009). In this particular group, which is one of the lineages most derived with the family, EFNs were observed in aggregates/concentrated, as well as

isolated and scattered EFNs. We suggest that the presence of EFNs in this pattern may be related to this diversity of the tribe. The greater the number of EFNs per unit area in specific regions, greater was the production capacity of nectar in large quantities (McDade and Turner, 1997). Abundant nectar can attract different animals, especially ants that patrol the leaves in search of food, providing protection against herbivores and pathogens (Koptur *et al*., 1998; Vesprini *et al.,* 2003; Heil *et al*., 2004; Oliveira and Freitas, 2004; Nascimento and Del-Claro, 2010; Marazzi *et al.*, 2013*a*). In fact, ants of the genera *Crematogaster*, *Camponotus*, *Brachymyrmex* and *Linepithema* were observed visiting the foliar nectaries and forming nests near to individuals of *Calolisianthus speciosus* (Delgado *et al.*, 2011*a*), a species belonging to the tribe Helieae. This species presents agglomerated/concentrated nectaries on the leaf base and apex, easily identified in the field and with apparent abundant secretion. Ants of the genus *Crematogaster* are potentially defensive against herbivore action (Baccaro *et al.,* 2010) and recruit other ants (McDade and Turner, 1997). Besides the defense function, these ants can play an essential role in plant nutrition, since ant nests near *C. speciosus* can provide additional sources of nutrients (Wagner, 1997). It is possible that the protection and additional nutrition conferred by ants visiting the nectaries has allowed that plants of this lineage occupy different environments, which may ultimately have been a stimulus for morphological diversification of Helieae.

On the other hand, EFNs are absent in species of the tribe Gentianeae, sister group of Helieae. Nogueira *et al*. (2012*a*) emphasized that extrinsic factors such as abiotic changes associated with the geographic distribution of plants may significantly affect the evolution of EFNs and the ant-guarding system. As observed in the present study, no secretory structure occurs on the leaves of Gentianeae species and the great evolutionary success of this group (ca. 58% of species from the family belong to this

tribe) is probably related to the distribution of a large portion of its species in alpine or temperate regions (Struwe *et al*., 2002). Ant diversity is lower in these regions compared with Neotropical regions (Moreau and Bell, 2013), which may be related to the absence of EFNs in these species.

The presence of EFNs in the common ancestor of Gentianaceae suggests that mutualistic association with ants must have existed since early diversification of the family. This is indeed possible since the estimated date for the common ancestor of Gentianaceae (70 Mya; 95% HPD, 83.9-56.7 Mya) coincides with the estimated data for mass diversifications among current ant groups (Moreau *et al.* 2006, 2013).

CONCLUDING REMARKS

This work is the first to present a study on the diversity and evolution of foliar EFNs from the family Gentianaceae in a phylogenetic context. Foliar EFNs represent an ancestral condition for Gentianaceae and a potential synapomorphy of the family. The presence or absence of EFNs in particular lineages within the family as well as diversity with regards to the type and arrangement of such structures is information that can assist in the delineation and characterization of groups. The geographical distribution pattern of Gentianaceae species was correlated with the presence of EFNs and also with the occurrence and interactions of EFNs with the ants. Thus, the importance of EFNs in the evolution and diversification of Gentianaceae as a whole must be considered.

SUPPLEMENTARY INFORMATION

Supplementary data is available online and includes: **Appendix 1.** Taxa and GenBank accession numbers analyzed for this study. **Appendix 2.** Listing with species of Gentianaceae used in anatomic studies with the respective numbers of collectors and

herbaria. **Fig. S1.** Phylogenetic tree of Gentianaceae derived from the Bayesian analysis of the *trnL* dataset with posterior probabilities indicated on the branches. **Fig. S2.** Phylogenetic tree of Gentianaceae derived from the Bayesian analysis of the *matK* dataset with posterior probabilities indicated on the branches. **Fig. S3.** Phylogenetic tree of Gentianaceae derived from the Bayesian analysis of the ITS dataset with posterior probabilities indicated on the branches. **Fig. S4.** Phylogenetic tree of Gentianaceae derived from the Bayesian analysis of the combined dataset with posterior probabilities indicated on the branches. **Fig. S5.** Ancestral character state reconstruction for presence of foliar EFNs based on maximum parsimony and a sample of 1000 post-burn-in BEAST trees. The character states assigned to each species are given next to the species names. Branches are colored according to the inferred state using parsimony (grey lines indicate ambiguous parsimony reconstructions). Pie charts on the nodes show the proportion of trees in the post-burn-in sample for which a particular reconstruction was recovered; black indicates that a node is absent and grey indicates that the reconstruction is equivocal in a proportion of the trees. **Fig. S6.** Ancestral character state reconstruction for geographic distribution based on maximum parsimony and a sample of 1000 post-burn-in BEAST trees. The character states assigned to each species are given next to the species names. Branches are colored according to the inferred state using parsimony (grey lines indicate ambiguous parsimony reconstructions). Pie charts on the nodes show the proportion of trees in the post-burn-in sample for which a particular reconstruction was recovered; black indicates that a node is absent and grey indicates that the reconstruction is equivocal in a proportion of the trees. **Fig. S7.** Ancestral character state reconstruction for geographic distribution based on maximum parsimony and statistical dispersal-vicariance analysis (S-DIVA). The character states assigned to each species are given next to the species names. Branches are colored

according to the parsimony reconstruction (grey lines indicate ambiguous parsimony reconstructions), and the pie charts on the nodes show the results of the S-DIVA reconstruction (areas with frequencies below 0.05 are collectively represented in black).

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LIST OF LEGENDS

Table 1. Species of Gentianaceae analyzed, organized by tribe/subtribe, characteristics of the leaf nectaries and geographical distribution pattern of the species.

Table 2. Age estimates and ancestral state reconstructions for key nodes.

Fig. 1. Maximum clade credibility tree from the BEAST analysis for Gentianaceae. The tree is a chronogram with branches proportional to time. Posterior probabilities greater than 0.5 are indicated on the branches and 95% highest posterior densities of age estimates are indicated with bars. Numbered nodes are discussed in the text.

Fig. 2. Extrafloral nectaries isolated and dispersed over the leaf blade of species of Gentianaceae. (A) *Symbolanthus brittonianus* (B) *Aripuana cullmaniorum.* (C) *Chelonanthus albus*. (D) *Chelonanthus acutangulus.* (E) *Tachia loretensis*. (F) *Curtia verticillaris*. (G) *Coutoubea ramosa.* (H) *Potalia amara*. (I) *Celiantha chimantensis*. (J) *Chelonanthus viridiflorus*. (K) *Chelonanthus albus.* Black arrows indicate nectary. Scale bars (A-H) = 100 μm and (I-K) = 50 μm.

Fig. 3. Extrafloral nectaries in aggregates at the leaf base of species of Gentianaceae. (A, B) *Chelonanthus albus.* (C) *Chelonanthus angustifolius*. (D) *Chelonanthus*

viridiflorus. (E) *Chelonanthus angustifolius*. (F) *Chelonanthus grandiflorus.* Scale bars: (A, D-F) = 500 μm and (B, C) = 100 μm.

Fig. 4. Extrafloral nectaries in aggregates at the leaf apex of species of Gentianaceae. (A) *Neblinantha parviflora*. (B, C) *Adenolisianthus arboreus.* (D, E) *Chelonanthus angustifolius*. (F) *Irlbachia nemorosa.* Scale bars: (A, B, D) = 500 μm and (C, E, F) = 100 μm.

Fig. 5. Ancestral character state reconstruction for presence of foliar EFNs based on maximum parsimony and the Bayesian binary Markov chain Monte Carlo (BBM) method. The character states assigned to each species are given next to species names. Branches are colored according to the parsimony reconstruction (grey lines indicate ambiguous parsimony reconstructions), and the pie charts on the nodes show the results of the Bayesian reconstruction (areas with frequencies below 0.05 are collectively represented in black).

Fig. 6. Ancestral character state reconstruction for geographic distribution based on maximum parsimony and the Bayesian binary Markov chain Monte Carlo (BBM) method. The character states assigned to each species are given next to species names. Branches are colored according to the parsimony reconstruction (grey lines indicate ambiguous parsimony reconstructions), and the pie charts on the nodes show the results of the Bayesian reconstruction (areas with frequencies below 0.05 are collectively represented in black).

Table 1. Species of Gentianaceae analyzed, organized by tribe/subtribe, characteristics of the leaf nectaries and geographical distribution pattern of the species.

Táxon	EFNs	Distribution pattern of the EFNs	Geographical distribution pattern of the species (References)
Chironieae - Canscorinae			
**Canscora andrographioides* Griff. ex C.B.Clarke	Absent	-	Paleartics (Struwe *et al*., 2002; Mansion and Struwe, 2004)
**Canscora diffusa* (Vahl) R.Br. ex Roem. and Schult.	Absent	-	Australian, Paleartics, Tropical Africa (Struwe *et al*., 2002; Thiv and Kadeiret, 2002; Mansion and Struwe, 2004)
**Hoppea dichotoma* Willd.	Absent	-	Orient (Struwe *et al*., 2002; Thiv and Kadeiret, 2002)
**Schinziella tetragona* (Schinz) Gilg	Absent	-	Tropical Africa ((Thiv and Kadeiret, 2002)
Chironieae - Chironiinae			
**Bisgoeppertia scandens* (Spreng.) Urb.	Absent	-	Neotropics (Liogier, 1989; Struwe *et al*., 2002)
**Blackstonia perfoliata* (L.) Huds.	Absent	-	Paleartics (Struwe *et al*., 2002; Mansion and Struwe, 2004)
**Centaurium erythraea* Rafn	Absent	-	Paleartics (Mansion and Struwe, 2004)
**Centaurium littorale* (Turner) Gilmour	Absent	-	Paleartics (Mansion and Struwe, 2004)
**Centaurium maritimum* (L.) Fritsch ex E.Jansen	Absent	-	Paleartics (Mansion and Struwe, 2004)
**Centaurium pulchellum* (Sw.) Druce	Absent	-	Paleartics (Mansion and Struwe, 2004)
**Chironia baccifera* L.	Absent	-	Tropical Africa (Mansion and Struwe, 2004)
**Cicendia filiformis* (L.) Delarbre	Absent	-	Paleartics (Mansion and Struwe, 2004)
**Cicendia quadrangularis* (Lam.) Griseb.	Absent	-	Neotropics, Paleartics, Tropical Africa (Mansion and Struwe, 2004)
**Eustoma exaltatum* (L.) Salisb. ex G.Don	Absent	-	Neotropics (Mansion and Struwe, 2004)
**Eustoma grandiflorum* (Raf.) Shinners	Absent	-	Neotropics (Mansion and Struwe, 2004)
**Ixanthus viscosus* Griseb.	Absent	-	Paleartics (Mansion and Struwe, 2004)
**Orphium frutescens* E.Mey.	Absent	-	Tropical Africa (Mansion and Struwe, 2004)
**Sabatia angularis* Pursh	Absent	-	Neoartics (voucher)
Sabatia brevifolia Raf.	Absent	-	Neoartics (voucher)
Sabatia campanulata (L.) Torr.	Absent	-	Neoartics (voucher)
Sabatia campestris Nutt.	Absent	-	Neoartics (Mansion and Struwe, 2004)
**Sabatia dodecandra* (L.) Britton, Sterns and Poggenb.	Absent	-	Neoartics (Mansion and Struwe, 2004)
**Sabatia gentianoides* Elliott	Absent	-	Neoartics (voucher)
Sabatia grandiflora (A.Gray) Small	Absent	-	Neoartics (voucher)
**Sabatia stellaris* Pursh	Absent	-	Neoartics (Mansion and Struwe, 2004)
**Schenkia spicata* (L.) G.Mans.	Absent	-	Neoartics, Paleartics (Mansion, 2004; Mansion and Struwe, 2004)

Zeltnera maryanna (B.L.Turner) G.Mans.	Absent	-	Neoartics (Mansion, 2004)
**Zeltnera stricta* (Schiede) G.Mans.	Absent	-	Neotropics (Mansion, 2004; Mansion and Struwe, 2004)
Zygostigma australe Griseb.	Absent	-	Neotropics (Struwe *et al*., 2002)
Chironieae - Coutoubeinae			
**Coutoubea ramosa* Aubl.	Present	Isolated	Neotropics (Guimaraes and Klein, 1985)
**Coutoubea spicata* Aubl.	Present	Isolated	Neotropics (Guimaraes and Klein, 1985)
#*Deianira damazioi* E.F.Guim.	Present	Aggregates	Neotropics (Guimarães, 1977)
**Deianira pallescens* Cham. and Schltdl.	Absent	-	Neotropics (Guimarães, 1977)
#*Schultesia australis* Griseb.	Present	Isolated	Neotropics (Guimarães, 2002)
#*Schultesia brachyptera* Cham.	Present	Isolated	Neotropics (Guimarães, 2002)
Schultesia doniana Progel	Present	Isolated	Neotropics (Guimarães, 2002)
**Schultesia guianensis* (Aubl.) Malme	Pres/Absent	Isolated	Neotropics (Guimarães, 2002)
Schultesia pachyphylla Griseb.	Present	Isolated	Neotropics Guimarães, 2002)
**Symphyllophytton caprifolioides* Gilg	Present	Isolated	Neotropics (Mansion and Struwe, 2004)
**Xestaea lisianthoides* Griseb.	Present	Isolated	Neotropics (Guimarães, 2002)
Exaceae			
**Exacum affine* Balf.f.	Absent	-	Paleartics (Kissling, 2007)
**Exacum macranthum* Arn.	Absent	-	Orient (Kissling, 2007)
**Exacum tetragonum* Roxb.	Absent	-	Paleartics (Kissling, 2007)
**Gentianothamnus madagascariensis* Humbert	Absent	-	Tropical Africa (Yuan *et al*., 2003)
**Sebaea sedoides* Gilg	Absent	-	Tropical Africa (Kissling, 2007)
**Tachiadenus carinatus* Griseb.	Absent	-	Tropical Africa (Yuan *et al*., 2003; Kissling, 2007)
**Tachiadenus gracilis* Griseb.	Absent	-	Tropical Africa (Yuan *et al*., 2003; Kissling, 2007)
Gentianeae - Gentianinae			
**Crawfurdia speciosa* Wall.	Absent	-	Orient (Ho and Pringle, 1995)
Crawfurdia trailliana Forrest	Absent	-	Orient (Ho and Pringle, 1995)
**Gentiana asclepiadea* L.	Absent	-	Paleartics (voucher)
Gentiana bigevolii Gray	Absent	-	Neoartics (voucher)
Gentiana campestris L.	Absent	-	Paleartics (voucher)
Gentiana catesbai Walt	Absent	-	Neoartics (voucher)
Gentiana ciliata L.	Absent	-	Paleartics (voucher)
**Gentiana cruciata* L.	Absent	-	Paleartics (Zhang et al., 2009)
Gentiana inthurniana Kern.	Absent	-	Paleartics (voucher)
**Gentiana lutea* L.	Absent	-	Paleartics (Yuan *et al*., 1996)
**Gentiana nivalis* L.	Absent	-	Paleartics (voucher)
**Gentiana pneumonanthe* L.	Absent	-	Paleartics (voucher)

**Gentianeae - Swertiinae*			
**Gentiana punctata* L.	Absent	-	Paleartics (voucher)
**Gentiana pyrenaica* L.	Absent	-	Paleartics (Yuan *et al*., 1996)
**Gentiana sedifolia* Kunth	Absent	-	Neotropics (voucher)
Gentiana spathaceae Kunth	Absent	-	Neotropics (voucher)
**Gentiana terglouensis* Hacq.	Absent	-	Neotropics (voucher)
**Gentiana verna* L.	Absent	-	Paleartics (voucher)
**Tripterospermum cordatum* (Marquand) Harry Sm.	Absent	-	Paleartics, Orient (Struwe *et al*., 2002)
**Tripterospermum taiwanense* (Masam.) Satake	Absent	-	Paleartics, Orient (Struwe *et al*., 2002)
**Bartonia paniculata* subsp. *iodandra* (B.L.Rob.) J.M.Gillett	Absent	-	Neoartics (Gillett, 1959)
**Bartonia paniculata* (Michx.) Muhl. subsp. *paniculata*	Absent	-	Neoartics (Gillett, 1959)
**Bartonia virginica* (L.) Britton, Sterns and Poggenb.	Absent	-	Neoartics (Gillett, 1959)
**Frasera parryi* Torr.	Absent	-	Neoartics (Struwe *et al*., 2002)
**Frasera tubulosa* Coville	Absent	-	Neoartics (Struwe *et al*., 2002)
**Gentianella amarella* L. (Borner)	Absent	-	Paleartics (von Hagen and Kadereit, 2001)
Gentianella cerastioides (Kunth) Fabris	Absent	-	Neotropics (von Hagen and Kadereit, 2001)
**Gentianella foliosa* (Kunth) Fabris	Absent	-	Neotropics (voucher)
Gentianella helianthemoides (Gil.) Fabris	Absent	-	Neotropics (voucher)
**Gentianella propinqua* (Richardson) J.M.Gillett	Absent	-	Neoartics (von Hagen and Kadereit, 2001)
Gentianella ranunculoides (Willd. ex Schult.) Pringle	Absent	-	Neotropics (voucher)
**Halenia conzattii* Greenm.	Absent	-	Neotropics (von Hagen and Kadereit, 2003)
**Halenia corniculata* (L.) Cornaz	Absent	-	Paleartics (von Hagen and Kadereit, 2003)
**Halenia palmeri* A.Gray	Absent	-	Neotropics (von Hagen and Kadereit, 2003)
Halenia weddelliana Gilg	Absent	-	Neotropics (von Hagen and Kadereit, 2003)
**Jaeschkea oligosperma* Knobl.	Absent	-	Orient (voucher)
**Lomatogonium carinthiacum* (Wulfen) Rchb.	Absent	-	Paleartics (voucher)
**Lomatogonium oreocharis* C.Marquand	Absent	-	Paleartics (voucher)
**Megacodon stylophorus* (C.B.Clarke) Harry Sm.	Absent	-	Paleartics (voucher)
**Obolaria virginica* L.	Absent	-	Neoartics (Gillett, 1959)
**Swertia bimaculata* Hook.f. and Thomson ex C.B.Clarke	Absent	-	Neoartics, Paleartics (voucher)
**Swertia perennis* L.	Absent	-	Neoartics, Paleartics (John, 1941)
Swertia pseudochinensis H.Hara f. *alba* Y.N.Lee	Absent	-	Paleartics, Orient (voucher)
**Swertia tetrapetala* Pall.	Absent	-	Neoartics, Paleartics, Orient (voucher)
**Veratrilla baillonii* Franch.	Absent	-	Orient (Smith, 1970; Ho and Pringle, 1995)

Helieae

**Adenolisianthus arboreus* (Spruce ex Progel) Gilg	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Lepis, 2009)
**Aripuana cullmaniorum* Struwe, Maas and V.A.Albert	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Lepis, 2009)
**Calolisianthus pedunculatus* (Cham. and Schltdl.) Gilg	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Calió, 2009)
**Calolisianthus pendulus* (Mart.) Gilg	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Calió, 2009)
**Calolisianthus speciosus* Gilg	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Calió, 2009)
Celiantha bella Maguire and Steyerm.	Present	Isolated	Neotropics (Struwe *et al*., 2002; Struwe *et al*., 2009)
Celiantha chimantensis (Steyerm. and Maguire) Maguire	Present	Isolated	Neotropics (Struwe *et al*., 2002; Struwe *et al*., 2009)
Celiantha imthurniana (Oliv.) Maguire	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Struwe *et al*., 2009)
Chelonanthus acutangulus (Ruiz and Pav.) Gilg	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Lepis, 2009)
**Chelonanthus alatus* Pulle	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Lepis, 2009)
**Chelonanthus albus* (Spruce ex Progel) V.M.Badillo	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Lepis, 2009)
**Chelonanthus angustifolius* (Kunth) Gilg	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Lepis, 2009)
**Chelonanthus grandiflorus* (Aubl.) E.Hassl.	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Lepis, 2009)
Chelonanthus matogrossensis (J.G.M.Pers. and Maas) Struwe and V.A.Albert	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Lepis, 2009)
Chelonanthus pterocaulis Lepis	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Lepis, 2009)
**Chelonanthus purpurascens* (Aubl.) Struwe, S.Nilsson and V.A.Albert	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Lepis, 2009)
**Chelonanthus viridiflorus* (Mart.) Gilg	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Lepis, 2009)
Chorisepalum ovatum Gleason	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
**Chorisepalum psychotrioides* Ewan	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
**Chorisepalum sipapoanum* (Maguire) Struwe and V.A.Albert	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
Helia brevifolia Cham.	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Calió, 2009)
**Helia oblongifolia* Mart.	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Calió, 2009)
Irlbachia cardonae (Gleason) Maguire	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
**Irlbachia nemorosa* (Willd. ex Roem. and Schult.) Merr.	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Guimarães and Saavedra, 2013)
**Irlbachia poeppigii* (Griseb.) L.Cobb and Maas	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
**Irlbachia pratensis* (Kunth) L.Cobb and Maas	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
**Irlbachia pumila* (Benth.) Maguire	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
**Lehmanniella splendens* (Hook.) Ewan	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
**Macrocarpaea angelliae* J.R.Grant and Struwe	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
**Macrocarpaea arborescens* Gilg	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
**Macrocarpaea bangiana* Gilg	Present	Isolated and aggregates	Neotropics (Ewan, 1948; Struwe *et al*., 2002)

**Macrocarpaea domingensis* Urb. and Ekman	Present	Isolated and aggregates	Neotropics (Ewan, 1948; Struwe *et al*., 2002)
Macrocarpaea glaziovii Gilg	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Guimarães *et al*., 2013)
**Macrocarpaea obtusifolia* (Griseb.) Gilg	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Guimarães *et al.*, 2013)
**Macrocarpaea rubra* Malme	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Guimarães *et al.*, 2013)
**Macrocarpaea valerioi* Standl.	Present	Isolated and aggregates	Neotropics (Ewan, 1948; Struwe *et al*., 2002)
**Neblinantha parvifolia* Maguire	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
Neblinantha neblinae Maguire	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
Prepusa alata Porto and Brade	Present	Isolated and aggregates	Neotropics (Calió *et al.,* 2008)
Prepusa connata Gardner	Present	Isolated and aggregates	Neotropics (Calió *et al.,* 2008)
Prepusa hookeriana Gardner	Present	Isolated and aggregates	Neotropics (Calió *et al.,* 2008)
**Prepusa montana* Mart.	Present	Isolated and aggregates	Neotropics (Calió *et al.,* 2008)
Prepusa viridiflora Brade	Present	Isolated and aggregates	Neotropics (Calió *et al.,* 2008)
**Purdieanthus pulcher* (Hook.) Gilg	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
Rogersonanthus arboreus (Britton) Maguire and B.M.Boom	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Lepis, 2009)
Rogersonanthus quelchii (N.E.Br.) Maguire and B.M.Boom	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Lepis, 2009)
Roraimaea aurantiaca Struwe, S.Nilsson and V.A.Albert	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Struwe *et al*., 2008)
Senaea coerulea Taub.	Present	Isolated and aggregates	Neotropics (Calió *et al*., 2008)
Senaea janeirensis Brade	Present	Isolated and aggregates	Neotropics (Calíó *et al*., 2008)
Sipapoantha ostrina Maguire and B.M.Boom	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002; Lepis, 2009)
**Symbolanthus australis* Struwe	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
Symbolanthus brittonianus Gilg	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
Symbolanthus calygonus Griseb.	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
Symbolanthus elisabethae Gilg	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
**Symbolanthus frigidus* (Sw.) Struwe and K.Gould	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
**Symbolanthus mathewsii* (Griseb.) Ewan	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
Symbolanthus yaviensis Steyermark	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
**Symbolanthus pulcherrimus* Gilg	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)
**Tachia grandiflora* Maguire and Weaver	Present	Isolated and aggregates	Neotropics (Cobb and Maas, 1998; Struwe *et al*., 2002)
**Tachia guianensis* Aubl.	Present	Isolated and aggregates	Neotropics (Cobb and Maas, 1998; Struwe *et al*., 2002)
**Tachia loretensis* Maguire and Weaver	Present	Isolated and aggregates	Neotropics (Cobb and Maas, 1998; Struwe *et al*., 2002)
**Tachia occidentalis* Maguire and Weaver	Present	Isolated and aggregates	Neotropics (Cobb and Maas, 1998; Struwe *et al*., 2002)
**Tachia parviflora* Maguire and Weaver	Present	Isolated and aggregates	Neotropics (Cobb and Maas, 1998; Struwe *et al*., 2002)
**Tetrapollinia caerulescens* (Aubl.) Maguire and B.M.Boom	Present	Isolated and aggregates	Neotropics (Struwe *et al*., 2002)

Potalieae - Faroinae			
**Enicostema axillare* (Lam.) A.Raynal	Absent	-	Tropical Africa (Struwe *et al*., 2002)
**Enicostema verticillatum* Engl. ex Gilg	Absent	-	Neotropics (Struwe *et al*., 2002)
Faroa malaissei Bamps	Absent	-	Não registrada para os neotrópicos (Struwe *et al*., 2002)
**Neurotheca loeselioides* (Spruce ex Progel) Baill.	Present	Isolated	Neotropics, Tropical Africa (Struwe *et al*., 2002)
Potalieae - Lisianthiinae			
Lisianthius axillaris Kuntze	Present	Isolated	Neotropics (Struwe *et al*., 2002)
**Lisianthius jefensis* A.Robyns and T.S.Elias	Present	Isolated	Neotropics (Struwe *et al*., 2002)
**Lisianthius laxiflorus* Urb.	Present	Isolated	Neotropics (Struwe *et al*., 2002)
Lisianthius saponarioides Cham. and Schltdl.	Present	Isolated	Neotropics (Struwe *et al*., 2002)
Potalieae - Potaliinae			
**Fagraea ceilanica* Thunb.	Absent	-	Neoartics, Orient (Struwe *et al*., 2002)
**Potalia amara* Aubl.	Present	Isolated	Neotropics (Struwe *et al*., 2002)
**Potalia resinifera* Mart.	Present	Isolated	Neotropics (Struwe *et al*., 2002)
Saccifolieae			
#*Curtia conferta* (Mart.) Knobl.	Present	Isolated	Neotropics (Crespo and Marcondes-Ferreira, 2009)
#*Curtia diffusa* (Mart.) Cham.	Present	Isolated	Neotropics (Crespo and Marcondes-Ferreira, 2009)
#*Curtia tenella* (Mart.) Cham.	Present	Isolated	Neotropics (Crespo and Marcondes-Ferreira, 2009)
*#*Curtia tenuifolia* Knobl.	Present	Isolated	Neotropics (Crespo and Marcondes-Ferreira, 2009)
*# *Curtia verticillaris* (Spreng.) Knobl.	Present	Isolated	Neotropics (Crespo and Marcondes-Ferreira, 2009)
Hockinia montana Gardner	Present	Isolated	Neotropics (Struwe *et al*., 2002)
#*Tapeinostemon longiflorum* var. *longiflorum* Maguire and Steyerm.	Present	Isolated	Neotropics (Struwe *et al*., 2002)
#*Tapeinostemon spenneroides* Benth.	Present	Isolated	Neotropics (Struwe *et al*., 2002)
**Voyriella parviflora* Miq.	Present	Isolated	Neotropics (Maguire and Boom, 1989)
Voyrieae			
**Voyria aphylla* (Jacq.) Pers.	Present	Isolated	Neotropics (Struwe *et al*., 2002)
**Voyria aurantiaca* Splitg.	Present	Isolated	Neotropics (Struwe *et al*., 2002)

1 * Species used in the reconstruction of phylogeny and character optimization. # Anatomical data on EFNs obtained only from the literature.

Table 2. Age estimates and ancestral state reconstructions for key nodes.

		Probabilities for nodal reconstructions				
		EFNs absence/presence		Geographic distribution of species		
	Age estimate and 95% HPD	**Parsimony**	**BBM**	**Parsimony**	**BBM**	**S-DIVA**
Gentianaceae (Node 1)	70.4	A: 0.17	B: 0.93	A: 1.00	A: 0.98	ACDE: 0.96
	83.9-56.7	Equivocal: 0.83				
Saccifolieae (Node 2)	36.4					
	49.6-9.7	B: 1.00	B: 0.99	A: 1.00	A: 0.99	A: 1.00
Exaceae + *Voyrieae* + Chironieae + Potalieae + Gentianeae + Helieae (node 3)	53.1	A: 0.17	B: 0.48	A: 1.00	A: 0.89	ACDE: 0.95
	61.7-44.9	Equivocal: 0.83	A: 0.39		D: 0.05	
			AB: 0.13			
Exaceae (Node 4)	23.2	A: 1.00	A: 0.99	D: 0.99	D: 0.93	CDE: 0.97
	18.59-2.4			Equivocal: 0.01		
Voyrieae + Chironieae + Potalieae + Gentianeae + Helieae (Node 5)	49.8	A: 0.17	B: 0.74	A: 1.00	A: 0.99	A: 0.99
	58.4-42.0	B: 0.02	AB: 0.15			
		Equivocal: 0.80	A: 0.11			
Voyrieae (Node 6)	17.6	B: 1.00	B: 0.98	A: 1.00	A: 0.99	A: 1.00
	28.0-8.2					
Chironieae + Potalieae + Gentianeae + Helieae (Node 7)	45.4	A: 0.17	B: 0.55	A: 1.00	A: 0.99	A: 0.99
	51.6-39.6	B: 0.02	A: 0. 25			
		Equivocal: 0.80	AB: 0.20			
Chironieae (Node 8)	36.1	A: 0.17	A: 0.69	A: 1.00	A: 0.99	A: 0.93

	43.3-29.2	B: 0.02	AB: 0.19			
		Equivocal: 0.80	B: 0.12			
Chiroiinae (Node 9)	33.8	A: 1.00	A: 0.99	A: 0.97	A: 0.96	AC: 1.00
	41.4-26.1			Equivocal: 0.02		
Coutoubeinae (Node 10)	19.7	B: 1.00	B: 0.69	A: 1.00	A: 0.99	A: 1.00
	29.6-11.1		AB: 0.27			
Canscorinae (Node 11)	23.0	A: 1.00	A: 0.99	Equivocal: 1.00	A: 0.521	CE: 0.34
	32.3-13.6				E: 0.21	CDE: 0.32
					D: 0.12	DE: 0.30
Potalieae + Gentianeae +Helieae (Node 12)	41.0	A: 0.17	B: 0.70	A: 1.00	A: 0.99	A: 1.00
	45.7-36.8	B: 0.02	AB: 0.20			
		Equivocal: 0.80	A: 0.10			
Potalieae (Node 13)	36.3	A: 0.17	B: 0.81	A: 1.00	A: 0.98	A: 0.83
	38.3-34.4	B: 0.02	AB: 0.15			AE : 0.17
		Equivocal: 0.81				
Potaliinae (Node 14)	29.2	B: 0.01	B: 0.76	A: 0.57	A: 0.96	AE: 1.00
	37.2-18.1	Equivocal: 0.56	AB: 0.18	Absent: 0.43		
		Absent: 0.43	A: 0.06			
Faroinae (Node 15)	25.3	B: 0.18	B: 0.86	A: 0.98	A: 0.63	A: 1.00
	32.8-17.4	Equivocal: 0.81	AB: 0.12	Absent: 0.02	AD: 0.36	
		Absent: 0.01				
Lisianthiinae (Node 16)	3.2					
	5.8-1.0	B: 1.00	B: 0.99	A: 1.00	A: 0.99	A: 1.00
Gentianeae + Helieae (Node 17)	35.2	A: 0.17	B: 0.66	A: 1.00	A: 0.98	AE: 0.16

	41.1-29.3	B: 0.02 Equivocal: 0.80	AB: 0.23 A: 0.11			AC: 0.16 ABCE: 0.13 ACE: 0.13 ABE: 0.13 AB: 0.13 ABC: 0.13
Gentianeae (Node 18)	30.7 36.6-24.7	A: 1.00	A: 0.94 AB: 0.05	Equivocal: 1.00	A: 0.54 B: 0.18 C: 0.13	BC: 0.33 BE: 0.33 BCE: 0.33
Swertinae (Node 19)	16.3 18,1-14.6	A: 1.00	A: 0.99	B: 0.51 Equivocal: 0.49	B: 0.95	B: 0.65 BC: 0.10 BE: 0.14 BCE: 0.10
Gentianinae (Node 20)	22.9 28.4-17.6	A: 1.00	A: 0.99	Equivocal: 1.00	C: 0.73 CE: 0.11 E: 0.09	C: 0.49 CE: 0.49
Helieae (Node 21)	29.4 38.0-20.2	B: 1.00	B: 0.97	A: 1.00	A: 0.99	A: 1.00

1 BBM. Bayesian binary Markov chain Monte Carlo; S-DIVA. statistical dispersal–vicariance analysis.

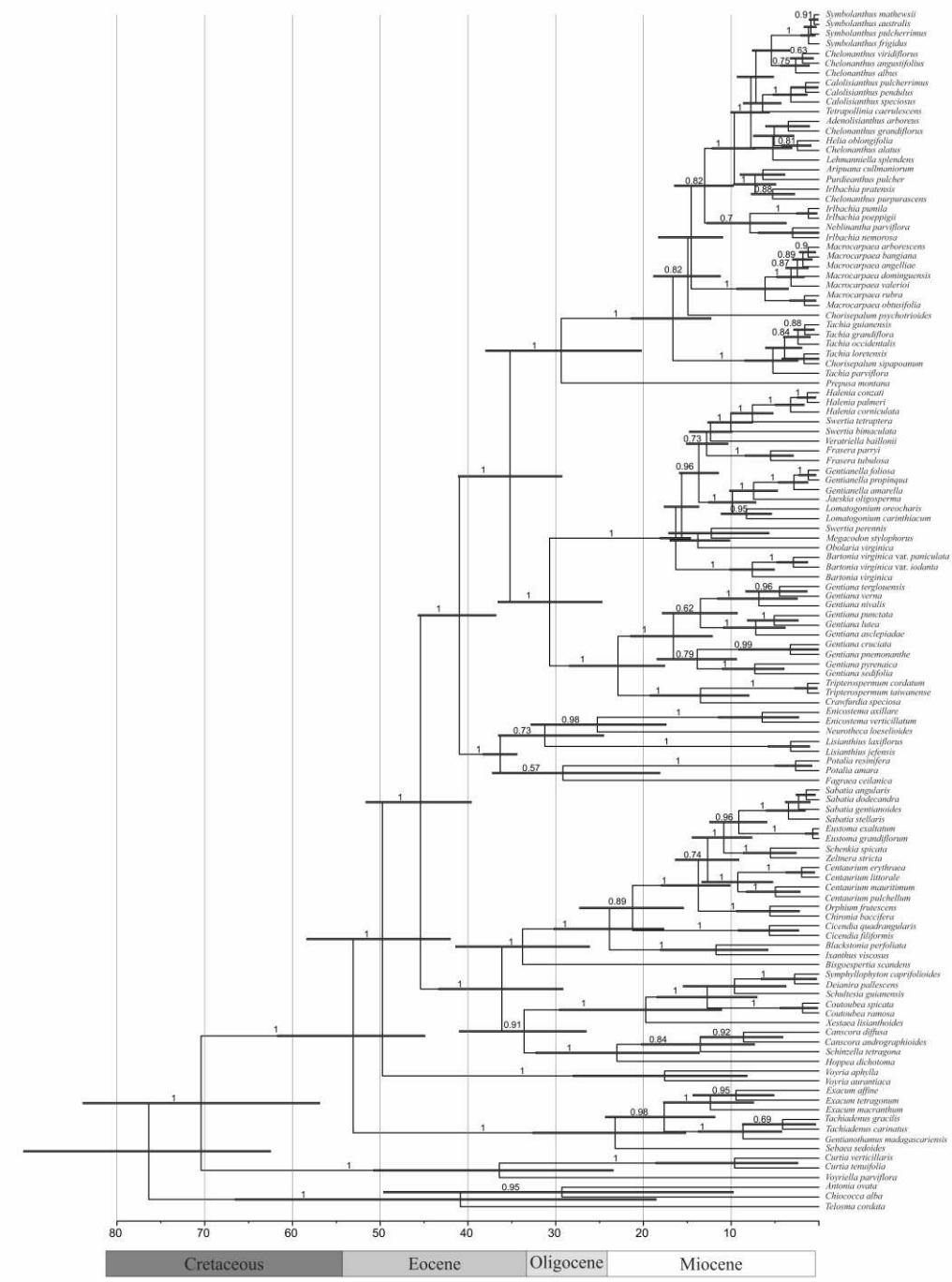

Fig. 1. Maximum clade credibility tree from the BEAST analysis for Gentianaceae. The tree is a chronogram with branches proportional to time. Posterior probabilities greater than 0.5 are indicated on the branches and 95% highest posterior densities of age estimates are indicated with bars. Numbered nodes are discussed in the text.

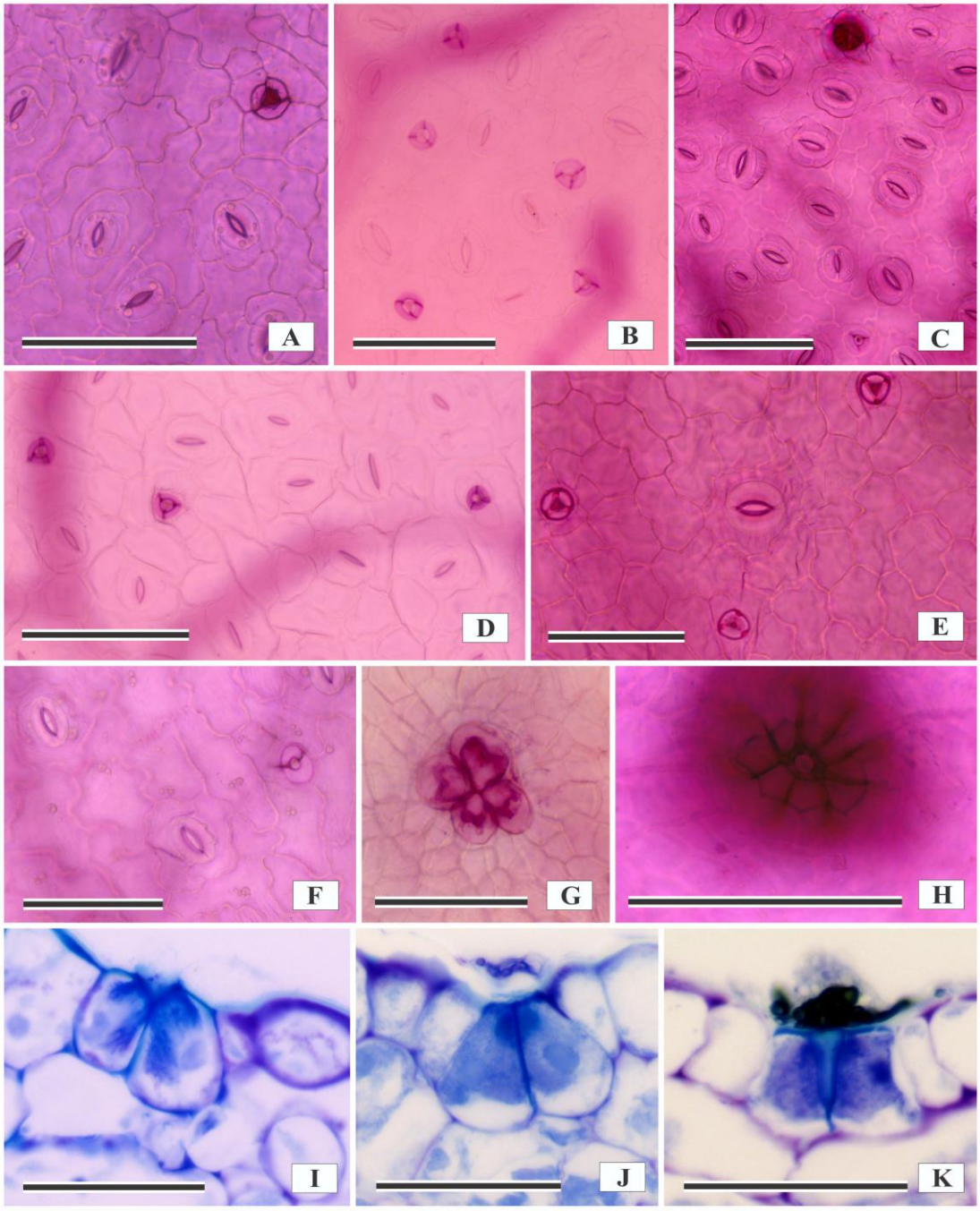

Fig. 2. Extrafloral nectaries isolated and dispersed over the leaf blade of species of Gentianaceae. (A) *Symbolanthus brittonianus* (B) *Aripuana cullmaniorum.* (C) *Chelonanthus albus*. (D) *Chelonanthus acutangulus.* (E) *Tachia loretensis*. (F) *Curtia verticillaris*. (G) *Coutoubea ramosa.* (H) *Potalia amara*. (I) *Celiantha chimantensis*. (J) *Chelonanthus viridiflorus*. (K) *Chelonanthus albus.* Black arrows indicate nectary. Scale bars (A-H) = 100 µm and (I-K) = 50 µm.

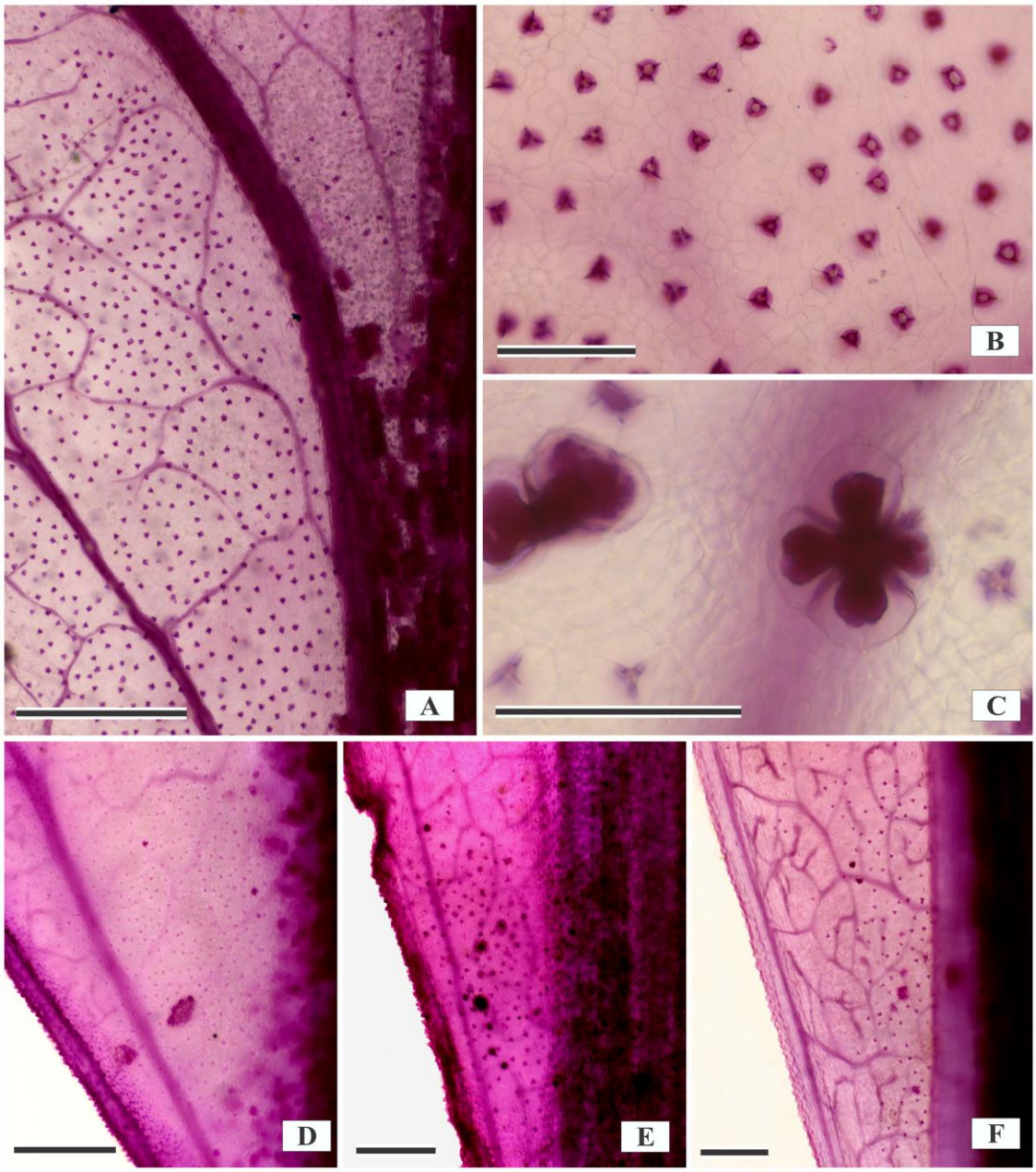

Fig. 3. Extrafloral nectaries in aggregates at the leaf base of species of Gentianaceae. (A, B) *Chelonanthus albus.* (C) *Chelonanthus angustifolius*. (D) *Chelonanthus viridiflorus.* (E) *Chelonanthus angustifolius*. (F) *Chelonanthus grandiflorus.* Scale bars: (A, D-F) = 500 μm and (B, C) = 100 μm.

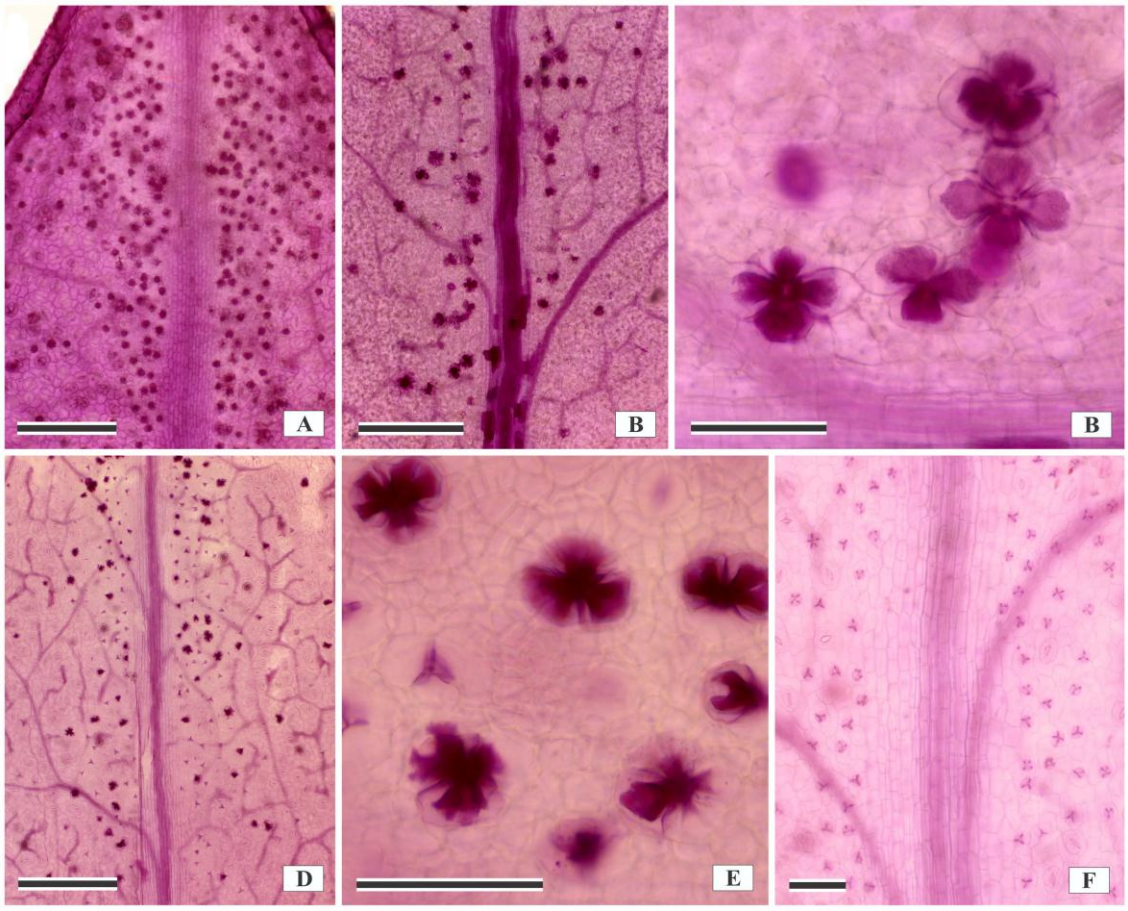

Fig. 4. Extrafloral nectaries in aggregates at the leaf apex of species of Gentianaceae. (A) *Neblinantha parviflora*. (B, C) *Adenolisianthus arboreus.* (D, E) *Chelonanthus angustifolius*. (F) *Irlbachia nemorosa.* Scale bars: (A, B, D) = 500 µm and (C, E, F) = 100 µm.

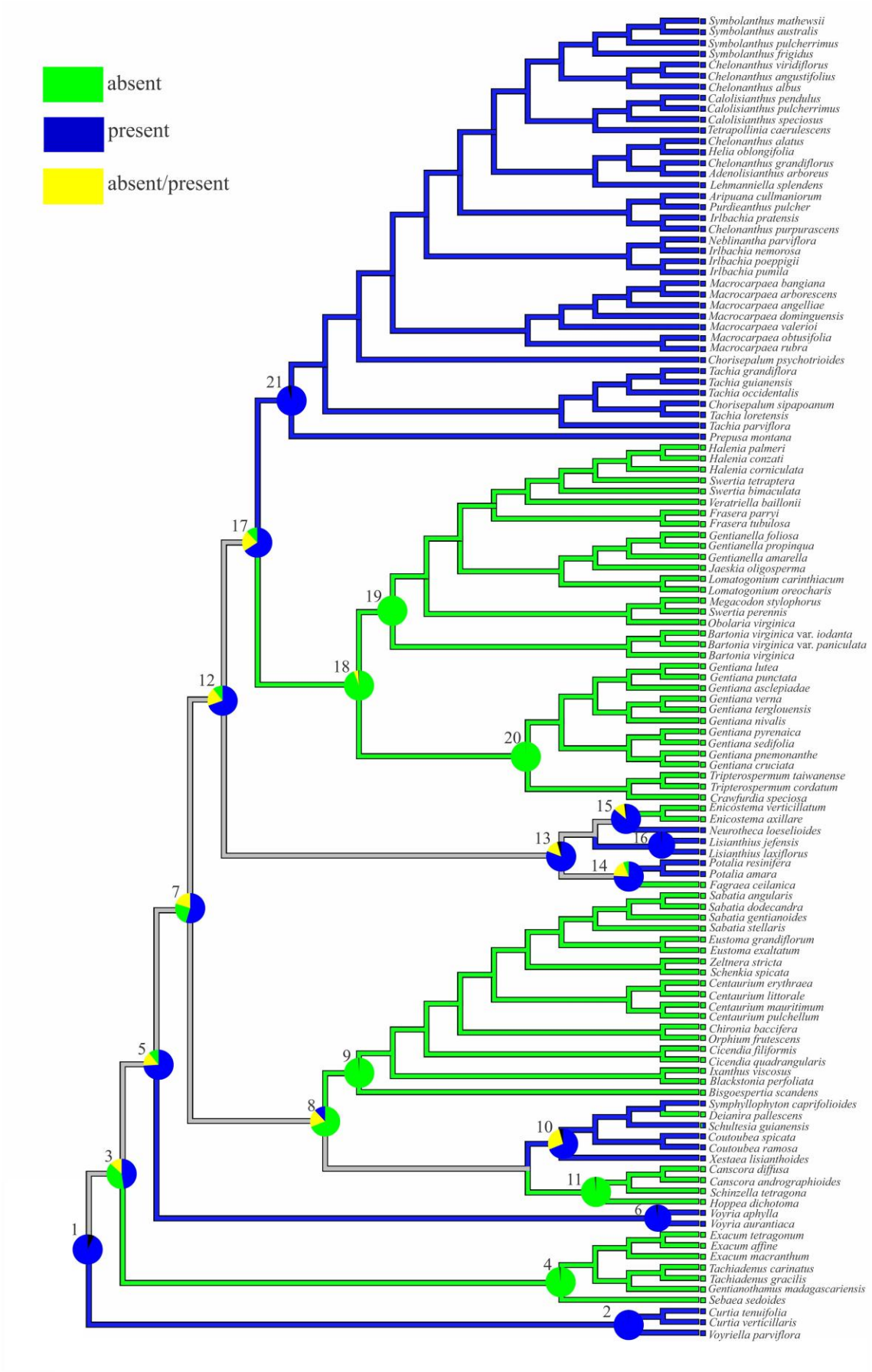
absent
present
absent/present
Symbolanthus mathewsii
Symbolanthus australis
Symbolanthus pulcherrimus
Symbolanthus frigidus
Chelonanthus viridiflorus
Chelonanthus angustifolius
Chelonanthus albus
Calolisianthus pendulus
Calolisianthus pulcherrimus
Calolisianthus speciosus
Tetrapollinia caerulescens
Chelonanthus alatus
Helia oblongifolia
Chelonanthus grandiflorus
Adenolisianthus arboreus
Lehmanniella splendens
Aripuana cullmaniorum
Purdieanthus pulcher
Irlbachia pratensis
Chelonanthus purpurascens
Neblinantha parviflora
Irlbachia nemorosa
Irlbachia poeppigii
Irlbachia pumila
Macrocarpaea bangiana
Macrocarpaea arborescens
Macrocarpaea angelliae
Macrocarpaea dominguensis
Macrocarpaea valerioi
Macrocarpaea obtusifolia
Macrocarpaea rubra
Chorisepalum psychotrioides
Tachia grandiflora
Tachia guianensis
Tachia occidentalis
Chorisepalum sipapoanum
Tachia loretensis
Tachia parviflora
Prepusa montana
Halenia palmeri
Halenia conzati
Halenia corniculata
Swertia tetraptera
Swertia bimaculata
Veratriella baillonii
Frasera parryi
Frasera tubulosa
Gentianella foliosa
Gentianella propinqua
Gentianella amarella
Jaeskia oligosperma
Lomatogonium carinthiacum
Lomatogonium oreocharis
Megacodon stylophorus
Swertia perennis
Obolaria virginica
Bartonia virginica var. iodanta
Bartonia virginica var. paniculata
Bartonia virginica
Gentiana lutea
Gentiana punctata
Gentiana asclepiadae
Gentiana verna
Gentiana terglouensis
Gentiana nivalis
Gentiana pyrenaica
Gentiana sedifolia
Gentiana pneumonanthe
Gentiana cruciata
Tripterospermum taiwanense
Tripterospermum cordatum
Crawfurdia speciosa
Enicostema verticillatum
Enicostema axillare
Neurotheca loeselioides
Lisianthius jefensis
Lisianthius laxiflorus
Potalia resinifera
Potalia amara
Fagraea ceilanica
Sabatia angularis
Sabatia dodecandra
Sabatia gentianoides
Sabatia stellaris
Eustoma grandiflorum
Eustoma exaltatum
Zeltnera stricta
Schenkia spicata
Centaurium erythraea
Centaurium littorale
Centaurium mauritimum
Centaurium pulchellum
Chironia baccifera
Orphium frutescens
Cicendia filiformis
Cicendia quadrangularis
Ixanthus viscosus
Blackstonia perfoliata
Bisgoespertia scandens
Symphyllophyton caprifolioides
Deianira pallescens
Schultesia guianensis
Coutoubea spicata
Coutoubea ramosa
Xestaea lisianthoides
Canscora diffusa
Canscora andrographioides
Schinzella tetragona
Hoppea dichotoma
Voyria aphylla
Voyria aurantiaca
Exacum tetragonum
Exacum affine
Exacum macranthum
Tachiadenus carinatus
Tachiadenus gracilis
Gentianothamus madagascariensis
Sebaea sedoides
Curtia tenuifolia
Curtia verticillaris
Voyriella parviflora
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Fig. 5. Ancestral character state reconstruction for presence of foliar EFNs based on maximum parsimony and the Bayesian binary Markov chain Monte Carlo (BBM) method. The character states assigned to each species are given next to species names. Branches are colored according to the parsimony reconstruction (grey lines indicate ambiguous parsimony reconstructions), and the pie charts on the nodes show the results of the Bayesian reconstruction (areas with frequencies below 0.05 are collectively represented in black).

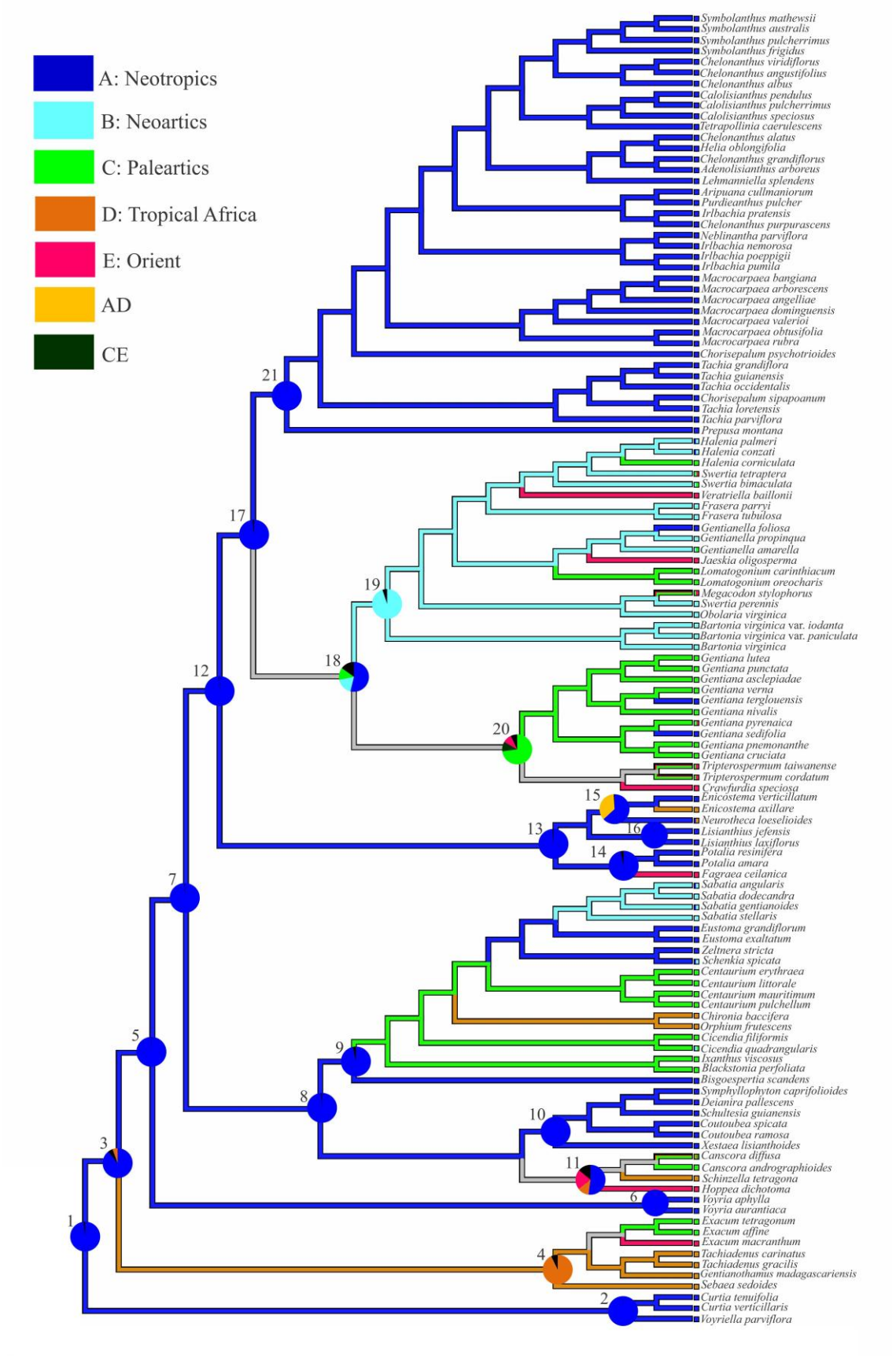
A: Neotropics
B: Neoartics
C: Paleartics
D: Tropical Africa
E: Orient
AD
CE
Symbolanthus mathewsii
Symbolanthus australis
Symbolanthus pulcherrimus
Symbolanthus frigidus
Chelonanthus viridiflorus
Chelonanthus angustifolius
Chelonanthus albus
Calolisianthus pendulus
Calolisianthus pulcherrimus
Calolisianthus speciosus
Tetrapollinia caerulescens
Chelonanthus alatus
Helia oblongifolia
Chelonanthus grandiflorus
Adenolisianthus arboreus
Lehmanniella splendens
Aripuana cullmaniorum
Purdieanthus pulcher
Irlbachia pratensis
Chelonanthus purpurascens
Neblinantha parviflora
Irlbachia nemorosa
Irlbachia poeppigii
Irlbachia pumila
Macrocarpaea bangiana
Macrocarpaea arborescens
Macrocarpaea angelliae
Macrocarpaea dominguensis
Macrocarpaea valerioi
Macrocarpaea obtusifolia
Macrocarpaea rubra
Chorisepalum psychotrioides
Tachia grandiflora
Tachia guianensis
Tachia occidentalis
Chorisepalum sipapoanum
Tachia loretensis
Tachia parviflora
Prepusa montana
Halenia palmeri
Halenia conzati
Halenia corniculata
Swertia tetraptera
Swertia bimaculata
Veratriella baillonii
Frasera parryi
Frasera tubulosa
Gentianella foliosa
Gentianella propinqua
Gentianella amarella
Jaeskia oligosperma
Lomatogonium carinthiacum
Lomatogonium oreocharis
Megacodon stylophorus
Swertia perennis
Obolaria virginica
Bartonia virginica var. iodanta
Bartonia virginica var. paniculata
Bartonia virginica
Gentiana lutea
Gentiana punctata
Gentiana asclepiadae
Gentiana verna
Gentiana terglouensis
Gentiana nivalis
Gentiana pyrenaica
Gentiana sedifolia
Gentiana pnemonanthe
Gentiana cruciata
Tripterospermum taiwanense
Tripterospermum cordatum
Crawfurdia speciosa
Enicostema verticillatum
Enicostema axillare
Neurotheca loeselioides
Lisianthius jefensis
Lisianthius laxiflorus
Potalia resinifera
Potalia amara
Fagraea ceilanica
Sabatia angularis
Sabatia dodecandra
Sabatia gentianoides
Sabatia stellaris
Eustoma grandiflorum
Eustoma exaltatum
Zeltnera stricta
Schenkia spicata
Centaurium erythraea
Centaurium littorale
Centaurium mauritimum
Centaurium pulchellum
Chironia baccifera
Orphium frutescens
Cicendia filiformis
Cicendia quadrangularis
Ixanthus viscosus
Blackstonia perfoliata
Bisgoespertia scandens
Symphyllophyton caprifolioides
Deianira pallescens
Schultesia guianensis
Coutoubea spicata
Coutoubea ramosa
Xestaea lisianthoides
Canscora diffusa
Canscora andrographioides
Schinzella tetragona
Hoppea dichotoma
Voyria aphylla
Voyria aurantiaca
Exacum tetragonum
Exacum affine
Exacum macranthum
Tachiadenus carinatus
Tachiadenus gracilis
Gentianothamus madagascariensis
Sebaea sedoides
Curtia tenuifolia
Curtia verticillaris
Voyriella parviflora
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Fig. 6. Ancestral character state reconstruction for geographic distribution based on maximum parsimony and the Bayesian binary Markov chain Monte Carlo (BBM) method. The character states assigned to each species are given next to species names. Branches are colored according to the parsimony reconstruction (grey lines indicate ambiguous parsimony reconstructions), and the pie charts on the nodes show the results of the Bayesian reconstruction (areas with frequencies below 0.05 are collectively represented in black).

SUPPLEMENTARY INFORMATION

Supplementary Information Appendix 1. Taxa and GenBank accession numbers analyzed for this study.

Táxon	*trnL*	*matK*	ITS
Chironieae - Canscorinae			
Canscora andrographioides Griff. ex C.B.Clarke	AJ490192	-	AJ489866
Canscora diffusa (Vahl) R.Br. ex Roem. and Schult.	AF102389	AJ388143, AJ388212	AY256386, AY256391
Hoppea dichotoma Willd.	AF102440	AJ388170, AJ388240	-
Schinziella tetragona (Schinz) Gilg	AF102479	AJ010527, AJ011456	-
Chironieae - Chroniinae			
Bisgoeppertia scandens (Spreng.) Urb.	-	-	FJ232556
Blackstonia perfoliata (L.) Huds.	AF402254	-	AY047793, AY047878
Centaurium erythraea Rafn	AY251729	-	AY251669, AY251699
Centaurium littorale (Turner) Gilmour	AY251732	-	AY251672, AY251702
Centaurium maritimum (L.) Fritsch ex E.Jansen	AF102394	AJ010508, AJ011437	AJ011466, AJ011476
Centaurium pulchellum (Sw.) Druce	AY251734	-	AY047787, AY047872
Chironia baccifera L.	AF102402	AJ010509, AJ011438	AJ011464, AJ011474
Cicendia filiformis (L.) Delarbre	AF102403	AJ010510, AJ011439	AJ011463, AJ011473
Cicendia quadrangularis (Lam.) Griseb.	AF102404	AJ388148, AJ388217	AY251682, AY251712
Eustoma exaltatum (L.) Salisb. ex G.Don	AY251752	-	AY251698, AY251728
Eustoma grandiflorum (Raf.) Shinners	AY251751	-	AY251696, AY251726
Ixanthus viscosus Griseb.	AY251741	AJ010521, AJ011450	AJ011481, AJ011471
Orphium frutescens E.Mey.	AY251748	AJ010525, AJ011454	AY251693, AY251723
Sabatia angularis Pursh	AF102476	AJ010526, AJ011455	AJ011467, AJ011477
Sabatia dodecandra (L.) Britton, Sterns and Poggenb.	AY255693	-	AY256383, AY256388
Sabatia gentianoides Elliott	AF102477	AJ388186, AJ388256	-
Sabatia stellaris Pursh	AY255694	-	AY256384, AY256389
Schenkia spicata (L.) G.Mans.	AF402253	-	AY047792, AY047877
Zeltnera stricta (Schiede) G.Mans.	AF402236	-	AY047758, AY047843
Chironieae - Coutoubeinae			
Coutoubea ramosa Aubl.	AF102408	AJ010511, AJ011440	-
Coutoubea spicata Aubl.	AF102409	AJ388150, AJ388219	EU709780
Deianira pallescens Cham. and Schltdl.	AF102410	AJ388153, AJ388222	-
Schultesia guianensis (Aubl.) Malme	AF102480	AJ388188, AJ388258	-
Symphyllophytton caprifolioides Gilg	AF102490	AJ010530, AJ011459	AJ011462, AJ011472
Xestaea lisianthoides Griseb.		AJ388199, AJ388269	-
Exaceae			
Exacum affine Balf.f.	AF102417	AJ010515, AJ011444	AJ489879
Exacum macranthum Arn.	AJ490216	-	AJ489891
Exacum tetragonum Roxb.	AF102418	AJ388156, AJ388225	AJ489907
Gentianothamnus madagascariensis Humbert	AJ490240	-	AJ489914
Sebaea sedoides Gilg	FJ014185	-	FJ665995
Tachiadenus carinatus Griseb.	AF102491	AJ011434, AJ011460	AJ489923
Tachiadenus gracilis Griseb.	FJ014194	-	-

Gentianeae - Gentianinae			
Crawfurdia speciosa Wall.	AJ242605	AJ010512, AJ011441	AJ294586, AJ294646
Gentiana asclepiadea L.	X77871	AJ388165, AJ388235	Z48083, Z48076
Gentiana cruciata L.	AF102434	AJ010519, AJ011448	DQ398634
Gentiana lutea L.	X75702	-	Z48122, Z48119
Gentiana nivalis L.	X75703	EF552121	-
Gentiana pneumonanthe L.	X77889	-	-
Gentiana punctata L.	X77894		Z48066, Z48088
Gentiana pyrenaica L.	X77895	-	Z48068, Z48087
Gentiana sedifolia Kunth	AF102436	AJ388167, AJ388237	Z71963, Z71964
Gentiana terglouensis Hacq.	X77897	EF552075	-
Gentiana verna L.	X75704	EF552088	GU251028
Tripterospermum cordatum (Marquand) Harry Sm.	AY563392	-	AY562172
Tripterospermum taiwanense (Masam.) Satake	-	-	GU251053
Gentianeae - Swertiinae			
Bartonia paniculata (Michx.) Muhl. subsp. *iodandra* (B.L.Rob.) J.M.Gillett	EU834126	-	EU812472
Bartonia paniculata (Michx.) Muhl. subsp. *paniculata*	EU834125	-	EU812470
Bartonia virginica (L.) Britton, Sterns and Poggenb.	EU834127	AJ388141, AJ388210	EU812474
Frasera parryi Torr.	-	AJ408029, AJ408022	AJ306083, AJ306111
Frasera tubulosa Coville	-	AJ408030, AJ408023	AJ306084, AJ306112
Gentianella amarella L. (Borner)	-	AJ406326, AJ406355	AJ294591, AJ294651
Gentianella cerastioides (Kunth) Fabris			
Gentianella foliosa (Kunth) Fabris	AJ315190	-	AJ318538, AJ410317
Gentianella helianthemoides (Gil.) Fabris			
Gentianella propinqua (Richardson) J.M.Gillett	-	AJ406340, AJ406368	AJ294619, AJ294679
Halenia conzattii Greenm.	-	-	AJ411747,AJ411716
Halenia corniculata (L.) Cornaz	-	AJ388168, AJ388238	AJ306087, AJ306114
Halenia palmeri A.Gray	AF102437	AJ388169, AJ388239	AJ294632, AJ294692
Halenia weddelliana Gilg			
Jaeschkea oligosperma Knobl.	AF102444	AJ388171, AJ388241	AJ294633, AJ294693
Lomatogonium carinthiacum (Wulfen) Rchb.	X77899	AJ406346, AJ406374	AJ294634, AJ294694
Lomatogonium oreocharis C.Marquand	AF102452	AJ388174, AJ388244	AJ294635, AJ294695
Megacodon stylophorus (C.B.Clarke) Harry Sm.	AF102458	AJ388177, AJ388247	AY858679
Obolaria virginica L.	AF102464	AJ388180, AJ388250	AJ306094, AJ306121
Swertia bimaculata Hook.f. and Thomson ex C.B.Clarke		AJ408033, AJ408026	AJ306097, AJ306124
Swertia perennis L.	-	AJ010528, AJ011457	-
Swertia tetrapetala Pall.	AJ315229	-	Z48115, Z48139
Veratrilla baillonii Franch.	AF102497	AJ388196, AJ388266	AJ294644, AJ294704
Helieae			
Adenolisianthus arboreus (Spruce ex Progel) Gilg	-	-	EU709784
Aripuana cullmaniorum Struwe, Maas and V.A.Albert	AJ242603	AJ388140, AJ388209	EU709785
Calolisianthus pedunculatus (Cham. and Schltdl.) Gilg	AF102388	AJ388142, AJ388211	EU709786
Calolisianthus pendulus (Mart.) Gilg	AF102387	-	-
Calolisianthus speciosus Gilg	-	-	EU709787

Chelonanthus alatus Pulle	AJ490194	-	AJ489868
Chelonanthus albus (Spruce ex Progel) V.M.Badillo	AF102397	-	EU709789
Chelonanthus angustifolius (Kunth) Gilg	AJ490195	-	AJ489869
Chelonanthus grandiflorus (Aubl.) E.Hassl.	-	-	EU709788
Chelonanthus purpurascens (Aubl.) Struwe, S.Nilsson and V.A.Albert	AF102398	AJ388146, AJ388215	FJ232584
Chelonanthus viridiflorus (Mart.) Gilg	AF102399	-	EU709792
Chorisepalum psychotrioides Ewan	-	-	EU709793
Chorisepalum sipapoanum (Maguire) Struwe and V.A.Albert	-	AJ388147, AJ388216	-
Helia oblongifolia Mart.	-	-	EU709794
Irlbachia nemorosa (Willd. ex Roem. and Schult.) Merr.	-	-	EU709795
Irlbachia poeppigii (Griseb.) L.Cobb and Maas	AF102441	-	EU709796
Irlbachia pratensis (Kunth) L.Cobb and Maas	AF102442	-	EU709797
Irlbachia pumila (Benth.) Maguire	-	-	EU709798
Lehmanniella splendens (Hook.) Ewan	-	AJ388172, AJ388242	-
Macrocarpaea angelliae J.R.Grant and Struwe	-	-	EU528073
Macrocarpaea arborescens Gilg	EU528051	-	EU528076
Macrocarpaea bangiana Gilg	-	-	EU528078
Macrocarpaea domingensis Urb. and Ekman	AF102454	AJ010523, AJ011452	EU709799
Macrocarpaea obtusifolia (Griseb.) Gilg	EU528057	-	EU528125
Macrocarpaea rubra Malme	EU528059	AJ388175, AJ388245	EU528138
Macrocarpaea valerioi Standl.	AF102456	AJ388176, AJ388246	EU528148
Neblinantha parvifolia Maguire	AF102461	AJ388179, AJ388249	-
Prepusa montana Mart.	-	-	EU709805
Purdieanthus pulcher (Hook.) Gilg	-	-	EU709800
Symbolanthus australis Struwe	F102489	-	EU709801
Symbolanthus frigidus (Sw.) Struwe and K.Gould	AF102498	AJ388198, AJ388268	EU709802
Symbolanthus mathewsii (Griseb.) Ewan	-	-	EU528152
Symbolanthus pulcherrimus Gilg	AF102488	-	EU709803
Tachia grandiflora Maguire and Weaver	-	AJ388193, AJ388263	DQ401415
Tachia guianensis Aubl.	-	AJ011433, AJ011461	DQ401419
Tachia loretensis Maguire and Weaver	AF102492	-	DQ401421
Tachia occidentalis Maguire and Weaver	-	-	DQ401423
Tachia parviflora Maguire and Weaver	-	-	DQ401424
Tetrapollinia caerulescens (Aubl.) Maguire and B.M.Boom	AF102494	AJ388194, AJ388264	EU709804
Potalieae - Faroinae			
Enicostema axillare (Lam.) A.Raynal	-	AJ010513, AJ011442	FJ232582
Enicostema verticillatum Engl. ex Gilg	AF102414	AJ388155, AJ388224	EU709781
Neurotheca loeselioides (Spruce ex Progel) Baill.	AF102463	AJ010524, AJ011453	FJ232570
Potalieae - Lisianthiinae			
Lisianthius jefensis A.Robyns and T.S.Elias	AF102448	AJ010522, AJ011451	EU709782
Lisianthius laxiflorus Urb.	AF102449	-	FJ232552
Potalieae - Potaliinae			
Fagraea ceilanica Thunb.	-	-	FJ232572
Potalia amara Aubl.	AF102470	AJ388183, AJ388253	DQ449926

Potalia resinifera Mart.	AF102471	AJ388184, AJ388254	DQ449922
Saccifolieae			
Curtia tenuifolia Knobl.	AJ242606	AJ388151, AJ388220	AJ242613, AJ242614
Curtia verticillaris (Spreng.) Knobl.	-	AJ388152, AJ388221	-
Voyriella parviflora Miq.	AJ242607	AJ388197, AJ388267	AJ242615, AJ242616
Voyrieae			
Voyria aphylla (Jacq.) Pers.	-	-	KC535866
Voyria aurantiaca Splitg.	-	-	KC535867
Out Group			
Apocynaceae - *Telosma cordata* (Burm. f.) Merr.	AF102493	DQ660551	-
Loganiaceae - *Antonia ovata* Pohl	AF102379	AJ388200, AJ388270	-
Rubiaceae - Chiococca alba (L.) Hitchc.	GQ852473	AY538378	-

Supplementary Information Appendix 2. Listing with species of Gentianaceae used in anatomic studies with the respective numbers of collectors and herbaria.

Tribe (subtribe) - *Taxon*: Voucher (Herbarium code). The herbaria acronyms: HUEFS = Universidade Estadual de Feira de Santana; INPA = Instituto Nacional de Pesquisas da Amazônia; MBM = Museu Botânico Municipal; NYBG = New York Botanical Garden; RB = Jardim Botânico do Rio de Janeiro; SJRP = Universidade Estadual Paulista Júlio de Mesquita Filho; SP = Instituto de Botânica; SPF = Universidade de São Paulo; UPCB = Universidade Federal do Paraná; UEC = Herbário da Universidade Estadual de Campinas; US = Smithsonian Institution; VIC = Universidade Federal de Viçosa.

Chironieae (Canscorinae) - *Canscora andrographioides* Griff. ex C.B.Clarke: H.I.Liang (NYBG), H.I.Liang (NYBG), N.K.Chun and C.L.Tso (NYBG); *Canscora diffusa* (Vahl) R.Br. ex Roem. & Schult.: D.A.Nangona and K.Kaunda 201 (NYBG), s.c. (NYBG), T.Koyama et al s.n. (NYBG); *Hoppea dichotoma* Willd.: M.Ramos s.n. (US), M.Ramos s.n. (US), C.J.Saldanha 15212 (US); *Schinziella tetragona* (Schinz) Gilg: E.A.Robinson 6464 (NYBG), J.M. Fay 7275 (US).

Chironieae (Chrinoiinae) - *Bisgoeppertia scandens* (Spreng.) Urb.: T.Zanoni et al s.n. (NYBG), B.A.H.Liogier s.n (NYBG), T.Zanoni et al s.n (NYBG); *Blackstonia perfoliata* (L.) Huds.: A.Schinini et al 30984 (MBM), s.c. (RB), J.Lewalle 8763 (NYBG); *Centaurium erythraea* Rafn: I.Cordeiro et al 2993 (SPF), I.Cordeiro et al 2993 (SP), J.Lovo 138 (SPF), H.Luederwaltd 51 (SP), T.Sendulsky 566 (SP), R.Kral 75992 (SP); *Centaurium littorale* (Turner) Gilmour: R.Lampinen (MBM), J.Walter 106 (MBM), F.H.Hermann 19553 (US); *Centaurium maritimum* (L.) Fritsch ex E.Jansen: Pajarón 53 (MBM), Pajarón 53 (NYBG); *Centaurium pulchellum* (Sw.) Druce: F.Xabier S.Gomes s.n. (MBM), I.Soto s.n. (MBM), G.Seijo et al 2424 (MBM), F.Diaconescu s.n.

(SP), C.Bircã s.n. (SP), A.W.Cusick 1133 (NYBG), A.W. Cusick 34481 (NYBG), W.D.Longbottom 13784 (NYBG); *Chironia baccifera* L.: s.c. (MBM), H.J.T.Venter 10676 (NYBG), R.Brand et al 175 (NYBG), s.c. (NYBG), P.Herman (US); *Cicendia filiformis* (L.) Delarbre: J.Stefani s.n. (SP), J.Stefani (US), s.c. (US), P.Aellen s.n. (US); *Cicendia quadrangularis* (Lam.) Griseb.: H.W.Camp 3516 (NYBG), B.Ertter et al s.n. (NYBG), s.c. (NYBG); *Eustoma exaltatum* (L.) Salisb. ex G.Don: W.D.Stevens and B.A.Krukoff s.n (MBM), E.Madrid and M.Arandia 1047 (MBM), D.E.Breedlove 51004 (NYBG), M.C.Carlson s.n. (NYBG), E.Cabrera and H.Cabrera 6948 (NYBG); *Eustoma grandiflorum* (Raf.) Shinners: L.Rossi s.n. (SP), P.Fryxell 1103 (NYBG), L.K.Magrath and R.R.Weedon 5753 (NYBG), P.Fryxell 2751 (NYBG); *Ixanthus viscosus* Griseb.: J.L.Panero and J.F.Ortega 7037 (MBM), J.R.Grant and J.Cortina 10-4636 (NYBG), S.Carquist 2708 (US); *Orphium frutescens* E.Mey.: B.S. 3051 (NYBG), D.Stevenson s.n. (NYBG), s.c. (NYBG); *Sabatia angularis* Pursh: H.N.Moldenke 20496 (SPF), J.B.Nelson and A.Aurrich 16714 (NYBG), F.W.H. 10462 (NYBG), R.D.Thomas 124348 (NYBG), L.B.Smith and A.R.Hodgdon s.n. (US), W.H.Duncan 8522 (US); *Sabatia brevifolia* Raf.: J.R.Bozeman and A.E.Radford 11580 (SP); *Sabatia campanulata* (L.) Torr.: R.Kral 43645 (MBM), M.Meyers and H.Iltis 2482 (MBM), S.L.Orzell and E.L.Bridges 14064 (MBM); G.L.Webster and R.L.Wilbur 3486 (US); *Sabatia campestris* Nutt.: P.A.Fryxel 5118 (MBM), G.L.Webster and R.L.Wilbur 3261 (NYBG), R.D.Thomas 100175 (NYBG), R.D.Thomas and C.Amason 160953 (NYBG), E.D.Seneca (RB), C.M.Allen 9083 (RB); *Sabatia dodecandra* (L.) Britton, Sterns & Poggenb.: R.P.Wunderlin and B.F.Hansen 10273 (MBM), V.Ducey 137 (MBM); *Sabatia gentianoides* Elliott: R.D.Thomas 129561 (NYBG), R.D.Thomas 100738 (NYBG), R.D.Thomas and J.Kessler 81701 (NYBG), R.F.Thorne s.n. (US), D.Demaree and J.County s.n. (US), s.c. (US); *Sabatia grandiflora* (A.Gray) Small:

R.P.Wunderlin and B.F.Hansen 10267 (MBM), F.S.Blanton and H.O'Neill (US), J.P.Standley 446 (US); *Sabatia stellaris* Pursh: S.W.Leonard and A.E.Radford 1681 (SP); *Schenkia spicata* (L.) G.Mans.: M.Lazãr and E. Marin s.n. (SP), J.Risler and L.A.Kerrigan 403 (NYBG), D.E.Symon 15270 (NYBG), H.P.Vonow 911 (NYBG); *Zeltnera maryanna* (B.L.Turner) G.Mans.: L.C.Higgins 8689 (NYBG), L.C.Higgins 17698 (NYBG), L.C.Higgins 7468 (NYBG); *Zeltnera stricta* (Schiede) G.Mans.: C.R.Broome and R.M.Lloyd 634 (NYBG), C.R.Broome 746 (NYBG), C.R.Broome and R.M.Lloyd 620 (NYBG); *Zygostigma australe* Griseb.: S.Isabel and R.Camaquá s.n. (MBM), G.Hatschbach et al 71812 (MBM), F.C.Hoehne s.n. (SP), Dr.A.Usteri s.n. (SP), A.C.Cervi et al 6368 (UPCB), Herter 94108 (RB).

Chironieae (Coutoubeinae) – *Coutoubea ramosa* Aubl.: G.F.Árbocz et al 4290 (SP), A.S.L.da Silva et al 3528 (SP), C.Ferreira 9584 (RB), R.Neto et al 493 (RB), Silva et al 3804 (RB), Marquete et al s.n. (RB); *Coutoubea spicata* Aubl.: G.Hatschbach et al 75629 (MBM 285632), R.L.Froes 29329 (SP), R.Mello-Silva et al 3137 (SPF), D.Sucre 9382 (RB), Heiden et al 960 (RB), Ratter et al s/n (INPA); *Deianira pallescens* Cham. & Schltdl.: G.E.Valente et al 2500 (VIC), V.C.Dalvi et al s.n. (VIC), V.C.Dalvi and D.M.T.Francino s.n. (VIC); *Schultesia doniana* Progel: G.L.Esteves et al 1770 (SPF), s.c. (US), S.Tavares 496 (US); *Schultesia guianensis* (Aubl.) Malme: G.Eiten and L.T.Eiten 4675 (SP), W.Ganev 3006 (SPF), G.Hatschbach et al 63056 (MBM); *Schultesia pachyphylla* Griseb.: M.G.L.Wanderley et al 2661 (SP), A.M.Giulietti et al s.n. (SPF), J.R.Pirani et al 5386 (SPF), V.C.Dalvi et al 51 (VIC), V.C.Dalvi et al 74 (VIC), V.C.Dalvi et al 75 (VIC); *Xestaea lisianthoides* Griseb.: P.H.Gentle 9052 (US), P.C.Standley 30379 (US), E.P.Killip 3362 (US); *Symphyllophytton caprifolioides* Gilg: J.A.Ratter et al s.n. (MBM), I.Gottsberger and G.Gottsberger 16-25771 (SP).

Exaceae – *Exacum affine* Balf.f. : L.Struwe 1080 (NYBG), R.L.Froes 29329 (SP), s.c. (RB); *Exacum macranthum* Arn.: A.G.Robyns 6960 (SP), L.H.Cramer 5157 (US), B.Bremer 875 (US), A.G.Robyns 6960 (US); *Exacum tetragonum* Roxb.: P.van Royen 3807 (NYBG), P.van Royen 3941 (NYBG), R.Torres 4822 (NYBG), F.M.Jarrett and T.P.Ramamoorty s.n. (US); *Gentianothamnus madagascariensis* Humbert: F. Rasoavimbahoaka et al 102 (US); *Sebaea sedoides* Gilg: James L.Sidey (US), James L.Sidey (US), E.Werdermann et al 2218 (US); *Tachiadenus carinatus* Griseb.: S.K.Pell et al 606 (NYBG), J.S.Miller 3691 (US), F.R.Fosterg 52578 (US); *Tachiadenus gracilis* Griseb.: F.R.Fosberg 52572 (US), R.Razakamala et al s.n. 110 (US), s.c. (US).

Gentianeae (Gentianinae) – *Crawfurdia speciosa* Wall.: s.c. (NYBG), s.c. (NYBG); *Crawfurdia trailliana* Forrest: s.c. (NYBG); *Gentiana asclepiadea* L.: Franzén et al 811 (MBM), T.Barta 250 (MBM), K.Domin s.n. (SP), s.c. (UPCB); *Gentiana bigevolii* Gray: E.Makings et al 2744 (MBM); *Gentiana campestris* L.: I.Kause s.n. (SP); *Gentiana catesbai* Walt: D.N.Wiggs and T.Bynum s.n. (SP); *Gentiana ciliata* L.: A.Zick s.n. (MBM), s.c. (MBM); *Gentiana cruciata* L.: J.Meister s.n. (MBM), S.L.Jury et al 6604 (MBM); *Gentiana inthurniana* Kern.: E.Strasseg s.n. (MBM); *Gentiana lutea* L.: s.c. (NY), W.Koch s.n. (NY); *Gentiana nivalis* L.: R.Camoletto s.n. (SP); *Gentiana pneumonanthe* L.: O.Furnrohr s.n. (MBM), G.H.Leute s.n. (MBM), s.c. (SP), J.V. Kováts s.n. (RB); *Gentiana punctata* L.: W.Repetzky s.n. (MBM); *Gentiana pyrenaica* L.: A.C.Cervi et al 4996 (MBM), A.C.Cervi et al 4996 (UPCB), K.H.Rechinger and H.Sleumer s.n. (MBM); *Gentiana sedifolia* Kunth: L.B.Holm-Nielsen and J.Jaramillo s.n. (US), P.Acevedo-Rdzg et al s.n. (US), J.L.Clark 719 (US); *Gentiana spathaceae* Kunth.: J.L.Panero et al 5575 (MBM); *Gentiana terglouensis* Hacq.: Leute and Kosh s.n. (MBM); *Gentiana verna* L.: A.C.Cervi and A. Chautems

6707 (UPCB); *Tripterospermum cordatum* (Marquand) Harry Sm.: W.Zongh-Tao et al 870362 (NYBG), L.Zhen-yu et al 1422 (US); *Tripterospermum taiwanense* (Masam.) Satake: Li Zhen-yu et al 1422 (US); H.Tsung-Hsin and Chi-Hsing Hsio (NYBG), S.F.Huang 962 (NYBG), D.E.Boufford et al 25257 (NYBG), J.C.Wang et al 3972 (MBM), W.L.Wagner 6660 (US).

Gentianeae (Swertiinae) – *Bartonia paniculata* subsp. *iodandra* (B.L.Rob.) J.M.Gillett: J.Rousseau s.n. (NYBG), M.L.Fernald and K.M.Wiegand s.n. (NYBG), M.L.Fernald and K.M.Wiegand s.n. (NYBG); *Bartonia paniculata* (Michx.) Muhl. subsp. *paniculata*: M.L.Fernald and K.M.Wiegand s.n. (NYBG); *Bartonia paniculata* (Michx.) B.L.Rob.: L.K.Magrath et al 17310 (NYBG), H.E.Ahles 36920 (NYBG); *Bartonia virginica* (L.) Britton, Sterns & Poggenb.: F.C.Mackeever s.n. (SP), M.L.Fernald et al s.n. (NYBG), R.C.Bean and D.White s.n. (NYBG), M.L.Fernald and B.Long s.n. (NYBG); *Frasera parryi* Torr.: V.Duran 3488 (NYBG), R.Grade s.n. (NYBG); *Frasera tubulosa* Coville: J.T.Howell 38580 (NYBG); J.T.Howell and G.H.True 47953 (NYBG), B.Ertter et al 6351 (NYBG); *Gentianella amarella* L. (Borner): C.G.Alm s.n. (NYBG), N.Jacobsen and J. Svendsen s.n. (NYBG), H.Smith s.n. (NYBG), J.Chrtek and B.Deylová s.n. (SP); *Gentianella cerastioides* (Kunth) Fabris: B.Sparre s.n.(MBM); *Gentianella foliosa* (Kunth) Fabris: B.Sparre s.n. (MBM); *Gentianella helianthemoides* (Gil.) Fabris: A.Kaprovickas et al 47343 (MBM), B.G.E. et al 709 (SP); *Gentianella propinqua* (Richardson) J.M.Gillett: H.Lundsden s.n. (NYBG), J.Taylor et al s.n. (NYBG), S.McDaniel et al 21931 (NYBG); *Gentianella ranunculoides* (Willd. ex Schult.) Pringle: L. Holm-Nielsen et al 5314 (MBM); *Halenia conzattii* Greenm.: J.I.Calzada 21339 (SP); *Halenia corniculata* (L.) Cornaz: G.Murata et al s.n. (MBM), H.H.Iltis et al 636 (NYBG), N.Naruhashi 1143 (NYBG), H.H.Iltis et al 873 (NYBG); *Halenia palmeri* A.Gray: R.McVaugh 21741 (NYBG),

N.H.Holmgren and T.K.Lowrey 8070 (NYBG), F.W.Pennell s.n. (NYBG); *Halenia weddelliana* Gilg : B.Ollgaard and H.Balsiev s.n. (MBM), B.Ollgaard and H.Balsiev s.n. (MBM); *Jaeschkea oligosperma* Knobl.: M.Nath. s.n. (NY); *Lomatogonium carinthiacum* (Wulfen) Rchb.: V.Zuev s.n. (NYBG), G.Nakhutsrishvili and O.Abdaladze 160 (NYBG), O.Abdaladze et al 686 (NYBG), U.Hartmann s.n. (MBM); *Lomatogonium oreocharis* C.Marquand: J.F.Rock s.n. (NYBG), J.F.Rock s.n. (NYBG), J.F.Rock (US); *Megacodon stylophorus* (C.B.Clarke) Harry Sm.: G.Forrest (US), J.F.Rock s.n. (US); *Obolaria virginica* L.: R.Kral s.n. (MBM), R.D.Thomas s.n. (NYBG), R.D.Thomas s.n. (NYBG), M.L.Fernald and B.Long s.n. (NYBG); *Swertia bimaculata* Hook.f. & Thomson ex C.B.Clarke: H.Ohashi et al 9139 (SP); *Swertia perennis* L.: M.D.Windhan and M.B.Windhan 91-219 (MBM), B.Casaseca and J.Fernandez s.n. (MBM), R.Massati and A.Wells 8267 (NYBG), R.Massati 3393 (NYBG), W.Fertig 22811 (NYBG); *Swertia pseudochinensis* H.Hara f. *alba* Y.N.Lee: Guocheng-yong 20062 (MBM); *Swertia tetrapetala* Pall.: E.Pobedimovaet et al s.n. (SP); *Veratrilla baillonii* Franch.: J.F.Rock s.n. (US), J.F.Rock s.n. (US).

Helieae – *Adenolisianthus arboreus* (Spruce ex Progel) Gilg: B.G.S.Ribeiro 1060 (RB), R.E.Schultes 5614 (NYBG), S.Madrinán and C.Barbosa 867 (NYBG), J.J.Wurdack and L.S.Adderley 43722 (NYBG); *Aripuana cullmaniorum* Struwe, Maas & V.A.Albert: P.R.J.Bamps 5406 (NYBG) , C.D.A.Mota s.n. (INPA), Calder 2668 (INPA); *Calolisianthus pedunculatus* (Cham. & Schltdl.) Gilg: V.C.Dalvi et al 76 (VIC); V.C.Dalvi et al 102 (VIC), V.C.Dalvi et al 109 (VIC); *Calolisianthus pendulus* (Mart.) Gilg: V.C.Dalvi and D.M.T.Francino 05 (VIC), V.C.Dalvi and I.A.C.Coutinho 10 (VIC), V.C.Dalvi et al 96 (VIC), V.C.Dalvi et al 97 (VIC), V.C.Dalvi et al 98 (VIC); *Calolisianthus speciosus* Gilg: V.C.Dalvi et al 106 (VIC), V.C.Dalvi et al 107 (VIC), V.C.Dalvi et al 108 (VIC); *Celiantha bella* Maguire & Steyerm.: N.T.Silva and

U.Brazão s.n. (RB), B.Maguire et al s.n. (NYBG), J.A.Steyermark s.n. (NYBG); *Celiantha chimantensis* (Steyerm. & Maguire) Maguire: J.J.Wurdack s.n. (NYBG), J.A.Steyermark s.n. (NYBG), O.Huber et al s.n. (NYBG); *Celiantha imthurniana* (Oliv.) Maguire: J.A.Steyermark s.n. (NYBG), Vareschi et al 4917 (NYBG), R.Cardona 2664 (US); *Chelonanthus acutangulus* (Ruiz & Pav.) Gilg: M.Nee 48250 (NYBG), E.P.Killip and A.C.Smith 22534 (NYBG), s.c. (INPA), Benson 8302 (UEC); *Chelonanthus alatus* Pulle: E.Freire et al 12 (RB), L.Zarucchi et al 3028 (RB), A.Lisboa 66 (RB); *Chelonanthus albus* (Spruce ex Progel) V.M.Badillo: A.Ducke s.n. (RB), A.Ducke s.n. (RB), s.c. (INPA); *Chelonanthus angustifolius* (Kunth) Gilg: G.T.Prance et al s.n. (SP 225885), J.A.Silva et al 405 (INPA), Andrade 70 (SJRP), Silva 929 (SJRP); *Chelonanthus grandiflorus* (Aubl.) E.Hassl.: C.A.Todzia et al 2307 (INPA), D.W.Stevenson 952 (INPA), B.W.P.Alburquerque et al 695 (INPA), M.F.Silva et al 618 (INPA), Castellani 14098 (UEC); *Chelonanthus matogrossensis* (J.G.M.Pers. & Maas) Struwe & V.A.Albert: G. Hatschbach 31949 (MBM), O.S.Ribas and L.B.S.Pereira 2549 (MBM), D.L.Amaral s.n (RB), s.c. (INPA), Cunha et al 440 (UEC), Nave et al 1086 (UEC); *Chelonanthus pterocaulis* Lepis: J.A.Steyermark s.n. (NYBG), S.F.R.H.J.Valles s.n. (NYBG), G.T.Prance and E.Forero 4008 (INPA), W.A.Rodrigues s.n. (INPA), I.S.Mirandoa 404 (INPA); *Chelonanthus purpurascens* (Aubl.) Struwe, S.Nilsson & V.A.Albert: P.C.Porto. 2371 (RB), L.Montone 1007 (RB), W.Fonseca s.n. (RB), J.R.Pirani et al 5441 (SPF), J.R.Pirani et al 5456 (SPF), R.Mello-Silva and R.C.Forzza 2839 (SPF), R.Mello-Silva and R.C.Forzza 2845 (SPF), M.F.Calió et al 119 (SPF), M.L.O.Trovó et al 328 (SPF), V.C.Souza et al 22940 (SPF); V.C.Dalvi et al 30 (VIC), V.C.Dalvi et al 31 (VIC), V.C.Dalvi et al 32 (VIC), V.C.Dalvi et al 36 (VIC), V.C.Dalvi et al 37 (VIC), V.C.Dalvi et al 48 (VIC), V.C.Dalvi et al 52 (VIC),

V.C.Dalvi et al 60 (VIC); *Chelonanthus viridiflorus* (Mart.) Gilg: G. Hatschbach et al 36058 (MBM), G. Hatschbach and F.J.Zelma 49058 (MBM), M.R.P.Silva and I.Fernandes 3089 (MBM), J.A.Lombardi et al 3879 (RB), S.R.Neto et al 615 (SP), J.R.Pirani et al 4048 (SP), M.Alves et al 2250 (SP), M.F.A.Calió et al 38 (SPF), M.F.A.Calió et al 83 (SPF), A.Rapini et al 495 (SPF), M.F.Calió et al 204 (SPF); V.C.Dalvi et al 02 (VIC), V.C.Dalvi et al 03 (VIC); *Chorisepalum ovatum* Gleason: B.Maguire and L.Politi s.n. (RB), K.D.Phelps and C.B.Hitchocock s.n. (NYBG), B.K.Holst and R.L.Liesner 3313 (NYBG); *Chorisepalum psychotrioides* Ewan: S.S.Tillett and C.L.Tillett s.n. (NYBG), S.S.Tillett and C.L.Tillett s.n. (NYBG), H.D.Clarke et al 11682 (NYBG); *Chorisepalum sipapoanum* (Maguire) Struwe & V.A.Albert: B.Maguire and L.Politi s.n. (NYBG), B.Maguire and L.Politi s.n. (NYBG), B.Maguire and L.Politi s.n. (NYBG); *Helia brevifolia* Cham.: R Kummrow and J.Cordeiro 3391 (MBM), O.S.Ribas et al 5762 (MBM), W.Hoehne s.n. (SPF), W.Hoehne s.n. (SPF), G.Hatschbach and S.R.Ziller 64500 (SPF), H.Longhi et al s.n. (SPF), Luederwaldt s.n. (SPF), F.C.Hoehne s.n. (SPF), G.Gehrt s.n. (SPF), M.Kuhlmann s.n. (SPF), L.Krieger 9810 (SPF), G.C.T.Ceccantini and V. Alves 1480 (SPF), M.F.Calió et al 167 (SPF), M.F.Calió et al 168 (SPF), M.L.O.Trovó et al 316 (SPF), J.P.Souza et al 297 (SPF), R.Simão-Bianchini et al 889 (SPF), R.J.F.Garcia et al 1451 (SPF), C.M.Izumisaw et al 80 (SPF), H.Serafim 27 (SPF), E.P.Santos et al724 (UPCB), s.c. (HUEFS); *Helia oblongifolia* Mart.: A.C.Brade s.n. (SP), s.c. (SP), R.M.Harley et al 25648 (SPF), G.Ceccantini 180 (SPF), M.F.Calió et al 156 (SPF), M.F.Santos et al 108 (SPF), M.F.Calió et al 205 (SPF); *Irlbachia cardonae* (Gleason) Maguire: P.J.Maas et al s.n. (RB), P.J.Maas et al s.n. (RB), P.J.Maas and J.Steyermark s.n. (RB); *Irlbachia nemorosa* (Willd. ex Roem. & Schult.) Merr.: A.Ducke 11952 (RB), A.Ducke s.n. (RB), A.Ducke s.n. (RB), A.Ducke 12528 (RB), A. Loureiro et al

s.n. (RB), F.Barros 947 (SPF), W.Montovani and D.M.S.Rocha s.n. (SPF); *Irlbachia poeppigii* (Griseb.) L.Cobb & Maas: A.Ducke s.n. (RB); W.R.Anderson 11215 (INPA); P.L.B.Lisboa 793 (INPA); *Irlbachia pratensis* (Kunth) L.Cobb & Maas: J.J.Wurdack and L.S.Adderley s.n. (NYBG); I.Cordeiro et al 120 (INPA); *Irlbachia pumila* (Benth.) Maguire: C.Farney et al 1885 (RB 281371), James L. Zarucchi et al 3080 (RB 350583), P.J.M.Maas 6867 (INPA); *Lehmanniella splendens* (Hook.) Ewan: J.L.Luteyn and R.Callejas 12502 (MBM), R.Callejas et al 4191 (NYBG); R.C.Fonenegra and F.J.Roldán 2698 (NYBG); R.Romero-Castaneda 1519 (NYBG); *Macrocarpaea angelliae* J.R.Grant & Struwe: D.Neil and L.Jost 15345 (NYBG), J.R.Grant et al 02-4289 (NYBG); *Macrocarpaea arborescens* Gilg: J.Grant and L.Struwe 01-4066 (NYBG), J.E.Madsen and L.Elleman 75280 (NYBG), J.L.Luteyn et al 6669 (NYBG); *Macrocarpaea bangiana* Gilg: St.G.Back 8699 (MBM), St.G.Back 8699 (NYBG), F.F.Alfredo and H.Huaylla 13130 (NYBG); *Macrocarpaea domingensis* Urb. & Ekman: P.Acevedo-Rdzg. et al12583 (NYBG), P.Acevedo-Rdzg. et al13813 (NYBG); P.Acevedo-Rdzg. et al12583 (US); *Macrocarpaea glaziovii* Gilg: A.Ducke and J.G.Kuhlmann s.n. (RB),S.V.A.Pessoa et al 249 (RB), M.Nadruz et al 1992 (RB), A.Ducke and J.G.Kuhlmann (SP), J.Rossini and E.Bausen 539 (SPF); *Macrocarpaea obtusifolia* (Griseb.) Gilg: M.Barreto 8845 (MBM), I.Cordeiro and R.Mello-Silva 2785 (SPF), L.Kollmann et al 1986 (SPF), V.C.Dalvi et al 85 (VIC), V.C.Dalvi et al 86 (VIC); *Macrocarpaea rubra* Malme: J.M.Silva and E.Barbosa 3732 (MBM), P.I.Oliveira 686 (MBM), D.B.Falkenberg 3846 (MBM), E.J.Stange 93 (MBM), M.G.Bovini et al 2733 (RB), E.A.Anunciação et al 20 (SP), M.Sugiyana and M.Kirizawa 1000 (SP), M.F.R.Melo et al 1053 (SP), I.Cordeiro and R.Mello-Silva 2786 (SPF), P.Affonso et al 28 (SPF), M.F.Calió et al 169 (SPF), J.R.Grant et al 09-4599

(SPF), J.R.Grant et al 09-4599 (SPF), J.R.Grant et al 09-4602 (SPF), J.R.Grant et al 09-4598 (SPF), A.C.Cervi et al 6353 (UPCB), C.R.Sakagami et al 174 (UPCB), P.B.Schwartsburd and C.K.Peres 896 (UPCB); *Macrocarpaea valerioi* Standl.: R.W.Lent 26 (NYBG), A.Jimenez 967 (NYBG), Steven R.Hill et al 17751 (NYBG); *Neblinantha parvifolia* Maguire: C.Farney et al 876 (RB), C.Farney et al 892 (RB), J.A.Steyermark s.n.(RB); *Neblinantha neblinae* Maguire: J.A.Steyermark s.n. (NYBG), T.Plowman and W.W.Thomas s.n. (NYBG), J.A.Steyermark s.n. (US); *Prepusa alata* Porto & Brade: S.Lima and Brade 14101 (RB), C.Farley and J.M.Caruso 1195 (RB), C.G.Gomes et al 153 (SPF); *Prepusa connata* Gardner: G.Martinelli 602 (RB), G.Martinelli 6102 (RB), G.Martinelli 16216 (SPF); *Prepusa hookeriana* Gardner: C.Farrey et al795 (RB), C.G.Gomes et al 153 (MBM), C.G.Gomes et al 153 (RB), A.P.Duarte s.n. (RB), C.B.Costa et al 508 (SPF), C.B.Costa et al 508 (SP), M.Nadruz et al 1735 (SPF), J.Meireles and M.K.Caddah 433 (UPCB); *Prepusa montana* Mart.: V.C.Souza et al 26365 (SP), A.Furlan et al s.n. (MBM), G.Martinelli et al 5259 (RB), G.Martinelli et al 5425 (RB), H.C.Lima et al 3900 (RB), N.L.de Menezes et al s.n. (SPF), A.Furlan et al s.n. (SPF), G.Hatschbach and O.Guimarães 42398 (SPF), R.M.Harley 22878 (SPF), R.M.Harley 22755 (SPF), A.Freire-Fierro et al 1749 (SPF), A.Freire-Fierro et al 1767 (SPF), W.Ganev 816 (SPF), N.Hind et al 3165 (SPF), A.Oliveira et al 64 (SPF), T.B. Cavalcanti et al 3214 (SPF), M.F.Calió et al 116 (SPF), J.L.Hage et al 2330 (SPF), Andrade-Lima 6118 (SPF), E.Melo et al 4794 (SPF), C.N.Fraga et al 2691 (SPF), G.Hatschbach and R. Kummarow 47930 (UPCB); *Prepusa viridiflora* Brade: C.N.Fraga and L.Kollmann 722 (RB), Brade 14782 (RB), L.Kollmann and C.N.Fraga 3188 (SPF), P.H.Labiak et al 4209 (UPCB), C.N.Fraga et al 2233 (UPCB), R.C.Forzza et al 4955 (UPCB), L.Kollmann and A.P.Fontana 11081 (UPCB); *Purdieanthus pulcher* (Hook.) Gilg: M.L.Grant 10519 (NYBG), H.St John

20673 (NYBG), M.L.Grant 10519 (NYBG); *Rogersonanthus arboreus* (Britton) Maguire & B.M.Boom: O.Huber et al s.n. (NYBG), J.L.Luteyn and J.A.Steyermarck 9606 (NYBG), J.A.Steyermarck and J.J.Wurdack s.n. (NYBG); *Rogersonanthus quelchii* (N.E.Br.) Maguire & B.M.Boom: B.Maguire s.n. (NYBG), B.Maguire s.n. (NYBG), G.H.H.Tate 397 (NYBG), P.Fiaschi and G.M.Plunklet 3192 (SPF); *Roraimaea aurantiaca* Struwe, S.Nilsson & V.A.Albert: C.A/C/Ferreira 9125 (INPA); *Senaea coerulea* Taub.: M.C.E.Amaral et al s.n. (SP), M.C.E.Amaral et al s.n. (SPF), Dora Ramariz 127 (RB); *Senaea janeirensis* Brade: S.Lima and Brade 14215 (RB), J.Santos Lima s.n. (RB), G.Martinelli et al 12003(RB), *Sipapoantha ostrina* Maguire & B.M.Boom: B.Maguire and L.Politi s.n. (NYBG), B.Maguire et al s.n. (NYBG), J.A. Steyermark et al s.n. (NYBG); *Symbolanthus australis* Struwe: Yunga 339 (US), C.Davidson 4962 (US), H.W.Hodge 6032 (US); *Symbolanthus brittonianus* Gilg: O.Buchtien 5523 (SP), R.S.Willians 1446 (US), O.Buchtien s.n. (US), R.D.Metcalf s.n. (US); *Symbolanthus calygonus* Griseb. Ou Gilg: S.Knapp et al 2111 (MBM), G.Harling and L.Andersson s.n. (MBM), M.Y.Rimachi 3711 (MBM), P.A.Loizeau et al 713 (MBM), St.G.Beck 8740 (MBM), R.Ferreyra 6852 (US), H.A.Hallard 21095 (US); *Symbolanthus elisabethae* Gilg: S.E.Tillet et al s.n. (NYBG), J.A.Steyermark s.n. (US), E.S.S.Silva 11 (INPA); *Symbolanthus frigidus* (Sw.) Struwe & K.Gould: J.A. Steyermark et al s.n. (NYBG-D106), s.c. (US), H.Stehle 1595 (US), s.c. (US); *Symbolanthus mathewsii* (Griseb.) Ewan: F.Woitkowski 8288 (US), P.C.Hutchison and J.K.Wright 5565 (US), F.R.Fosberg and M.A.Giler 23172 (US), A.Lopez et al s.n. (US); *Symbolanthus yaviensis* Steyermark: B.Maguire and C.K.Maguire s.n. (NYBG), B.Maguire and C.K.Maguire s.n. (NYBG), O.Huber s.n. (NYBG); *Symbolanthus pulcherrimus* Gilg: F.Almeda and T.F.Daniel 7213 (MBM), A.Chacón 317 (NYBG), R.L.Wilbur et al

13476 (NYBG); *Tachia grandiflora* Maguire & Weaver: Ducke s.n. (RB), L.A.Pereira and J.O.Cardoso 1247 (RB), L.A.Pereira et al 1186 (RB), J.E.L.S. Ribeiro et al 839 (SP), J.C.Ongley and J.F.Ramos s.n. (NYBG), A.A.Oliveira et al s.n. (NYBG), C.Sastre et al 3878 (NYBG), J.H.E.Rova et al s.n. (NYBG), A.M.Pohlit s.n. (INPA); *Tachia guianensis* Aubl.: P.A. Loizeau et al 766 (MBM), T.G.Tutin 303 (RB), B.Maguire s.n. (NYBG), Oldeman 2182 (NYBG), Lescure 236 (NYBG); *Tachia loretensis* Maguire & Weaver: M.Y.Rimachi 6243 (RB), G. Klug s.n. (NYBG), L.Hendrix 300 (NYBG), P.Acevedo et al 1624 (NYBG); *Tachia occidentalis* Maguire & Weaver: S.McDaniel et al 22106 (MBM), M.C.Ferreira and A.P.de Araújo 52 (RB), M.F.Silva and P.Machado 1499 (INPA), G.T.Prance et al 12098 (INPA); *Tachia parviflora* Maguire & Weaver: A.Rodr. et al 6993 (NYBG), S.Knapp 7888 (NYBG); *Tetrapollinia caerulescens* (Aubl.) Maguire & B.M.Boom: L.O.A.Teixeira et al 1293 (RB), H.S.Irwin et al s.n. (RB), F.Barros 862 (RB), R.M.Harley et al 25990 (SP), R.C.Forzza and R.Mello-Silva 3748 (SPF), M.F.Calió et al 154 (SPF), P.G.Windisch et al 7836 (SPF), P.G.Windisch et al 7191 (SPF).

Potalieae (Faroinae) – *Enicostema axillare* (Lam.) A.Raynal: R.Spellenberg s.n. (NYBG), A.Rahman s.n. (NYBG), S.U.Abedin 3431 (NYBG); *Enicostema verticillatum* Engl. ex Gilg: W.D.Stevens and B.A.Krukoff s.n. (MBM), S.R. Hill 22020 (NYBG), L.M.Andrews s.n. (NYBG), N.A.Harriman 17572 (NYBG); *Faroa malaissei* Bamps: s.c. (MBM); *Neurotheca loeselioides* (Spruce ex Progel) Baill.: A.Ducke s.n. (RB), A.Janssen 131 (RB), A.C.Black 50-10007 (RB), O.P.Monteiro and J.F.Ramos 832 (INPA), M.N.Silva et al 182 (INPA), B.W.Nelson et al 1483 (INPA).

Potalieae (Lisianthiinae) – *Lisianthius axillaris* Kuntze: C.Soto and D.Álvarez 23568 (SP), L.R.Landrum and S.Landrum 6464 (MBM), E. Martinez S. 27262 (MBM),

D.Álvarez 1548 (MBM), C.H.Ramos et al 2107 (MBM); *Lisianthius jefensis* A.Robyns & T.S.Elias: J.P.Folsom et al 5655 (MBM), J.L.Luteyn 14796 (NYBG), R.E.Weaver and R.L.Wilbur 2243 (NYBG), J.H.Kirkbride et al 16 (NYBG); *Lisianthius laxiflorus* Urb.: L.Struwe and C.Specht s.n. (NYBG), J.A.Cedenõ and N.Cabezudo 258 (NYBG), J.L.Luteyn 11464 (NYBG); *Lisianthius saponarioides* Cham. & Schltdl.: J.Calónico S. 24433 (MBM), E.Martinéz S. 35659 (MBM), G.Aguilar M. and D.Alvaréz M. 3513 (MBM).

Potalieae (Potaliinae) – *Fagraea ceilanica* Thunb.: H.Lorenzi s.n. (SP); *Potalia amara* Aubl.: S.A.Mori et al 24073 (NYBG), S.A.Mori et al 24107 (NYBG), R.S.Cowan s.n. (NYBG); *Potalia resinifera* Mart.: G.T.Prance et al s.n. (NYBG), I.L.Amaral et al 218 (NYBG), A.Henderson and F.G.Padilha 2034 (NYBG), A.Knapp and J.Mallet 8492 (NYBG).

Saccifolieae – *Hockinia montana* Gardner: V.C.Dalvi et al 101 (VIC); *Voyriella parviflora* Miq.: M.J.Pires and N.T.Silva 1641 (NYBG), E.A.Christenson and S.R.George 1796 (NYBG), B.Maguire et al s.n. (NYBG), A.Ducke s.n. (INPA).

Voyrieae - *Voyria aphylla* (Jacq.) Pers.: J.S.Silva 542 (SP), P.Fiashi et al 708 (SPF), G.T.Prance et al s.n. (NYBG), D.G.Campbell et al s.n. (NYBG), A.S.Tavares et al 93 (NYBG); *Voyria aurantiaca* Splitg.: J.J.Wurdack and J.C.Monachino s.n. (US), G.Cremes and J.J.Granville 13677 (US), C.Jativa and C.Epling s.n. (US).

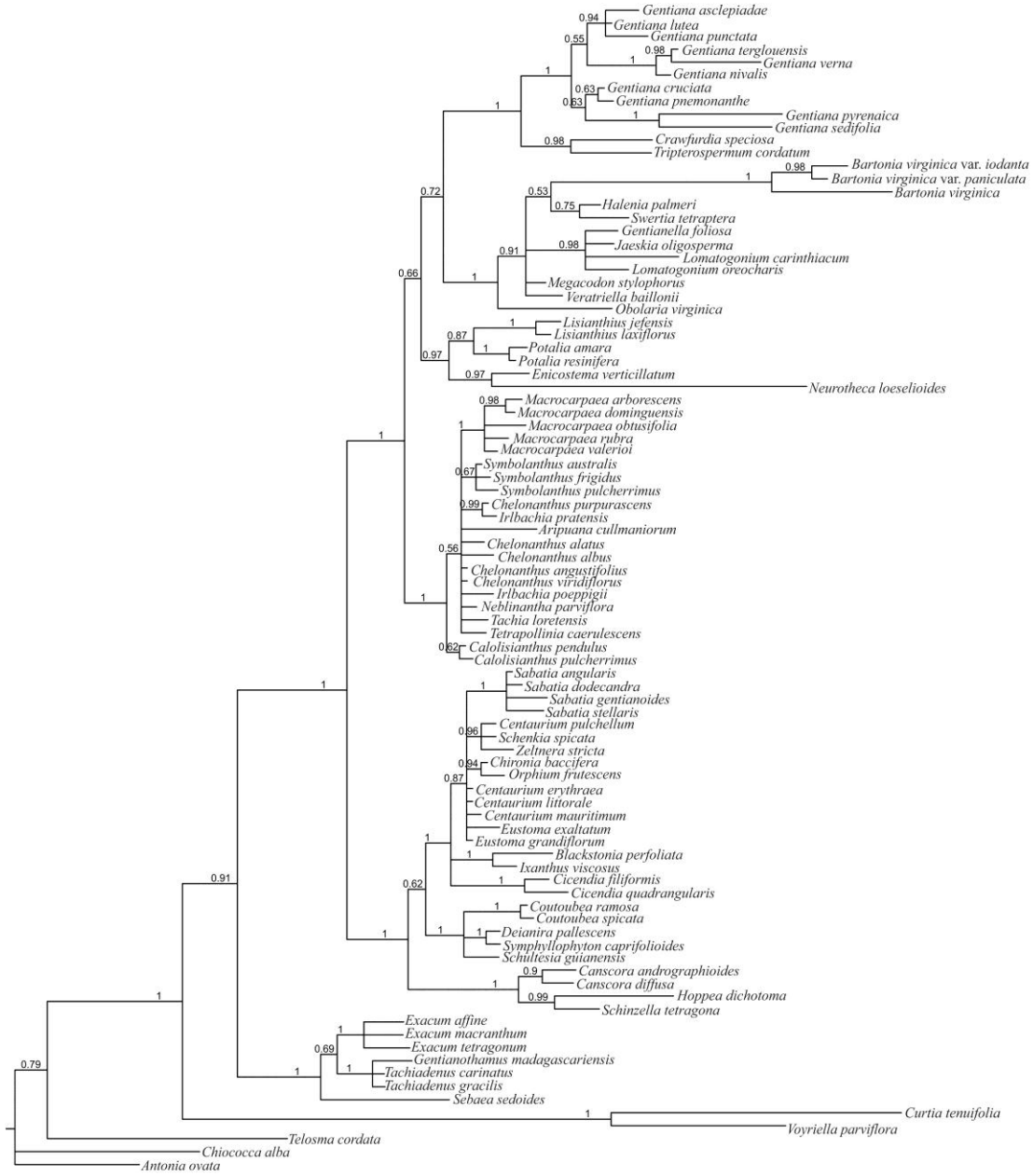

Fig. S1. Phylogenetic tree of Gentianaceae derived from the Bayesian analysis of the *trnL* dataset with posterior probabilities indicated on the branches.

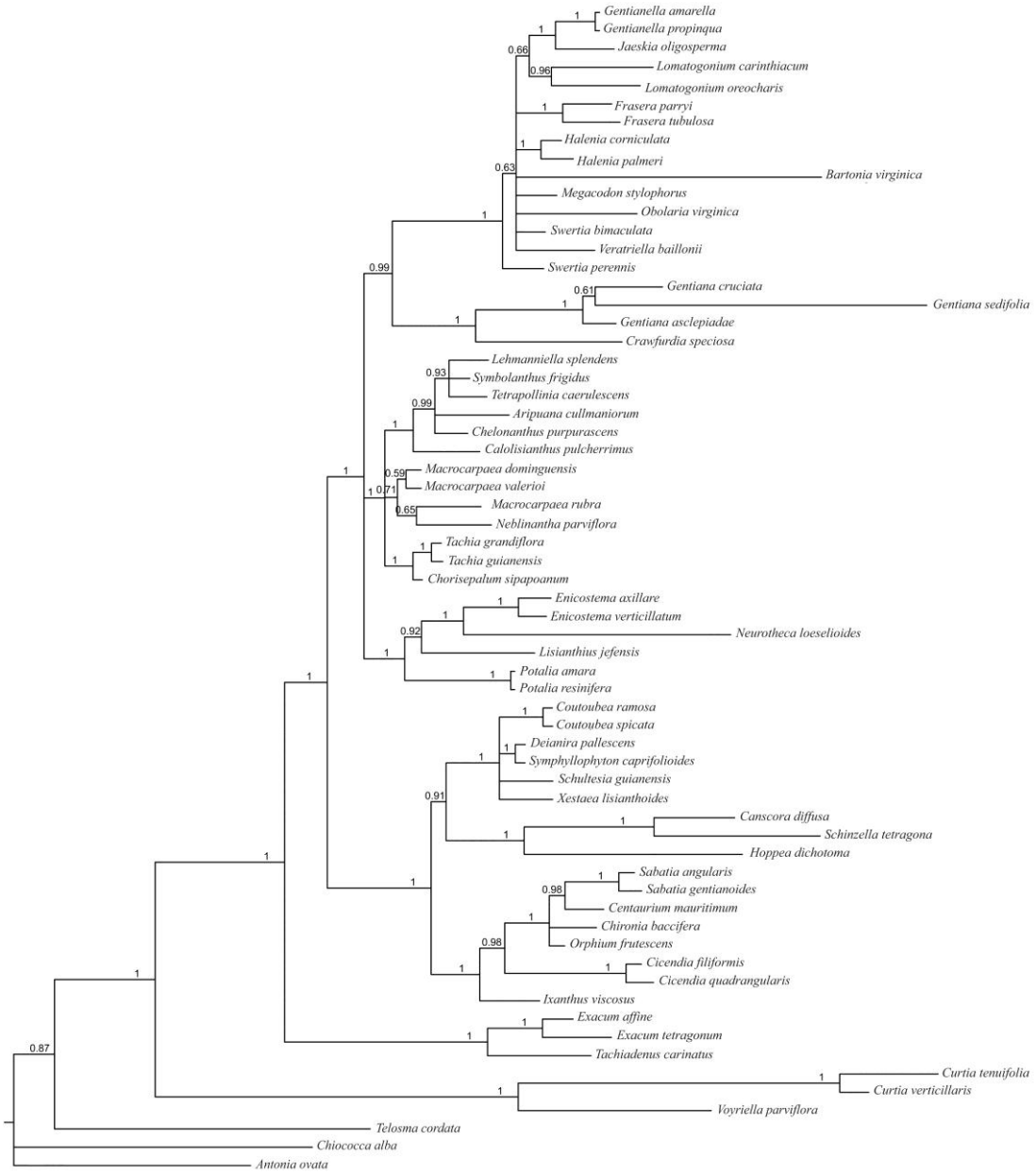

Fig. S2. Phylogenetic tree of Gentianaceae derived from the Bayesian analysis of the *matK* dataset with posterior probabilities indicated on the branches.

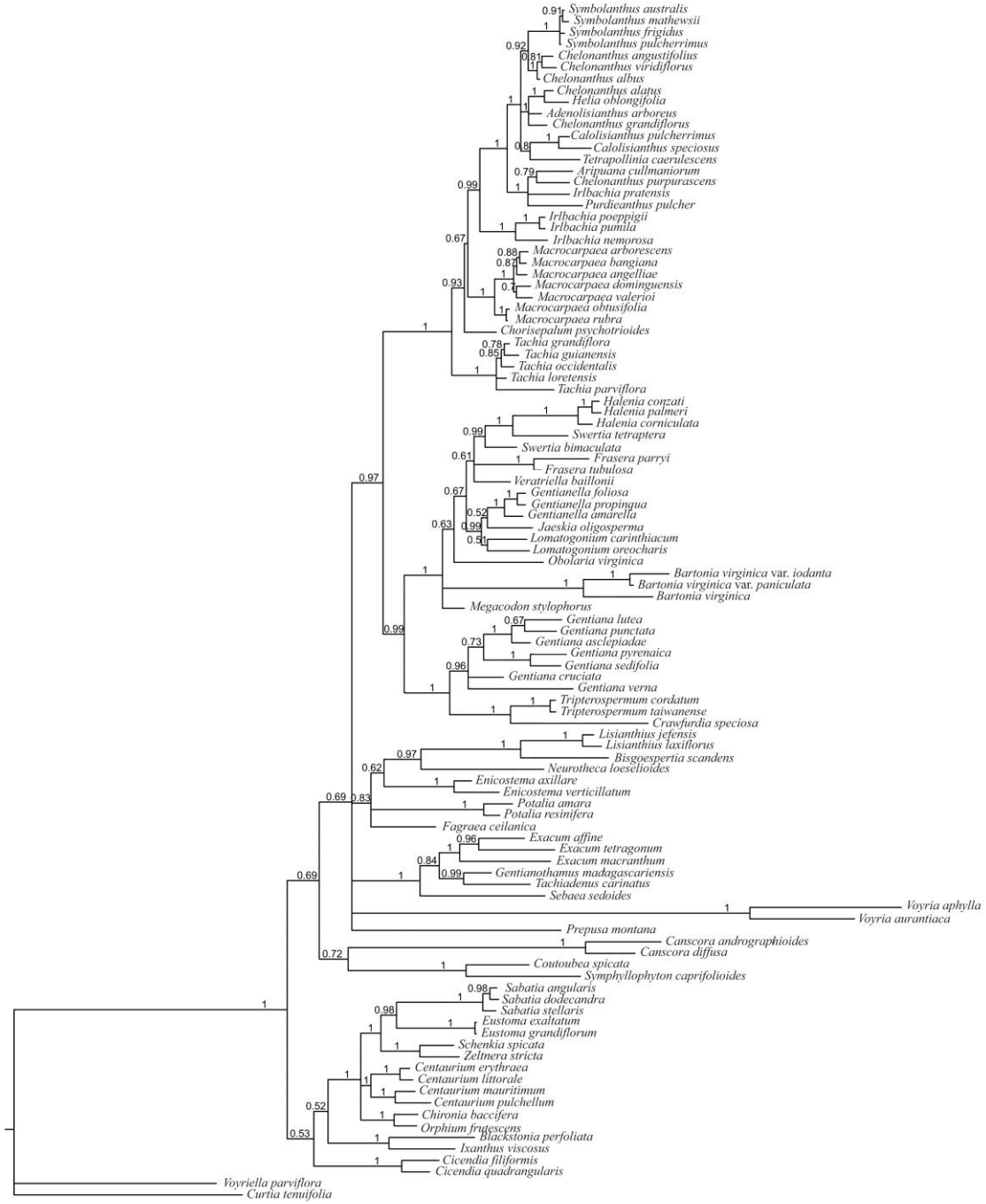

Fig. S3. Phylogenetic tree of Gentianaceae derived from the Bayesian analysis of the ITS dataset with posterior probabilities indicated on the branches.

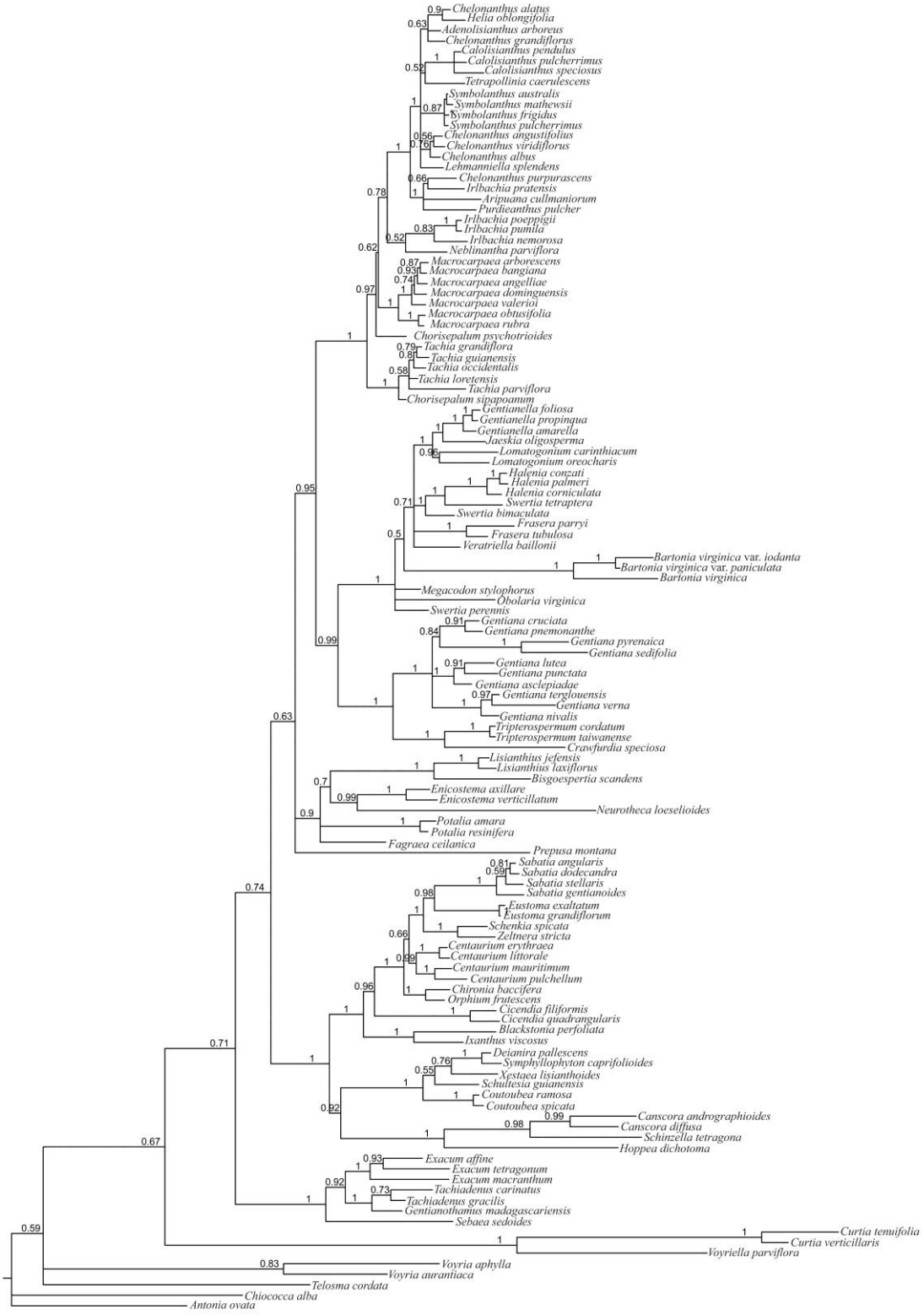

Fig. S4. Phylogenetic tree of Gentianaceae derived from the Bayesian analysis of the combined dataset with posterior probabilities indicated on the branches.

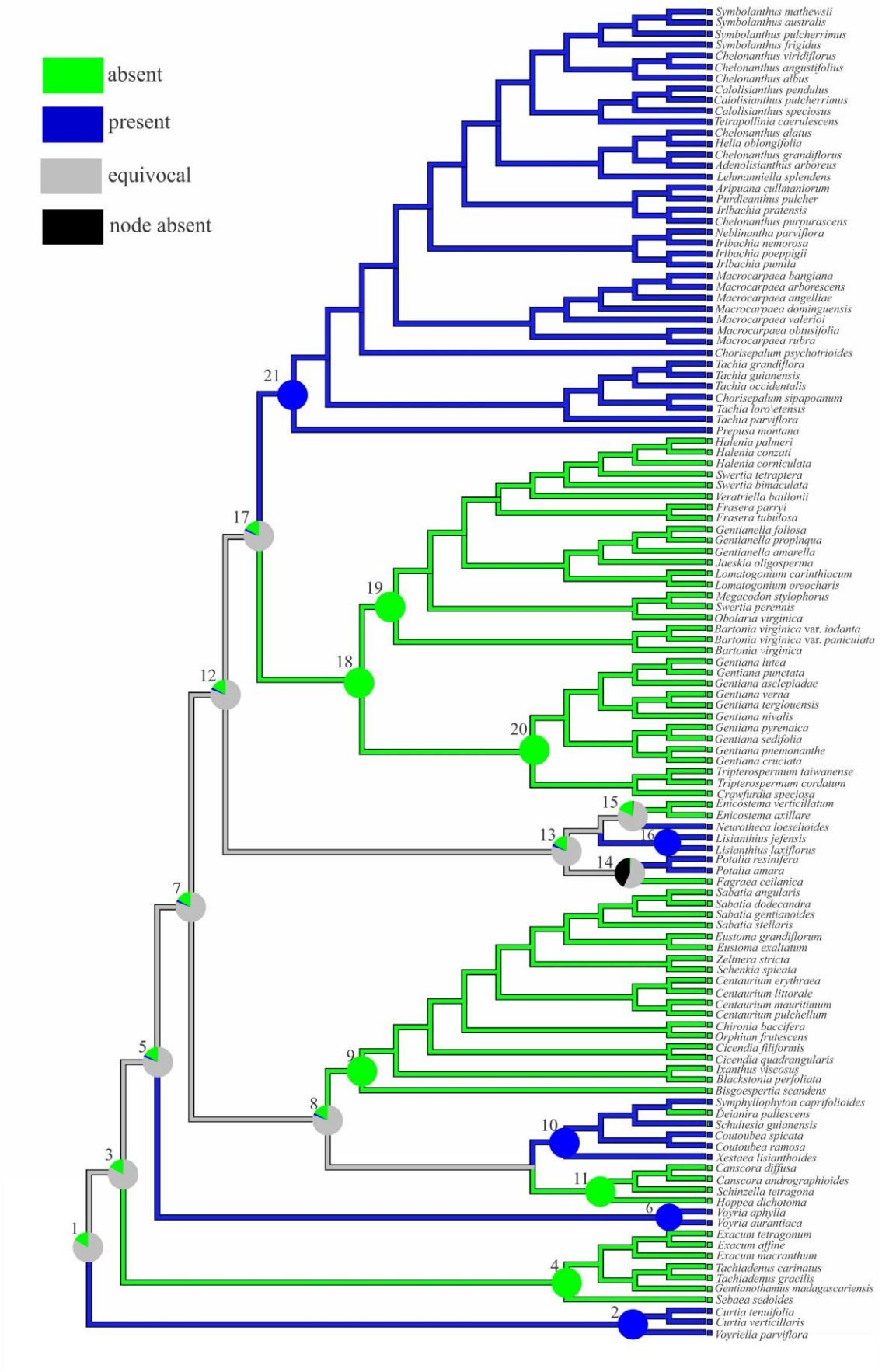
absent
present
equivocal
node absent
Symbolanthus mathewsii
Symbolanthus australis
Symbolanthus pulcherrimus
Symbolanthus frigidus
Chelonanthus viridiflorus
Chelonanthus angustifolius
Chelonanthus albus
Calolisianthus pendulus
Calolisianthus pulcherrimus
Calolisianthus speciosus
Tetrapollinia caerulescens
Chelonanthus alatus
Helia oblongifolia
Chelonanthus grandiflorus
Adenolisianthus arboreus
Lehmanniella splendens
Aripuana cullmaniorum
Purdieanthus pulcher
Irlbachia pratensis
Chelonanthus purpurascens
Neblinantha parviflora
Irlbachia nemorosa
Irlbachia poeppigii
Irlbachia pumila
Macrocarpaea bangiana
Macrocarpaea arborescens
Macrocarpaea angelliae
Macrocarpaea dominguensis
Macrocarpaea valerioi
Macrocarpaea obtusifolia
Macrocarpaea rubra
Chorisepalum psychotrioides
Tachia grandiflora
Tachia guianensis
Tachia occidentalis
Chorisepalum sipapoanum
Tachia loro\etensis
Tachia parviflora
Prepusa montana
Halenia palmeri
Halenia conzati
Halenia corniculata
Swertia tetraptera
Swertia bimaculata
Veratriella baillonii
Frasera parryi
Frasera tubulosa
Gentianella foliosa
Gentianella propinqua
Gentianella amarella
Jaeskia oligosperma
Lomatogonium carinthiacum
Lomatogonium oreocharis
Megacodon stylophorus
Swertia perennis
Obolaria virginica
Bartonia virginica var. iodanta
Bartonia virginica var. paniculata
Bartonia virginica
Gentiana lutea
Gentiana punctata
Gentiana asclepiadae
Gentiana verna
Gentiana terglouensis
Gentiana nivalis
Gentiana pyrenaica
Gentiana sedifolia
Gentiana pnemonanthe
Gentiana cruciata
Tripterospermum taiwanense
Tripterospermum cordatum
Crawfurdia speciosa
Enicostema verticillatum
Enicostema axillare
Neurotheca loeselioides
Lisianthius jefensis
Lisianthius laxiflorus
Potalia resinifera
Potalia amara
Fagraea ceilanica
Sabatia angularis
Sabatia dodecandra
Sabatia gentianoides
Sabatia stellaris
Eustoma grandiflorum
Eustoma exaltatum
Zeltnera stricta
Schenkia spicata
Centaurium erythraea
Centaurium littorale
Centaurium mauritimum
Centaurium pulchellum
Chironia baccifera
Orphium frutescens
Cicendia filiformis
Cicendia quadrangularis
Ixanthus viscosus
Blackstonia perfoliata
Bisgoespertia scandens
Symphyllophyton caprifolioides
Deianira pallescens
Schultesia guianensis
Coutoubea spicata
Coutoubea ramosa
Xestaea lisianthoides
Canscora diffusa
Canscora andrographioides
Schinzella tetragona
Hoppea dichotoma
Voyria aphylla
Voyria aurantiaca
Exacum tetragonum
Exacum affine
Exacum macranthum
Tachiadenus carinatus
Tachiadenus gracilis
Gentianothamus madagascariensis
Sebaea sedoides
Curtia tenuifolia
Curtia verticillaris
Voyriella parviflora
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Fig. S5. Ancestral character state reconstruction for presence of foliar EFNs based on maximum parsimony and a sample of 1000 post-burn-in BEAST trees. The character states assigned to each species are given next to the species names. Branches are colored according to the inferred state using parsimony (grey lines indicate ambiguous parsimony reconstructions). Pie charts on the nodes show the proportion of trees in the post-burn-in sample for which a particular reconstruction was recovered; black indicates that a node is absent and grey indicates that the reconstruction is equivocal in a proportion of the trees.

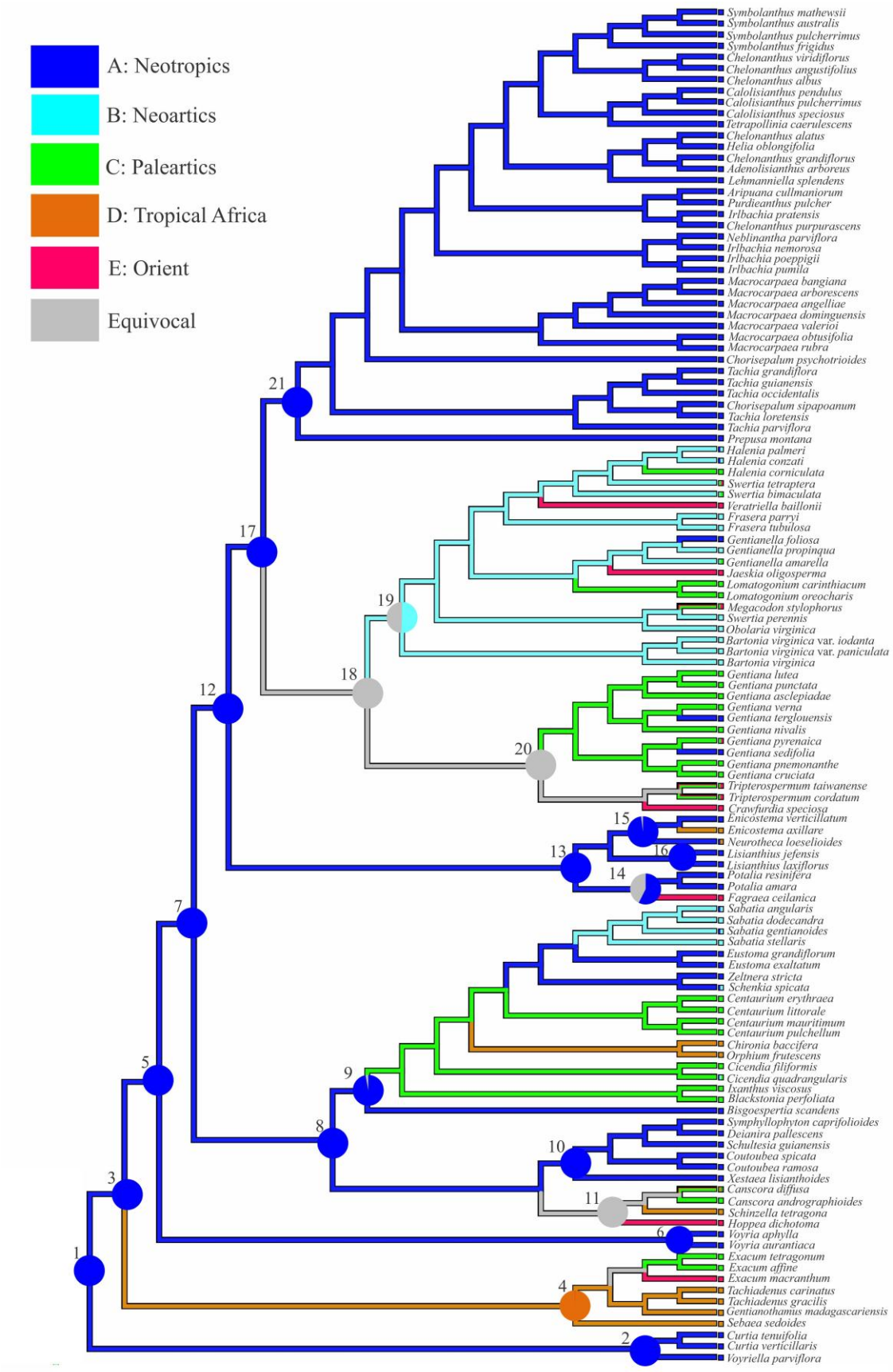
A: Neotropics
B: Neoartics
C: Paleartics
D: Tropical Africa
E: Orient
Equivocal
Symbolanthus mathewsii
Symbolanthus australis
Symbolanthus pulcherrimus
Symbolanthus frigidus
Chelonanthus viridiflorus
Chelonanthus angustifolius
Chelonanthus albus
Calolisianthus pendulus
Calolisianthus pulcherrimus
Calolisianthus speciosus
Tetrapollinia caerulescens
Chelonanthus alatus
Helia oblongifolia
Chelonanthus grandiflorus
Adenolisianthus arboreus
Lehmanniella splendens
Aripuana cullmaniorum
Purdieanthus pulcher
Irlbachia pratensis
Chelonanthus purpurascens
Neblinantha parviflora
Irlbachia nemorosa
Irlbachia poeppigii
Irlbachia pumila
Macrocarpaea bangiana
Macrocarpaea arborescens
Macrocarpaea angelliae
Macrocarpaea dominguensis
Macrocarpaea valerioi
Macrocarpaea obtusifolia
Macrocarpaea rubra
Chorisepalum psychotrioides
Tachia grandiflora
Tachia guianensis
Tachia occidentalis
Chorisepalum sipapoanum
Tachia loretensis
Tachia parviflora
Prepusa montana
Halenia palmeri
Halenia conzati
Halenia corniculata
Swertia tetraptera
Swertia bimaculata
Veratriella baillonii
Frasera parryi
Frasera tubulosa
Gentianella foliosa
Gentianella propinqua
Gentianella amarella
Jaeskia oligosperma
Lomatogonium carinthiacum
Lomatogonium oreocharis
Megacodon stylophorus
Swertia perennis
Obolaria virginica
Bartonia virginica var. iodanta
Bartonia virginica var. paniculata
Bartonia virginica
Gentiana lutea
Gentiana punctata
Gentiana asclepiadae
Gentiana verna
Gentiana terglouensis
Gentiana nivalis
Gentiana pyrenaica
Gentiana sedifolia
Gentiana pnemonanthe
Gentiana cruciata
Tripterospermum taiwanense
Tripterospermum cordatum
Crawfurdia speciosa
Enicostema verticillatum
Enicostema axillare
Neurotheca loeselioides
Lisianthius jefensis
Lisianthius laxiflorus
Potalia resinifera
Potalia amara
Fagraea ceilanica
Sabatia angularis
Sabatia dodecandra
Sabatia gentianoides
Sabatia stellaris
Eustoma grandiflorum
Eustoma exaltatum
Zeltnera stricta
Schenkia spicata
Centaurium erythraea
Centaurium littorale
Centaurium mauritimum
Centaurium pulchellum
Chironia baccifera
Orphium frutescens
Cicendia filiformis
Cicendia quadrangularis
Ixanthus viscosus
Blackstonia perfoliata
Bisgoespertia scandens
Symphyllophyton caprifolioides
Deianira pallescens
Schultesia guianensis
Coutoubea spicata
Coutoubea ramosa
Xestaea lisianthoides
Canscora diffusa
Canscora andrographioides
Schinzella tetragona
Hoppea dichotoma
Voyria aphylla
Voyria aurantiaca
Exacum tetragonum
Exacum affine
Exacum macranthum
Tachiadenus carinatus
Tachiadenus gracilis
Gentianothamus madagascariensis
Sebaea sedoides
Curtia tenuifolia
Curtia verticillaris
Voyriella parviflora
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Fig. S6. Ancestral character state reconstruction for geographic distribution based on maximum parsimony and a sample of 1000 post-burn-in BEAST trees. The character states assigned to each species are given next to the species names. Branches are colored according to the inferred state using parsimony (grey lines indicate ambiguous parsimony reconstructions). Pie charts on the nodes show the proportion of trees in the post-burn-in sample for which a particular reconstruction was recovered; black indicates that a node is absent and grey indicates that the reconstruction is equivocal in a proportion of the trees.

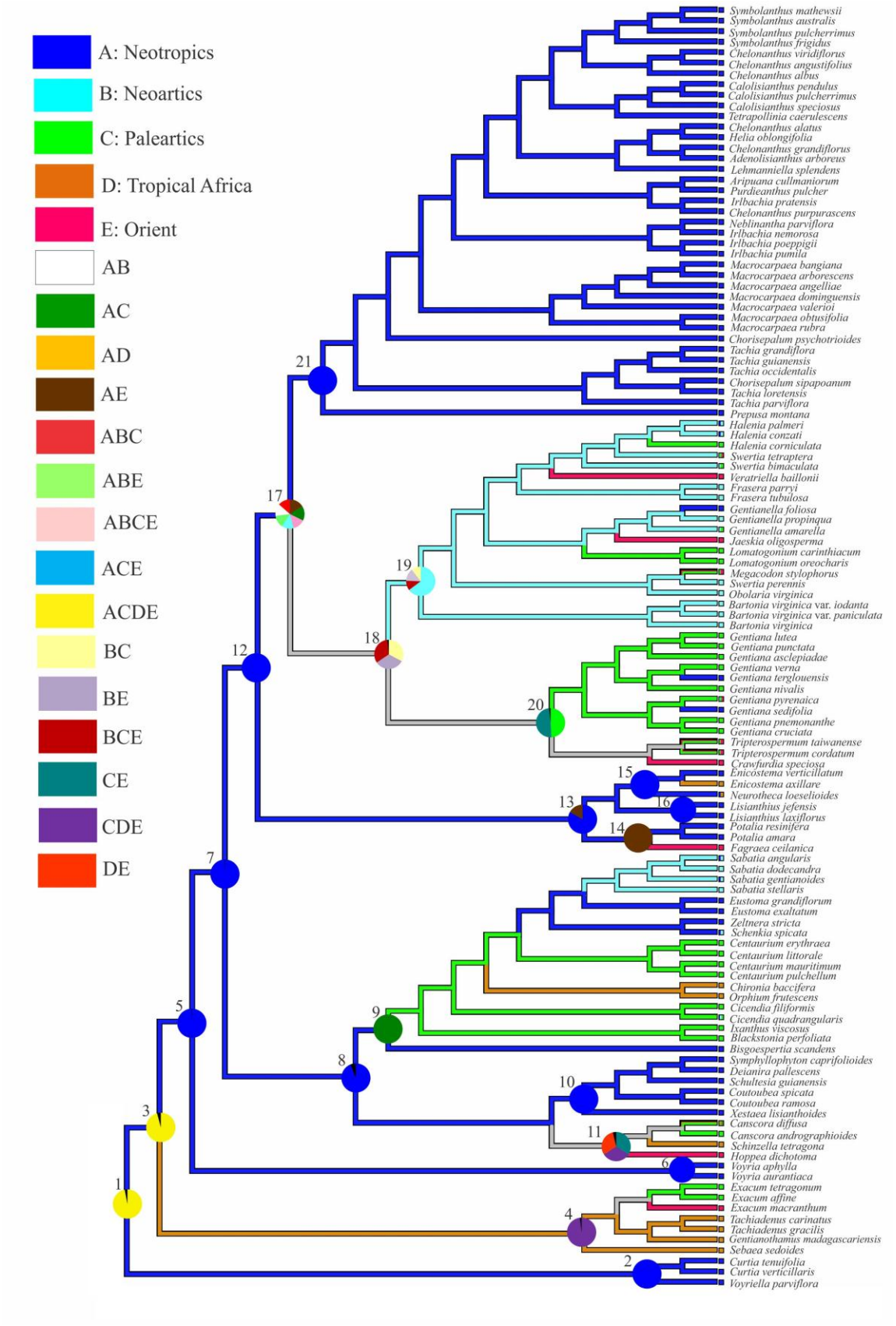
A: Neotropics
B: Neoartics
C: Paleartics
D: Tropical Africa
E: Orient
AB
AC
AD
AE
ABC
ABE
ABCE
ACE
ACDE
BC
BE
BCE
CE
CDE
DE
Symbolanthus mathewsii
Symbolanthus australis
Symbolanthus pulcherrimus
Symbolanthus frigidus
Chelonanthus viridiflorus
Chelonanthus angustifolius
Chelonanthus albus
Calolisianthus pendulus
Calolisianthus pulcherrimus
Calolisianthus speciosus
Tetrapollinia caerulescens
Chelonanthus alatus
Helia oblongifolia
Chelonanthus grandiflorus
Adenolisianthus arboreus
Lehmanniella splendens
Aripuana cullmaniorum
Purdieanthus pulcher
Irlbachia pratensis
Chelonanthus purpurascens
Neblinantha parviflora
Irlbachia nemorosa
Irlbachia poeppigii
Irlbachia pumila
Macrocarpaea bangiana
Macrocarpaea arborescens
Macrocarpaea angelliae
Macrocarpaea dominguensis
Macrocarpaea valerioi
Macrocarpaea obtusifolia
Macrocarpaea rubra
Chorisepalum psychotrioides
Tachia grandiflora
Tachia guianensis
Tachia occidentalis
Chorisepalum sipapoanum
Tachia loretensis
Tachia parviflora
Prepusa montana
Halenia palmeri
Halenia conzati
Halenia corniculata
Swertia tetraptera
Swertia bimaculata
Veratriella baillonii
Frasera parryi
Frasera tubulosa
Gentianella foliosa
Gentianella propinqua
Gentianella amarella
Jaeskia oligosperma
Lomatogonium carinthiacum
Lomatogonium oreocharis
Megacodon stylophorus
Swertia perennis
Obolaria virginica
Bartonia virginica var. iodanta
Bartonia virginica var. paniculata
Bartonia virginica
Gentiana lutea
Gentiana punctata
Gentiana asclepiadae
Gentiana verna
Gentiana terglouensis
Gentiana nivalis
Gentiana pyrenaica
Gentiana sedifolia
Gentiana pneumonanthe
Gentiana cruciata
Tripterospermum taiwanense
Tripterospermum cordatum
Crawfurdia speciosa
Enicostema verticillatum
Enicostema axillare
Neurotheca loeselioides
Lisianthius jefensis
Lisianthius laxiflorus
Potalia resinifera
Potalia amara
Fagraea ceilanica
Sabatia angularis
Sabatia dodecandra
Sabatia gentianoides
Sabatia stellaris
Eustoma grandiflorum
Eustoma exaltatum
Zeltnera stricta
Schenkia spicata
Centaurium erythraea
Centaurium littorale
Centaurium mauritimum
Centaurium pulchellum
Chironia baccifera
Orphium frutescens
Cicendia filiformis
Cicendia quadrangularis
Ixanthus viscosus
Blackstonia perfoliata
Bisgoespertia scandens
Symphyllophyton caprifolioides
Deianira pallescens
Schultesia guianensis
Coutoubea spicata
Coutoubea ramosa
Xestaea lisianthoides
Canscora diffusa
Canscora andrographioides
Schinzella tetragona
Hoppea dichotoma
Voyria aphylla
Voyria aurantiaca
Exacum tetragonum
Exacum affine
Exacum macranthum
Tachiadenus carinatus
Tachiadenus gracilis
Gentianothamus madagascariensis
Sebaea sedoides
Curtia tenuifolia
Curtia verticillaris
Voyriella parviflora
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Fig. S7. Ancestral character state reconstruction for geographic distribution based on maximum parsimony and statistical dispersal-vicariance analysis (S-DIVA). The character states assigned to each species are given next to the species names. Branches are colored according to the parsimony reconstruction (grey lines indicate ambiguous parsimony reconstructions), and the pie charts on the nodes show the results of the S-DIVA reconstruction (areas with frequencies below 0.05 are collectively represented in black).

CAPÍTULO 5

ANATOMIA FOLIAR DE HELIEAE (GENTIANACEAE): CONSIDERAÇÕES TAXONÔMICAS

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Anatomia foliar de Helieae (Gentianaceae): considerações taxonômicas

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Resumo

Helieae é a segunda maior tribo da família Gentianaceae, compreendendo 23 gêneros e cerca de 220 espécies. Destaca-se como um grupo de taxonomia complexa e controversa com difícil delimitação das espécies e dos gêneros. Estudos filogenéticos recentes sustentaram a monofilia da tribo e de diversos gêneros, entretanto, apontaram a necessidade de reclassificação de alguns gêneros parafiléticos. Objetivamos caracterizar a anatomia foliar de 60 espécies pertencentes a 21 gêneros, e identificar caracteres que possam subsidiar a delimitação dos gêneros, a identificação das espécies e a resolução dos problemas taxonômicos dentro da tribo. Amostras foliares, coletadas em campo ou provenientes de exsicatas, foram submetidas a técnicas usuais em anatomia para obtenção de cortes e diafanização. Análise de similaridade utilizando o Índice de Sorensen foi conduzida. Os caracteres anatômicos foliares possibilitaram o reconhecimento de espécies e a delimitação de 15 dos 21 gêneros estudados, com exceção dos gêneros *Chelonanthus, Celiantha, Helia, Irlbachia*, *Neblinantha e Symbolanthus.* Dentre os caracteres anatômicos foliares importantes para a taxonomia de Helieae destacam-se: presença/ausência de hipoderme bem como sua ocorrência; espessura das paredes periclinais externas e sinuosidade das paredes anticlinais das células epidérmicas; tipo e posição dos estômatos; conformação da nervura mediana; tipo e número de feixes vasculares presentes na nervura mediana; presença/ausência de cristais e; presença/ausência e distribuição das esclereides, papilas, tricomas e nectários. Os dados obtidos evidenciam que os caracteres anatômicos representam uma fonte de dados importante em um grupo de taxonomia complicada como Helieae.

Palavras-chave: coléteres - estruturas secretoras - nectários extraflorais – taxonomia - tricomas

Introdução

Gentianaceae Juss. é uma família de Angiospermas, incluída na ordem Gentianales juntamente com Apocynaceae, Loganiaceae, Gelsemiaceae e Rubiaceae (APG 2009). A família compreende sete tribos monofiléticas Chironieae, Exaceae, Gentianeae, Helieae, Potalieae, Saccifolieae e Voyrieae (Struwe et al. 2002; Merckx et al. 2013), com 91 gêneros e cerca de 1700 espécies (Gentian Research Network 2014).

Helieae Gilg é a segunda maior tribo, tanto em número de gênero como em número de espécies, compreendendo 23 gêneros e cerca de 220 espécies (Albert and Stuwe 2002) de taxonomia complexa e controversa (Weaver 1972; Struwe et al. 2002). A tribo é exclusiva dos neotrópicos e a maioria dos gêneros apresenta distribuição geográfica restrita (Struwe et al. 2002).

Embora os dados moleculares tenham contribuído substancialmente para o conhecimento da filogenia do grupo, ainda há dificuldades quanto à circunscrição dos gêneros e à distinção das espécies (Albert & Struwe 2002). A insuficiência de dados sobre a morfologia e, em especial, sobre a anatomia das espécies (Mészaros et al. 2002) constitui uma lacuna do conhecimento e obstáculo à identificação de possíveis sinapomorfias para a família, tribos e gêneros.

Dados moleculares acrescidos de dados morfológicos, micromorfológicos e palinológicos sustentaram a monofilia da tribo Helieae e de diversos gêneros, entretanto apontam para a necessidade de reclassificação de alguns gêneros parafiléticos como *Chelonanthus* Gilg e *Irlbachia* Mart. (Struwe et al. 2009). Nestes estudos, *Celiantha* Maguire, *Prepusa* Mart e *Senaea* Taub. aparecem como grupo-irmão do restante dos gêneros de Helieae que foram agrupados em dois subclados ("subclado *Symbolanthus*" e "subclado *Macrocarpaea*"). Tais resultados indicam a necessidade de aporte de dados

advindos de novas fontes com o intuito de esclarecer as relações filogenéticas entre os gêneros da tribo e delimitar adequadamente estes gêneros polifiléticos.

Estudos anatômicos com enfoque taxonômico têm evidenciado a importância dos caracteres anatômicos para a taxonomia e filogenia de Gentianaceae (Delgado et al. 2009; Delgado et al. 2011a,b; Dalvi et al. 2013, 2014; Guimarães et al. 2013). Assim, o presente trabalho objetivou caracterizar a anatomia foliar de espécies de Helieae e identificar caracteres que possam subsidiar a delimitação dos gêneros, a identificação das espécies e a resolução dos problemas taxonômicos dentro da tribo.

Material e métodos

Amostras foliares foram obtidas em excursões de campo, realizadas de março de 2008 a julho de 2012, nos estados de Minas Gerais e Bahia, Brasil, e de exsicatas de herbários nacionais e internacionais. Ao todo foram amostrados 21 dos 23 gêneros de Helieae, totalizando 60 espécies. Destes gêneros, seis são monoespecíficos (*Adenolisianthus* (Progel) Gilg, *Aripuana* Struwe, Maas, & V.A.Albert, *Lagenanthus* Gilg, *Purdieanthus* Gilg, *Tetrapollinia* Maguire & B.M. Boom e *Zonanthus* Griseb.). A listagem com as espécies estudadas, os respectivos coletores e vouchers encontram-se no apêndice 1.

Para os estudos anatômicos foram analisados, sempre que possível, três indivíduos de cada espécie. As amostras foliares coletadas em campo foram fixadas em FAA (formaldeído, ácido acético e etanol 50% 1:1:18 v/v) por cerca de 48 horas, submetidas à vácuo em dessecador e armazenadas em álcool etílico 70% (Johansen, 1940). O material retirado das exsicatas foi submetido ao processo de reversão de herborização (Meira & Martins 2003), posterior desidratação em série etílica e armazenamento em álcool etílico 70 %.

Para caracterização estrutural, amostras foliares foram incluídas em resina do tipo metacrilato (Historesin, Leica Instruments, Heidelberg, Alemanha) e submetidas à microtomia. Cortes transversais (7µm de espessura) da região mediana e basal da folha foram obtidos em micrótomo rotativo de avanço automático (modelo RM 2155, Leica Microsystems Inc.Deerfield, USA) utilizando-se navalhas de vidro. Os cortes foram corados com azul de toluidina pH 4.7 (O`Brien & McCully 1965) e montados em resina sintética (Permount-Fisher).

Folhas inteiras ou fragmentadas foram submetidas ao processo de diafanização utilizando-se solução de hidróxido de sódio a 10%, por cerca de 2 horas, e de hipoclorito de sódio a 20%, por tempo variável, dependendo da espécie, até total diafanização (Johansen 1940, modificado). Os fragmentos foram lavados em água destilada, submetidos à desidratação em série etílica, corados com Fucsina básica alcoólica (0,001%) e montados em gelatina glicerinada. As lâminas foram vedadas com esmalte incolor. Os estômatos foram classificados de acordo com Metcalfe & Chalk (1950).

Toda a análise do material e a captura de imagens foram realizadas em microscópio de luz (modelo Olympus AX70TRF, Olympus Optical, Tóquio, Japão) com câmara digital acoplada (AxionCam HRc; Zeiss, Göttingen, Germany) no Laboratório de Anatomia Vegetal da Universidade Federal de Viçosa (UFV), Minas Gerais, Brasil.

Após descrição anatômica, os caracteres foram listados (Tabela 1) e codificados mediante uma matriz (caracteres x táxons) (Tabela 2). A matriz foi elaborada no Microsoft Excel e exportada para o programa PAST (PAleontological Statistics) (Hammer et al. 2001). As espécies foram comparadas entre si por meio de métodos estatísticos multivariados de análise de agrupamento (Cluster análises). O dendrograma

foi construído com base no Índice de Sorensen. Também foram testados demais algorítimos como índice de Jaccard e distância Euclidiana.

Resultados

Epiderme

As 60 espécies de Helieae apresentaram epiderme unisseriada em ambas as faces da folha, como observado em *Symbolanthus yaviensis* (Fig. 1A), *Chelonanthus matogrossensis* (Fig. 1B), *Chelonanthus angustifolius* (Fig. 1C), *Chorisepalum ovatum* (Fig. 1D) e *Chorisepalum pyichotrioides* (Fig. 1E). Muitas espécies apresentaram hipoderme com número variável de camadas. Com relação à hipoderme muitas espécies apresentaram número variável de camadas. Uma ou duas camadas, restritas à face adaxial, foram constatadas em *Calolisianthus pedunculatus, Celiantha chimantensis* (Fig. 1F), *Rogersonanthus arboreus*, *R. quelchii, Senaea coerulea*, *S. janeirensis, Zonanthus cubensis*, e em espécies de *Macrocarpaea*, como em *M. obtusifolia* (Fig. 1G), exceto *M. arborescens* (Fig. 1H) que apresentou três a quatro camadas em ambas as faces. Três a quatro camadas de hipoderme, também restritas à face adaxial, foram observadas em *Sipapoantha ostrina* (Fig. 1I) e nas espécies de *Prepusa*, exceto *P. montana* a qual apresentou três a quatro camadas em ambas as faces e *P. viridiflora* com cinco a sete camadas restritas a face adaxial.

Células epidérmicas com paredes periclinais externas não espessadas ou levemente espessadas foram observadas na maioria das espécies (Fig. 1A-C). Paredes espessas (com mais da metade da altura das células epidérmicas) foram observadas em ambas as superfícies foliares de: *Celiantha bella* e *C. chimantensis* (Fig. 1F), em todas as espécies de *Chorisepalum* (Fig. 1D,E), de *Prepusa* (exceto na face abaxial de *P.*

viridiflora) e de *Rogersonanthus*. Por outro lado, *Neblinantha parviflora* e *Senaea coerulea* apresentaram paredes periclinais espessas somente na face adaxial do limbo.

Quando observadas em vista frontal, as células epidérmicas apresentam contorno diferenciado podendo ser reto, ondulado ou sinuoso (Fig. 2). Paredes retas em ambas as faces da folha foram evidentes em *Calolisianthus pedunculatus, Symbolanthus anomalus*, *Zonanthus cubensis* e em todas as espécies de *Celiantha* (Fig. 2A,B), *Chorisepalum*, *Prepusa* e *Macrocarpaea.* Paredes onduladas (Fig. 2C) em ambas as faces foram encontradas em *Adenolisianthus arboreus*, *Aripuana cullmaniorum*, *Chelonanthus matogrossensis*, *Rogersonanthus quelchii* e *Tachia grandiflora* e *T. loretensis*. Paredes com contorno sinuoso (Fig. 2D) foram relatadas em ambas as faces de *Chelonanthus acutangulus*, *C. alatus*, *C. albus*, *C. grandiflorus*, *C. pterocaulis*, *Irlbachia poepigii*, *I. pratensis*, *I. pumila* e *Tetrapollinia caerulescens*. As demais espécies apresentaram contorno diferente quando se compara a epiderme adaxial com a abaxial.

Epiderme papilosa ocorreu na maioria das espécies. Em relação à distribuição das papilas, diferentes padrões foram encontrados: folhas totalmente papilosas, papilas restritas à margem foliar, papilas restritas à margem e às nervuras de maior porte e papilas restritas a face adaxial do limbo. Papilas por todo o limbo foram encontradas em *Chelonanths alatus*, *C. albus*, *C. angustifolius* (Fig. 2E-F), *C. viridiflorus*, *Chorisepalum psychotrioides*, *Irlbachia nemorosa*, *I. poepigii* e *I. pumila*. Papilas apenas na face adaxial foram relatadas apenas em *Calolisianthus pedunculatus*. Papilas restritas a margem foliar foram constatadas em *Adenolisianthus arboreus*, *Celiantha chimatensis*, *Chelonanthus matogrossensis*, *C. acutangulus*, *C. purpurascens, C. pterocaulis*, *Lagenanthus principes*, *Lehmaniella splendens*, *Neblinantha parviflora*, *N. neblinae*, *Symbolanthus anomalus*, *S. elisabethae* e *S. yaviensis.* Margem foliar papilosa

e papilas nas nervuras de maior porte foram encontradas em *Chelonanthus grandiflorus*, *Chorisepalum ovatum*, *C. rotundifolium* e *C. sipapoanum*.

Apesar de a maioria das espécies de Helieae serem glabras, tricomas tectores foram relatados para seis espécies estudadas variando a sua distribuição no limbo: dispersos por toda a folha em *Macrocarpaea obtusifolia* (Fig. 2G); restritos a face abaxial do limbo em *Celiantha bella*; restritos ao terço apical da folha em *Celiantha imthurniana* (Fig. 2H); e restritos às nervuras de maior porte em *Macrocarpaea bangiana*, *M. valerioi* e *Purdienathus pulcher.*

A maioria das espécies de Helieae é hipoestomática, mas folhas anfiestomáticas ocorrem em *Chelonanthus matogrossensis* (Fig. 1B), *Irlbachia pratensis* e *Tetrapollinia caerulescens*. Folhas anfihipoestomáticas foram observadas para *Adenolisianthus arboreus, Helia brevifolia* e na maioria das espécies de *Chelonanthus* (Fig. 2E,F), exceto *C. matogrossensis*.

O tipo de estômato predominante na maioria das espécies é o anisocítico (Fig. 2 B-D). Apenas *Calolisianthus pedunculatus* apresenta estômatos estaurocíticos e *Chelonanthus alatus* e *Tetrapollinia caerulescens* apresentam estômatos predominantemente do tipo anomocítico.

Mesofilo

Todas as espécies apresentaram mesofilo dorsiventral (Fig. 1). Cristais dispersos pelo limbo foliar e/ou na nervura mediana são comuns nas espécies de Helieae. Cristais estão ausentes em *Lagenanthus principes, Lehmaniella splendens, Aripuana cullmaniorum, Tetrapollinia caerulescens*, *Neblinantha neblinae*, *Macrocarpaea arborescens* e *M. obtusifolia* e em todas as espécies de *Tachia* e *Irlbachia.*

Nervura mediana

A nervura mediana na maioria das espécies de Helieae apresenta-se pronunciada apenas na face abaxial (Fig. 3A-D). Nervura contida no limbo ocorreu em *Irlbachia pratensis*, *Prepusa alata*, *P. connata*. *P. hookeriana*, *Rogersonanthus quelchii* e *Tetrapollinia caerulescens*. Nas espécies de *Tachia* (Fig. 3E) a nervura é proeminente em ambas as faces.

O tipo de feixe vascular predominante na nervura mediana de espécies de Helieae é o bicolateral (Fig. 3A,B, G). Entretanto, feixes colaterais ocorrem em *Irlbachia poepigii*, *I. pratensis*, *I. pumila* e *Tetrapollinia caerulescens* (Fig. 3F)e feixes anficrivais em *Chorisepalum* (Fig. 3C, I), *Macrocarpaea* (Fig. 3D) e *Tachia* (Fig. 3E). Além do tipo de feixe, o número de feixes vasculares na região da nervura mediana também apresentou variações: um único feixe foi comumente observado na maioria das espécies (Fig. 3A,B); um feixe central e dois feixes acessórios foram descritos para as espécies de *Chorisepalum* (Fig. 3C) e *Rogersonanthus*; cinco ou mais feixes vasculares com distribuição aleatória foram registrados para as espécies de *Macrocarpaea* (Fig. 3D); e três feixes principais e cinco ou seis acessórios, de menor porte, ocorrem nas espécies de *Tachia* (Fig. 3E).

Esclereídes

Diferentes padrões de ocorrência de esclereídes com paredes espessadas, lignificadas e com pontoações foram observados (Fig. 1I). Em *Celiantha bella* e nas espécies de *Chorisepalum* as esclereídes ocorrem por todo o limbo foliar. Em *Chelonanthus albus*, *C. angustifolius*, *C. grandiflorus*, *C. pterocaulis*, *C. viridiflorus*, *Irlbachia cardone*, *Lagenanthus princeps*, *Neblinantha neblinae*, *N. parviflora*, *Rogersonanthus*, *Symbolanthus australis*, *S. calygonus* e *S. frigidus* as esclereídes ocorrem restritas ao

terço basal da folha. Em *Celiantha chimatensis*, *Macrocarpaea arborescens*, *M. obtusifolia, Symbolanthus pterocaulis* e *Tachia guianensis* as esclereides estão restritas às nervuras de maior porte. Apenas nas duas espécies de *Senaea* e em *Chelonanthus matogrossensis* esclereides ocorrem ao longo da margem foliar e nas nervuras de maior porte (Fig. 3A,I). Em *Sipapoantha ostrina* ocorrem esclereides por entre as células do parênquima paliçádico (Fig. 1J).

Estruturas secretoras

Dois tipos de estruturas secretoras foram encontrados em todas as espécies de Helieae: coléteres e nectários (Fig 4). Os coléteres estão localizados na base foliar na região de inserção da folha com o caule (Fig. 4A-C). Não foi possível a caracterização anatômica dos coléteres pois na maioria dos exemplares amostrados, provenientes de herbários, os coléteres estavam senescentes. Os nectários são formados por células epidérmicas modificadas e se assemelham em tamanho aos estômatos (Fig. 4D,E). Ocorrem em unidades dispersas e isoladas ao longo do limbo foliar (Fig. 4D-F) e/ou em agregados formando estruturas conspícuas ou inconspícuas (Fig. 4G-I). Nectários apenas em unidades dispersas e isoladas são encontrados nas três espécies de *Celiantha* e em *Zonanthus cubensis*. Ocorrem geralmente em ambas as faces do limbo, entretanto nas espécies do gênero *Celiantha* e em *Chorisepalum ovatum*, *C. sipapoanum*, *Macrocarpaea arborescens*, *Neblinantha parviflora*, *Sipapoantha ostrina* estão restritos à face abaxial do limbo. As demais espécies apresentam além dos nectários em unidades isoladas e dispersas, nectários em agregados. Os nectários em agregados podem ocorrer próximo ao ápice foliar (Fig. 4G,H), junto à base (Fig. 4I) ou em ambas as regiões podendo ser observados apenas em material diafanizado, ao microscópio, e raramente observados em campo. Encontram-se tanto no ápice como na base foliar na maioria das

espécies embora ocorram restritos ao ápice ou à base. Em algumas espécies a presença de nectários em agregados não é constante em todos os espécimes estudados ou não está bem delimitado como em: *Aripuana cullmaniorum*, *Irlbachia pratensis*, *Lagenanthus princeps*, *Lehmaniella splendens*, *Macrocarpaea arborescens*, *Neblinantha neblinae*, *Tachia loretensis*, *Tetrapollinia caerulescens* e em todas as espécies de *Prepusa*, *Rogersonanthus* e *Senaea.*

Análise de similaridade – considerações taxonômicas

A análise de similaridade (Fig. 5) foi gerada a partir de 53 caracteres anatômicos selecionados (Tabela 1) os quais se encontram codificados na Tabela 2.

Todas as espécies de Helieae estudadas apresentam epiderme unisserida, mesofilo dorsiventral, coléteres e nectários foliares. Dois grupos (A e B) foram delimitados: o grupo A inclui apenas *Irlbachia pratensis* e *Tetrapollinia caerulescens* (gênero monoespecífico), com índice de similaridade de cerca de 90%. As duas espécies compartilham a maioria dos caracteres diferindo apenas em relação ao contorno das células epidérmicas e tipo de estômato. O grupo B inclui todos os demais gêneros sendo dividido em dois grandes grupos: C e D. O grupo C, com cerca de 70% de similaridade, compreende três gêneros incluindo todas as espécies estudadas dos gêneros *Chorisepalum*, *Prepusa* e *Celiantha* (exceto *C. inthurniana*). Todas as espécies destes gêneros compartilham a presença células epidérmicas, em vista frontal, com contorno reto em ambas as faces; folhas hipostomáticas com estômatos predominantemente do tipo anisocítico e cristais dipersos pelo limbo. *Chorisepalum* é reconhecido ainda pela ausência de hipoderme; nervura mediana com feixes anficrivais sendo um feixe vascular central e dois feixes acessórios; esclereídes dispersos por todo o limbo; e nectários em agregados não constantes em todos os espécimes. *Prepusa*, por sua vez, apresenta

hipoderme; nervura mediana proeminente apenas na face abaxial, com feixes anficrivais; e nectários em agregados não constantes em todos os espécimes. As duas espécies de *Celiantha* apresentam nervura mediana proeminente apenas na face abaxial, com um único feixe vascular e do tipo bicolateral; esclereides por todo o limbo foliar e nectários apenas em unidades isolados e dispersos em ambas as faces do limbo.

O grupo D, com cerca de 65% de similaridade, compreende os demais gêneros e e *Celiantha inthurniana*. Dois grupos menores são formados: E e F. O grupo E inclui todas as espécies dos gêneros *Tachia* e *Macrocarpaea* os quais apresentam células epidérmicas com paredes não espessadas em ambas as faces; nervura mediana com feixes anficrivais; folhas hipostomáticas com estômatos predominantemente do tipo anisocítico. *Tachia* é reconhecido ainda pela presença de nervura mediana proeminente em ambas as faces, com três feixes vasculares de maior porte e seis feixes acessórios e nectários restritos a face abaxial do limbo. *Macrocarpaea* apresenta nervura mediana proeminente apenas na face abaxial; células epidérmicas com contorno reto, em vista frontal, em ambas as faces e cinco ou mais feixes dispostos de maneira aleatória na região da nervura mediana. O grupo F inclui representantes de 16 gêneros (*Chelonanthus*, *Helia*, *Adenolisianthus*, *Calolisianthus*, *Celiantha*, *Zonanthus*, *Irlbachia*, *Purdieanthus, Symbolanthus*, *Sipapoantha*, *Senaea*, *Aripuana*, *Lehmanniella*, *Lagenanthus*, *Neblinantha* e *Rogersonanthus*), sendo cinco monoespecíficos (*Purdieanthus, Adenolisianthus*, *Zonanthus*, *Aripuana* e *Lagenanthus*). Os dados anatômicos permitiram a diferenciação dos cinco gêneros monoespecíficos, assim como de outros três gêneros dos quais apenas uma espécie foi analisada (*Calolisianthus, Lehmanniella* e *Sipapoantha*). Outros dois gêneros também foram delimitados por dados anatômicos: *Senaea* e *Rogersonanthus*. *Senaea* compartilha a presença de uma ou duas camadas de hipoderme restritas à face adaxial; células epidérmicas com paredes

espessadas apenas na face adaxial e contorno ondulado, em vista frontal, na face abaxial; folhas anfiestomáticas com estômatos anisocíticos; esclereídes restritos a margem foliar; nervura mediana proeminente apenas na face abaxial; com um único feixe do tipo bicolateral; nectários restritos a face abaxial do limbo e nectários em agregados não constantes em todos os espécimes. *Rogersonanthus* possui uma ou duas camadas de hipoderme restritas à face adaxial; células epidérmicas com paredes espessas em ambas as faces e contorno ondulado, em vista frontal, na face abaxial; folhas anfiestomáticas com estômatos anisocíticos; esclereides restritos a base foliar; nervura mediana com um único feixe e do tipo bicolateral; cristais dispersos pelo limbo; nectários restritos a face abaxial do limbo e nectários em agregados constantes em todos os espécimes.

Apenas os gêneros *Chelonanthus*, *Helia*, *Irlbachia*, *Symbolanthus* e *Neblinantha* não foram delimitados pelos caracteres anatômicos. As espécies de *Chelonanthus* aparecem distribuídas em diferentes grupos, entremeadas a outros gêneros. As duas espécies de *Helia* se encontram juntas com a maioria das espécies de *Chelonanthus*. *Symbolanthus* forma um grupo juntamente com *Purdieanthus*. As duas espécies de *Neblinantha* aparecem separadas assim como as espécies de *Irlbachia*.

Discussão

A anatomia vem sendo utilizada como uma ferramenta útil para a taxonomia desde o início do século XIX (Solereder 1908). O estudo anatômico juntamente com a análise de similaridade de 60 espécies de Helieae permitiu identificar caracteres importantes para a taxonomia da tribo, especialmente para a identificação/separação dos gêneros.

A presença de estômatos predominantemente do tipo anomocítico e anisocítico verificada em Heliae é característica da família Gentianaceae (Solereder 1908; Metcalfe

& Chalk 1950; Delgado et al. 2009; 2011a). Os estômatos do tipo estaurocítico observados em *Calolisianthus pedunculatus* constitui um caráter que permite diferenciar esta espécie das demais do gênero. Estômatos do tipo diacítico e paracítico foram relatados para espécies de *Curtia* (Saccifolieae) (Dalvi et al. 2014).

A distribuição dos estômatos tem sido ressaltada, em Gentianaceae, como importante para diferenciação de espécies, como em *Deianira* (Delgado et al. 2009) e *Curtia* (Dalvi et al. 2014). Folhas hipoestomáticas são frequentes dentro da família, sendo folhas anfiestomáticas menos comuns (Metcalfe & Chalk 1950). Este padrão se manteve dentro da tribo Helieae. No entanto, observamos folhas anfihipoestomáticas apenas para as espécies do gênero *Chelonanthus* (exceção de *C. matogrossensis)* e em *Adenolianthus arboreus* sugerindo uma proximidade anatômica entre estes gêneros como demonstrado pela análise de similaridade.

A presença, ocorrência e número de camadas de hipoderme dentro de alguns grupos de Helieae merecem destaque e pode ser usada na distinção de *Calolisianthus pedunculatus* e *Celiantha chimantensis* das demais espécies dentro dos seus respectivos gêneros. A presença de hipoderme também representa um caráter comum a todas as espécies dos gêneros *Macrocarpaea*, *Prepusa*, *Rogersonanthus*, *Senaea*, *Sipapoantha* e *Zonanthus*. Provavelmente a ocorrência de hipoderme está relacionada com fatores ambientais, como a restrição hídrica, a que estas espécies estão submetidas, pois a hipoderme atua como armazenadora de água, filtro de luz e desempenha papel na sustentação (Fahn & Cutler 1992). Apesar do caráter adaptativo atribuído à hipoderme, esta característica possui valor taxonômico em Helieae visto que muitas destas espécies/gêneros são endêmicas.

A sinuosidade das paredes anticlinais das células epidérmicas se apresentou variável, entretanto todas as espécies de *Celiantha*, *Chorisepalum*, *Macrocarpaea* e

Prepusa apresentaram paredes retas em ambas às faces do limbo foliar. Muitos autores destacam que fatores ambientais como a intensidade luminosa afetam a sinuosidade das paredes das células epidérmicas (Metcalfe & Chalk 1979; Pyyko 1979) o que representa uma limitação para a aplicação deste caráter na txonomia. Portanto, é imprescindível avaliar as condições ambientais dos locais de ocorrência das espécies estudadas o que é uma dificuldade quando se trabalha com materiais oriundos de herbário. Dalvi et al. (2014) destacam que a sinuosidade pode ser usada para a taxonomia de espécies de *Curtia* e *Hockinia* (Saccifolieae) que occorrem sob condições de luminosisdade semelhantes.

Outro caráter variável de acordo com as condições ambientais é a espessura da parede periclinal externa das células epidérmicas. Todas as espécies de *Celiantha* e *Chorisepalum, Rogersonanthus, Purdieanthus pulcher*, *Sipapoantha ostrina* e a maioria das espécies de *Prepusa* apresentaram células epidérmicas com paredes periclinais externas espessas em ambas as faces. Como a maioria destes gêneros são endêmicos (Struwe et al. 2002) a espessura de parede pode ser levada em consideração para fins taxonômicos.

Apesar das espécies de Gentianaceae serem caracterizadas usualmente como glabras (Struwe et al. 2002), tricomas já haviam sido relatados para *Bartonia* e *Obolaria* (Metcalfe & Chalk 1950). Tricomas podem variar em tamanho e densidade de acordo com as características ambientais, entretanto a presença e o tipo peculiar de tricoma podem delimitar uma espécie, um gênero ou uma família inteira (Metcalfe & Chalk 1950). A presença de tricomas em algumas espécies de *Celiantha*, *Macrocarpaea* e em *Purdieanthus pulcher* foi relevante para a identificação e delimitação de espécies em Helieae.

As papilas, comumente relatadas para Gentianaceae (Metcalfe & Chalk 1950; Greimler et al. 2004) ocorrem na tribo Helieae e a presença ou a ausência destas estruturas é constante na maioria dos gêneros, com exceção de *Celiantha*, *Symbolanthus* e *Irlbachia*. Estudos complementares de micromorfologia são necessários para classificar os tipos de papilas e verificar o valor diagnóstico para as diferentes espécies ou gêneros de Helieae.

Quanto à conformação da nervura mediana, o formato proeminente apenas na face abaxial foi característico para Helieae. Assim, a nervura mediana proeminente em ambas as faces em *Tachia* foi útil para reconhecimento do gênero. O número de feixes vasculares na região da nervura mediana foi significativo para a taxonomia sendo a ocorrência de um único feixe vascular o mais comum em Helieae. A presença de um feixe principal e dois acessórios caracterizou os gêneros *Chorisepalum* e *Rogersonanthus.* A presença de cinco ou mais feixes dispostos de maneira aleatória permite a identificação de *Macrocarpaea*; enquanto a presença de três feixes principais além de feixes acessórios permite o reconhecimento das espécies de *Tachia*.

Além do número de feixes vasculares na nervura mediana, o tipo de feixe é variável em Helieae podendo ser diagnóstico para alguns gêneros, como é o caso da presença de feixes vasculares anficrivais em todas as espécies de *Chorisepalum, Macrocarpaea* e *Tachia*. Os feixes colaterais também são restritos a *Tetrapollinia caerulescens* e *Irlbachia pratensis*. Os caracteres relacionados aos feixes vasculares na região da nervura mediana são promissores para a identificação em nível genérico dentro de Helieae e já foram ressaltados para outros grupos dentro da família (Dalvi et al. 2014). Destaca-se o cuidado em examinar o tipo de feixe vascular em diferentes regiões da folha (ápice, meio e base) pois este pode apresentar variação dependendo da região analisada (Delgado et al. 2011a).

Coléteres constituem uma das sinapomorfias da ordem Gentianales (Judd et al. 2009) e estão presentes em todas as espécies de Helieae. O número, a morfologia e o arranjo dos coléteres constituem dados relevantes para a taxonomia e filogenia (Lersten 1974, 1975), como registrado para diferentes espécies de *Forsteronia* (Rio et al. 2005), *Exothostemon*, *Mandevilla* (Simões et al. 2006); *Turnera* e *Piriqueta* (González 1998) e *Simira* (Klein et al. 2004). A caracterização anatômica dos coléteres em Helieae não foi possível pois a maioria dos exemplares estudados foram obtidos de amostras de exsicatas e durante a padronização das amostras foram retiradas folhas completamente expandidas, nas quais os coléteres, na maioria das amostras, estavam em fase de senescência. Investigações acerca do tipo de coléteres em Helieae utilizando amostras frescas de meristemas ou folhas em estádios iniciais de desenvolvimento podem contribuir para a taxonomia do grupo.

A presença universal de nectários dentro de Helieae merece destaque. Além da presença, o padrão de distribuição dos nectários ao longo do limbo e sua ocorrência em unidades isoladas e/ou em agregados são importantes para o reconhecimento de diferentes gêneros dentro de Helieae. A importância taxonômica e filogenética destas estruturas na família Gentianaceae já foi ressaltada (Delgado et al. 2011a; Dalvi et al. 2013, 2014).

Identificamos caracteres anatômicos compartilhados por todas as espécies da tribo e caracteres úteis para reconhecimento de 15 dos 21 gêneros estudados. Os caracteres anatômicos não contribuíram apenas para a delimitação dos gêneros *Celiantha, Chelonanthus*, *Helia*, *Irlbachia*, *Symbolanthus* e *Neblinantha*. Em estudos filogenéticos recentes *Chelonanthus* e *Irlbachia* compreendem um grupo polifilético (Struwe et al. 2009). Adicionalmente, Calió (2009) demonstra que a relação entre *Chelonanthus, Adenolisianthus* e *Helia* não está esclarecida sugerindo o

estabelecimento de um único gênero. Nossos dados também indicam semelhança anatômica entre *Chelonanthus, Adenolisianthus* e *Helia*. Em relação à *Neblinantha*, as duas espécies diferem anatomicamente confirmando os resultados de Calió (2009). Estudos adicionais reunindo diversas fontes de dados e com um esforço amostral maior para inclusão de maior número de espécies dos diversos gêneros serão essenciais para esclarecer as relações filogenéticas em Helieae.

Estes resultados não apenas constituem uma ferramenta, mas comprovam a importância da anatomia foliar como uma fonte de dados importante dentro um grupo de taxonomia complicada como Helieae e abrem novas perspectivas de trabalho especialmente em relação às características das estruturas secretoras, coléteres e nectários, presentes no grupo.

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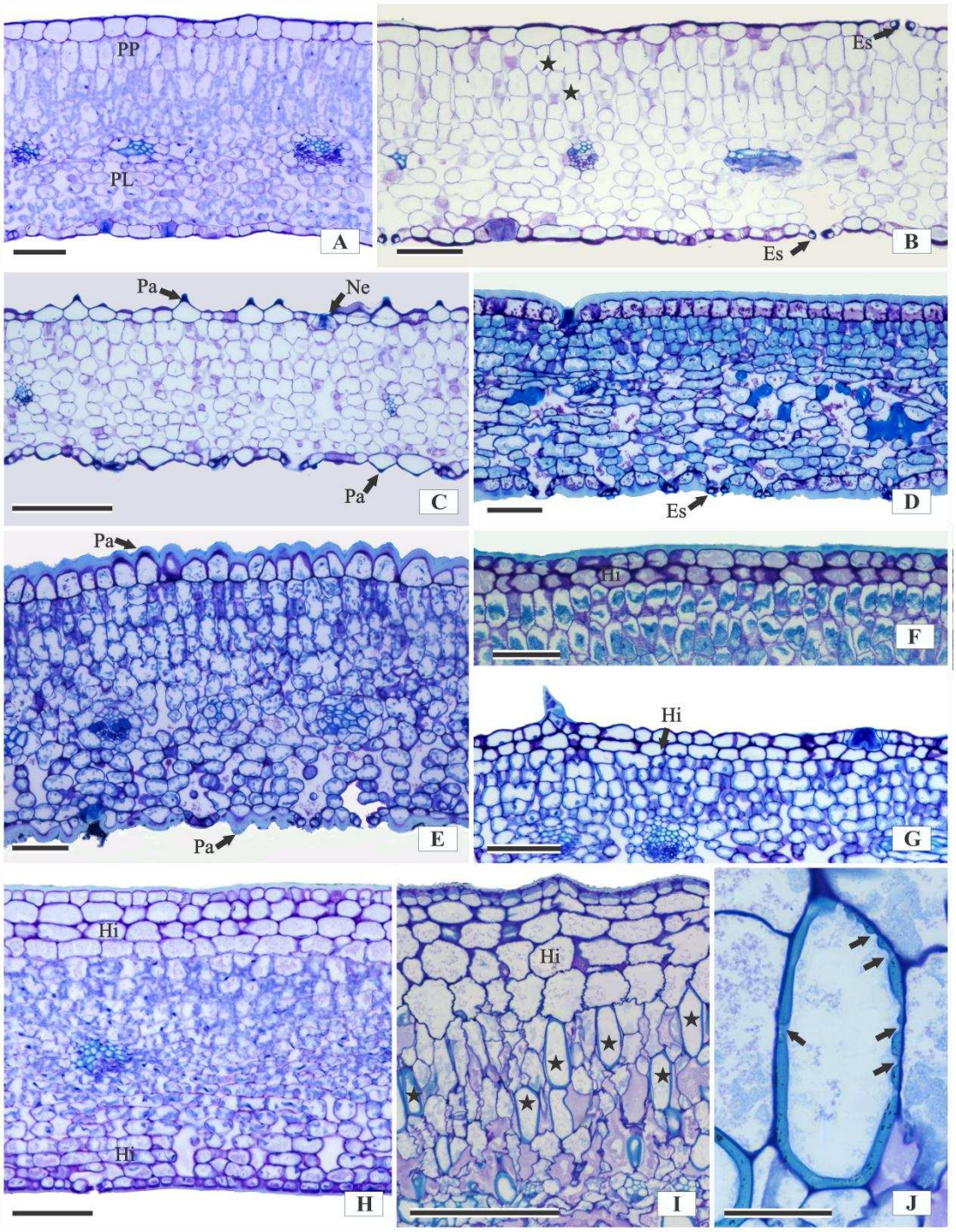

Fig. 1 Limbo foliar de espécies de Helieae (Gentianaceae). Secção transversal. **A** Mesofilo dorsiventral em *Symbolanthus yaviensis*. **B** Estômatos em ambas as faces em *Chelonanthus matogrossensis.* **C** Epiderme papilosa em ambas as faces em *Chelonanthus angustifolius.* **D** Células epidérmicas com paredes periclinais externas espessadas em *Chorisepalum ovatum.* **E** Epiderme papilosa com paredes espessadas em *Chorisepalum psychotrioides.* **F, G** Hipoderme voltada para a face adaxial em *Celiantha chimantensis* e *Macrocarpaea obtusifolia*, respectivamente. **H** Hipoderme em ambas as faces de *Macrocarpaea arborescens.* **I** Hipoderme e esclereídes entremeadas às células do parênquima paliçádico (★). **J** Detalhe das pontoações das esclereídes (seta). (Es, estômatos; Hi, hipoderme; Pa, papilas; PL, parênquima lacunoso; PP, parênquima paliçádico). *Barras das escalas:* A-I, 100 μm; J, 50 μm

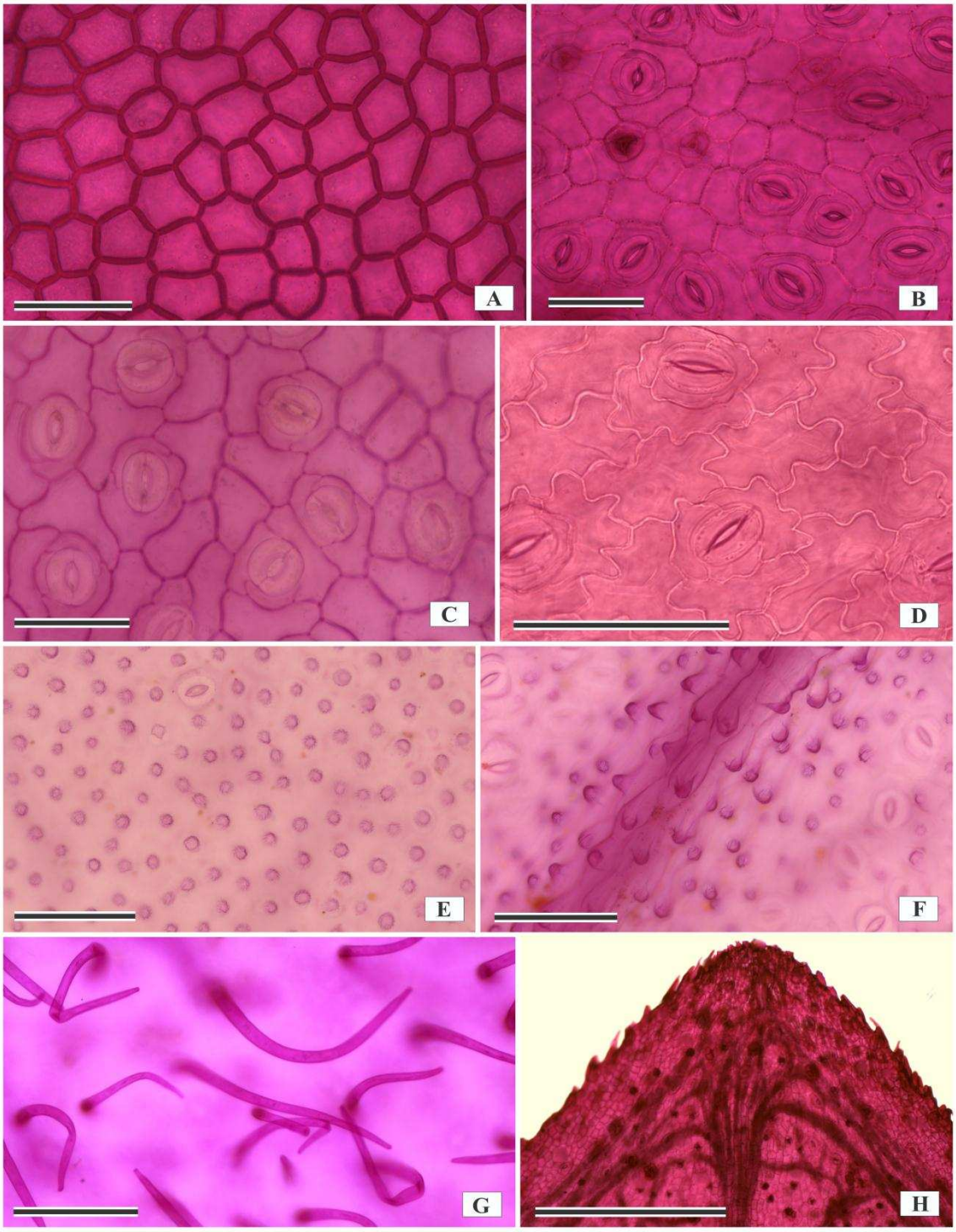

Fig. 2 Epiderme em vista frontal das espécies de Helieae (Gentianaceae). Material diafanizado. **A, B** Células epidérmicas com paredes anticlinais retas em *Celiantha imthurniana* (face adaxial e face abaxial respectivamente). **C** Células epidérmicas com paredes anticlinais onduladas e estômatos do tipo anisocítico em *Neblinantha parviflora* (face abaxial) **D** Células epidérmicas com paredes anticlinais sinuosas e estômatos do tipo anisocítico em *Irlbachia nemorosa* (face abaxial). **E, F** Papilas em *Chelonanthus angustifolius* (face adaxial e face abaxial respectivamente). **G** Tricomas em *Macrocarpaea obtusifolia*. **H** Tricomas restritos ao terço apical em *Celiantha imthurniana*. *Barras das escalas:* A-F e H, 100 μm; G, 500 μm

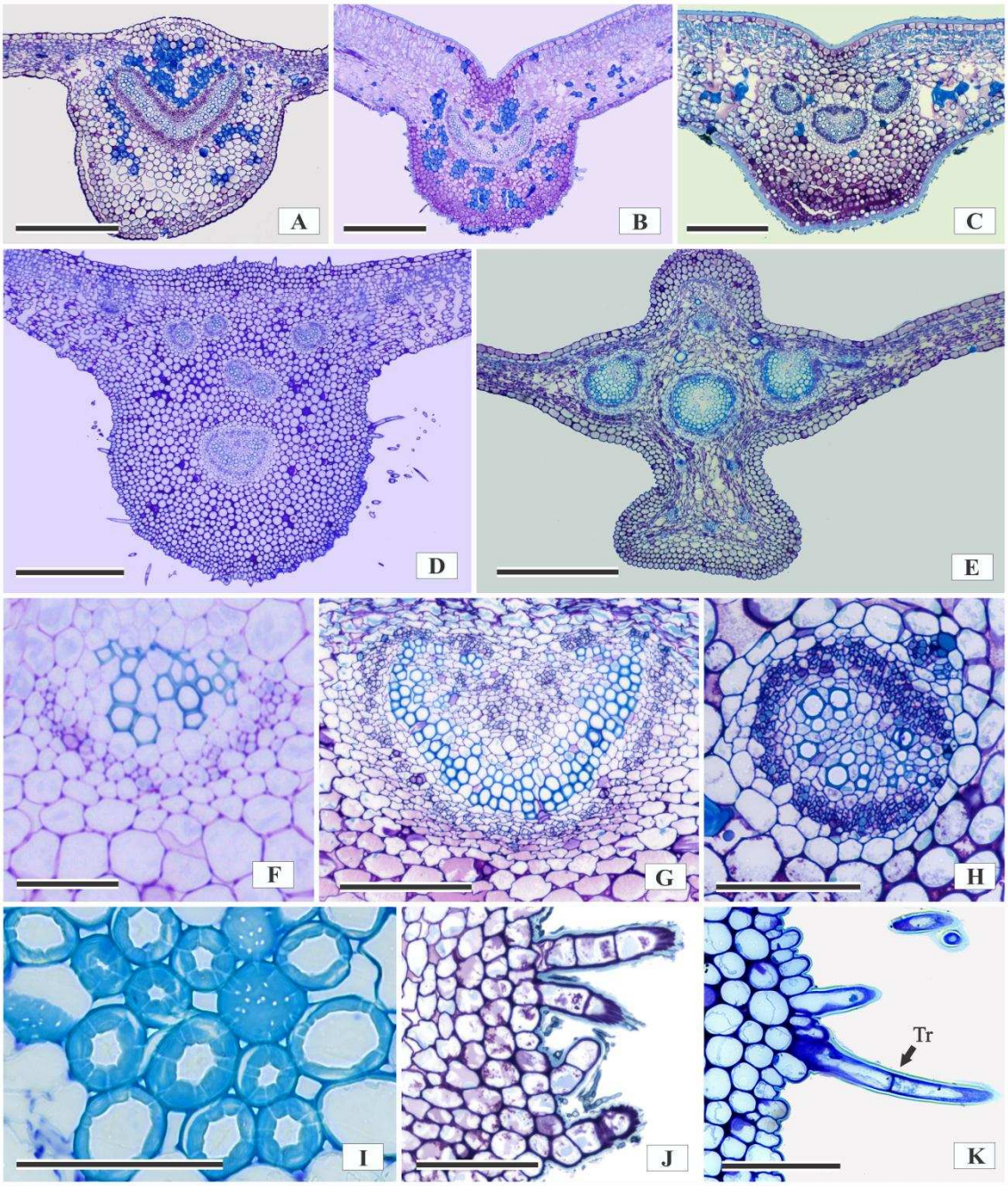

Fig. 3 Nervura mediana de espécies de Helieae (Gentianaceae). Secção transversal. **A-D** Nervura proeminente apenas na face abaxial. **E** Nervura proeminente em ambas as faces. **A,B** Um único feixe e esclereídes em *Chelonanthus matogrossensis* e *Celiantha bella*, respectivamente. **C** Um feixe central e dois feixes acessórios em *Chorisepalum ovatum*. **D** Vários feixes dispostos aleatoriamente em *Macrocarpaea obtusifolia.* **E** Três feixes principais e feixes acessórios em *Tachia grandiflora.* **F** Feixe colateral em *Tetrapollinia caerulescens.* **G** Feixe bicolateral em *Prepusa montan*a. **H** Feixe anficrival em *Chorisepalum ovatum***. I** Esclereídes em *Chelonanthus matogrossensis.* **J,K** Tricomas na região da nervura mediana de *Purdieantthus pulcher* e *Macrocarpaeae obtusifolia*, respectivamente**.** *Barras das escalas:* A-E e G,H, 500 μm; F, 50 μm; I-K, 100 μm.

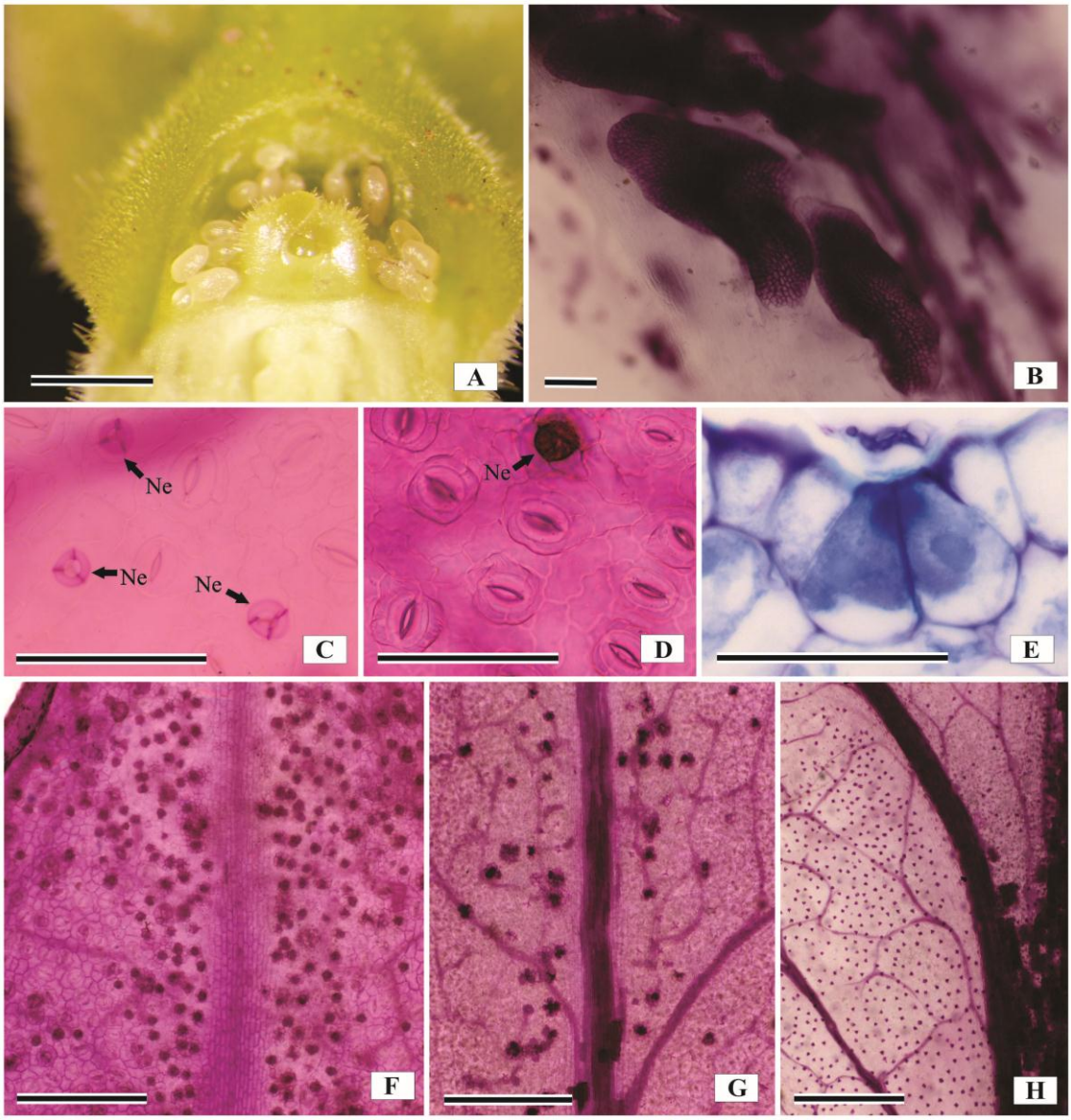

Fig 4 Estruturas secretoras em espécies de Helieae (Gentianaceae). **A-B** Coléteres. **C-H** Nectários. **A** Base foliar de *Macrocarpaea obtusifolia.* **B** Coléteres de *Macrocarpaeae valerioi* (material diafanizado). **C-E** Nectários dispersos pelo limbo de *Aripuana cullmaniorum, Chelonanthus albus* (diafanização) e *Chelonnathus viridiflorus* (corte transversal). **F,G** Nectários aglomerados no ápice foliar de *Neblinantha parviflora* e *Adenolisianthus arboreus*. **H** Nectários em agregados na base foliar de *Chelonanthus albus. Barras das escalas:* A, 2mm; B-D, 100 μm; E, 50 μm; F-H, 500 μm.

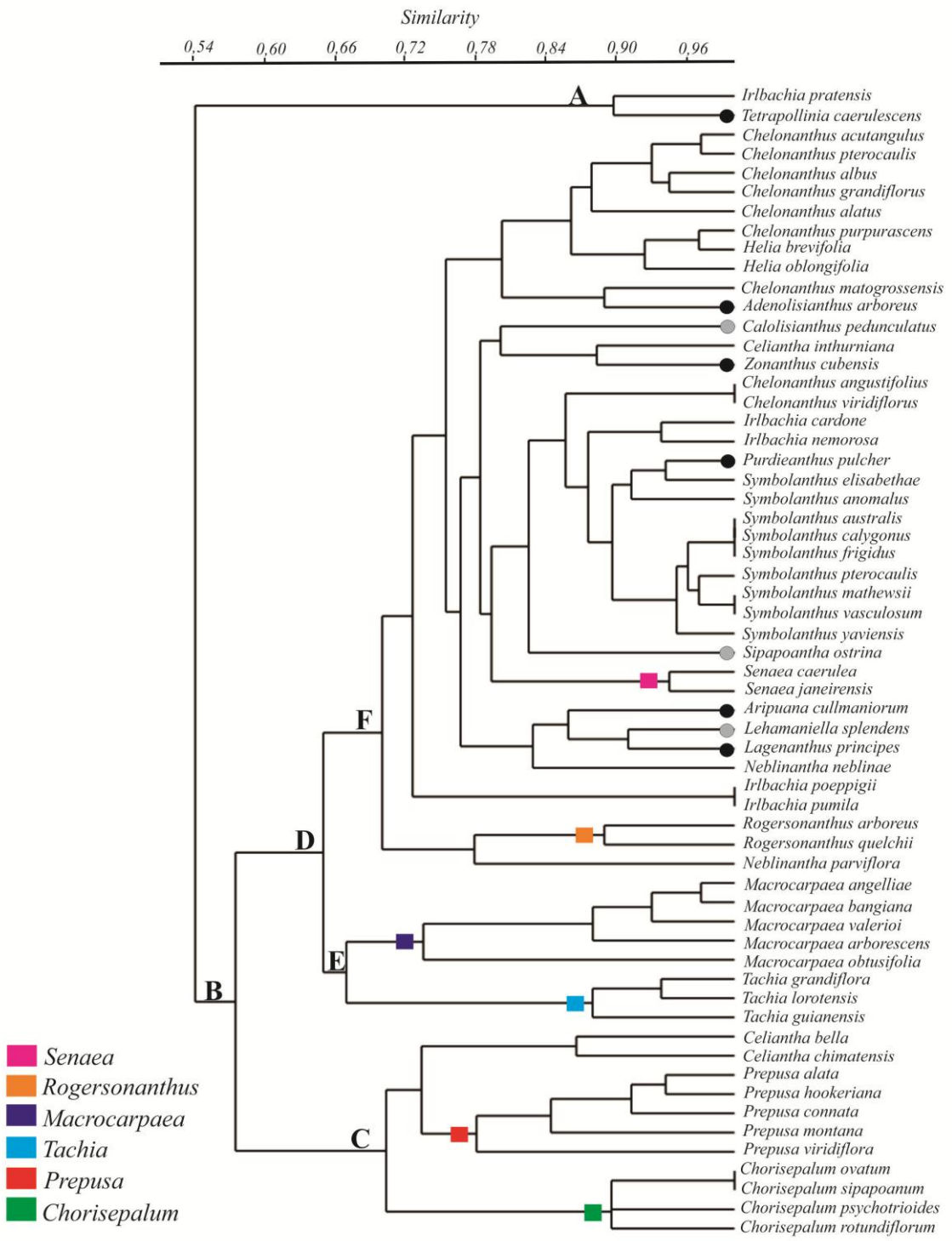

Fig 5 Análise de similaridade de espécies de Helieae (Gentianaceae) calculada usando o Índice de Sorensen`s. ● Gêneros monoespecíficos e ● gêneros com apenas uma espécie analisada.

Tabela 1: Lista dos caracteres anatômicos das espécies de Helieae (Gentianaceae) utilizados nas análises de similaridade.

1. Epiderme unisseriada em ambas as faces
2. Uma ou duas camadas de hipoderme restritas a face adaxial
3. Três ou quatro camadas de hipoderme restritas a face adaxial
4. Três ou quatro camadas de hipoderme em ambas as faces
5. Cinco ou mais camadas de hipoderme restritas a face adaxial
6. Células epidérmicas da face adaxial com paredes não espessadas
7. Células epidérmicas da face adaxial com paredes espessadas (mais da metade da célula)
8. Células epidérmicas da face abaxial com paredes não espessadas
9. Células epidérmicas da face abaxial com paredes espessadas (mais da metade da célula)
10. Mesofilo dorsiventral
11. Nervura mediana proeminente em ambas as faces
12. Nervura mediana proeminente apenas na face abaxial ou com leve elevação também na face adaxial
13. Nervura mediana contida no limbo
14. Nervura mediana com feixes vasculares colaterais
15. Nervura mediana com feixes vasculares bicolaterais
16. Nervura mediana com feixes vasculares anficrivais
17. Nervura mediana com um único feixe vascular
18. Nervura mediana com um feixe vascular central e dois acessórios
19. Nervura mediana com cinco ou mais feixes vasculares de tamanhos semelhantes
20. Nervura mediana com três feixes vasculares de maior porte e seis feixes acessórios
21. Cristais dispersos pelo limbo
22. Células epidérmicas, da face adaxial, em vista frontal, com contorno reto
23. Células epidérmicas, da face adaxial, em vista frontal, com contorno ondulado
24. Células epidérmicas, da face adaxial, em vista frontal, com contorno sinuoso
25. Células epidérmicas, da face abaxial, em vista frontal, com contorno reto
26. Células epidérmicas, da face abaxial, em vista frontal, com contorno ondulado
27. Células epidérmicas, da face abaxial, em vista frontal, com contorno sinuoso
28. Papilas por todo o limbo
29. Papilas restritas às margens
30. Papilas restritas às nervuras de maior calibre
31. Papilas restritas a face abaxial
32. Tricomas por todo o limbo
33. Tricomas restritos a face abaxial
34. Tricomas restritos ao ápice foliar
35. Tricomas restritos as nervuras de maior porte
36. Folhas hipoestomáticas
37. Folhas anfiestomáticas
38. Folhas anfihipoestomáticas
39. Estômatos predominantemente do tipo anomocítico
40. Estômatos predominantemente do tipo anisocítico
41. Estômatos predominantemente do tipo staurocítico
42. Esclereídes dispersas por todo o limbo
43. Esclereídes restritas as nervuras de maior porte
44. Esclereídes restritas a base foliar

45. Esclereídes ao longo da margem foliar
46. Esclereídes entremeadas às células do parênquima palicádico
47. Coléteres
48. Nectários
49. Nectários restritos a face abaxial
50. Nectários em ambas as faces
51. Nectários apenas em unidades isoladas dispersas pelo limbo foliar
52. Nectários em unidades isoladas dispersas pelo limbo foliar e em agregados formando áreas nectaríferas conspícuas ou pouco conspícuas (constante em todas as amostras)
53. Nectários em unidades isoladas dispersas pelo limbo foliar e em agregados formando áreas nectaríferas conspícuas ou pouco conspícuas (não constante em todas as amostras)

Tabela 2: Matriz com os 60 táxons de Helieae (Gentianaceae) e 53 caracteres anatômicos codificados na tabela 1. Para cada característica foi empregado (0) ausência e (1) presença.

	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**
Adenolisianthus arboreus	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	0	0	1	0
Aripuana cullamaniorum	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	0	0	1	0
Calolisianthus pedunculatus	1	1	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	1	0	0
Celiantha bella	1	0	0	0	0	0	1	0	1	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	1	0	0
Celiantha chimatensis	1	1	0	0	0	0	1	0	1	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	1	0	0
Celiantha imthurniana	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	1	0	0
Chelonanthus acutangulus	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	0	0	1	0	0	1
Chelonanthus alatus	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	0	0	1	0	0	1
Chelonanthus albus	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	0	0	1	0	0	1
Chelonanthus angustifolius	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0
Chelonanthus grandiflorus	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	0	0	1	0	0	1
Chelonanthus matogrossensis	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	0	0	1	0
Chelonanthus pterocaulis	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	0	0	1	0	0	1
Chelonanthus purpurascens	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	0	0	0	1
Chelonanthus viridiflorus	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0
Chorisepalum ovatum	1	0	0	0	0	0	1	0	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0	0	1	0	0
Chorisepalum psychotrioides	1	0	0	0	0	0	1	0	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0	0	1	0	0
Chorisepalum rotundifolium	1	0	0	0	0	0	1	0	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0	0	1	0	0
Chorisepalum sipapoanum	1	0	0	0	0	0	1	0	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0	0	1	0	0
Helia brevifolia	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	0	0	0	1
Helia oblongifolia	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	0	0	0	1
Irlbachia cardone	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	0	1	0	0	0	0	1
Irlbachia nemorosa	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	0	1	0	0	0	0	1

Irlbachia poeppigii	1	0	0	0	0	1	0	1	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	1	0	0	1
Irlbachia pratensis	1	0	0	0	0	1	0	1	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	1	0	0	1
Irlbachia pumila	1	0	0	0	0	1	0	1	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	1	0	0	1
Lagenanthus princeps	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	0	1	0	0	0	0	1
Lehmanniella splendens	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	0	0	0	1
Macrocarpaea angelliea	1	1	0	0	0	1	0	1	0	1	0	1	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0
Macrocarpaea arborescens	1	0	0	1	0	1	0	1	0	1	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0
Macrocarpaea bangiana	1	1	0	0	0	1	0	1	0	1	0	1	0	0	0	1	0	0	1	0	1	1	0	0	1	0	0
Macrocarpaea obtusifolia	1	1	0	0	0	1	0	1	0	1	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0
Macrocarpaea valerioi	1	1	0	0	0	1	0	1	0	1	0	1	0	0	0	1	1	0	0	0	1	1	0	0	1	0	0
Neblinantha neblinae	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	0	1	0	0	0	1	0
Neblinantha parviflora	1	0	0	0	0	0	1	1	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	0	1	0	0
Prepusa alata	1	0	1	0	0	0	1	0	1	1	0	0	1	1	0	0	1	0	0	0	1	1	0	0	1	0	0
Prepusa connata	1	0	1	0	0	0	1	0	1	1	0	0	1	0	1	0	1	0	0	0	1	1	0	0	1	0	0
Prepusa hookeriana	1	0	1	0	0	0	1	0	1	1	0	0	1	0	1	0	1	0	0	0	1	1	0	0	1	0	0
Prepusa montana	1	0	0	1	0	0	1	0	1	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	1	0	0
Prepusa viridiflora	1	0	0	0	1	0	1	1	0	1	0	1	0	1	0	0	1	0	0	0	1	1	0	0	1	0	0
Purdieanthus pulcher	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0	0	1
Rogersonanthus arboreus	1	1	0	0	0	0	1	0	1	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0
Rogersonanthus quelchii	1	1	0	0	0	0	1	0	1	1	0	0	1	0	1	0	1	0	0	0	1	0	1	0	0	1	0
Senaea coerulea	1	1	0	0	0	0	1	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0
Senaea janeirensis	1	1	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0
Sipapoantha ostrina	1	0	1	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0
Symbolanthus anomalus	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	1	0	0
Symbolanthus australis	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0
Symbolanthus calygonus	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0
Symbolanthus elisabethae	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0	0	1
Symbolanthus frigidus	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0

Symbolanthus mathesii	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0
Symbolanthus pterocaulis	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0
Symbolanthus vasculosus	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0
Symbolanthus yaviensis	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0
Tachia grandiflora	1	0	0	0	0	1	0	1	0	1	1	0	0	0	0	1	0	0	0	1	0	0	1	0	1	0	0
Tachia guianensis	1	0	0	0	0	1	0	1	0	1	1	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1
Tachia lorotensis	1	0	0	0	0	1	0	1	0	1	1	0	0	0	0	1	0	0	0	1	0	0	1	0	1	0	0
Tetrapollinia caerulescens	1	0	0	0	0	1	0	1	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	1
Zonanthus cubensis	1	1	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	0	0	1	0	0

	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	53
Adenolisianthus arboreus	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	1	1	1	0	0	1	0
Aripuana cullamaniorum	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	1
Calolisianthus pedunculatus	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	1	0
Celiantha bella	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1	0	0	0	0	1	1	0	1	1	0	0
Celiantha chimatensis	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	1	0	1	1	0	0
Celiantha imthurniana	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	0	0
Chelonanthus acutangulus	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0
Chelonanthus alatus	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	1	0	0	1	0
Chelonanthus albus	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	1	1	1	0	0	1	0
Chelonanthus angustifolius	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	1	1	1	0	0	1	0
Chelonanthus grandiflorus	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	1	1	1	0	0	1	0
Chelonanthus matogrossensis	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	1	1	1	0	0	1	0
Chelonanthus pterocaulis	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	1	1	1	0	0	1	0
Chelonanthus purpurascens	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0
Chelonanthus viridiflorus	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	1	1	1	0	0	1	0
Chorisepalum ovatum	0	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0	0	1	1	0	1	0	0	1
Chorisepalum psychotrioides	1	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0	0	1	1	1	0	0	0	1
Chorisepalum rotundifolium	0	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	0	1	0	1	1	1	0	0	0	1
Chorisepalum sipapoanum	0	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0	0	1	1	0	1	0	0	1
Helia brevifolia	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0
Helia oblongifolia	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0
Irlbachia cardone	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1	1	1	0	0	1	0
Irlbachia nemorosa	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0
Irlbachia poeppigii	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0
Irlbachia pratensis	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	1
Irlbachia pumila	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0
Lagenanthus princeps	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1	1	1	0	0	0	1

Lehmanniella splendens	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	1
Macrocarpaea angelliea	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0
Macrocarpaea arborescens	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	1	0	1	0	0	1
Macrocarpaea bangiana	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0
Macrocarpaea obtusifolia	0	0	0	0	1	0	0	0	1	0	0	0	1	0	1	0	0	0	0	1	1	1	0	0	1	0
Macrocarpaea valerioi	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0
Neblinantha neblinae	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1	1	0	1	0	0	1
Neblinantha parviflora	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1	1	1	0	0	1	0
Prepusa alata	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	1
Prepusa connata	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	1	0	0	1
Prepusa hookeriana	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	1
Prepusa montana	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	1
Prepusa viridiflora	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	1
Purdieanthus pulcher	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0
Rogersonanthus arboreus	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1	1	1	0	0	1	0
Rogersonanthus quelchii	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1	1	1	0	0	1	0
Senaea coerulea	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	1	1	0	0	0	1
Senaea janeirensis	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	1	1	0	0	0	1
Sipapoantha ostrina	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	1	1	0	1	0	1	0
Symbolanthus anomalus	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0
Symbolanthus australis	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1	1	1	0	0	1	0
Symbolanthus calygonus	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1	1	1	0	0	1	0
Symbolanthus elisabethae	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0
Symbolanthus frigidus	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1	1	1	0	0	1	0
Symbolanthus mathesii	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0
Symbolanthus pterocaulis	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	1	1	0	0	1	0
Symbolanthus vasculosus	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0
Symbolanthus yaviensis	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0

Tachia gradiflora	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0
Tachia guianensis	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	1	1	1	0	0	1	0
Tachia lorotensis	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	1
Tetrapollinia caerulescens	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0	1
Zonanthus cubensis	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	1	0	1	0	0

0 = ausente e 1 = presente.

Apêndice 1: Espécies de Gentianaceae estudadas organizadas por tribos com os respectivos coletores e herbários.

Tribe - *Taxon*: Voucher (Código do herbário). Sigla dos herbários: HUEFS = Universidade Estadual de Feira de Santana; INPA = Instituto Nacional de Pesquisas da Amazônia; MBM = Museu Botânico Municipal; NYBG = New York Botanical Garden; RB = Jardim Botânico do Rio de Janeiro; SJRP = Universidade Estadual Paulista Júlio de Mesquita Filho; SP = Instituto de Botânica; SPF = Universidade de São Paulo; UPCB = Universidade Federal do Paraná; UEC = Herbário da Universidade Estadual de Campinas; US = Smithsonian Institution; VIC = Universidade Federal de Viçosa.

Helieae – *Adenolisianthus arboreus* (Spruce ex Progel) Gilg: B.G.S.Ribeiro 1060 (RB), R.E.Schultes 5614 (NYBG), S.Madrinán & C.Barbosa 867 (NYBG), J.J.Wurdack & L.S.Adderley 43722 (NYBG); *Aripuana cullmaniorum* Struwe, Maas & V.A.Albert: P.R.J.Bamps 5406 (NYBG) , C.D.A.Mota s.n. (INPA), Calder 2668 (INPA); *Calolisianthus pedunculatus* (Cham. & Schltdl.) Gilg: V.C.Dalvi et al 76 (VIC); V.C.Dalvi et al 102 (VIC), V.C.Dalvi et al 109 (VIC); *Celiantha bella* Maguire & Steyerm.: N.T.Silva & U.Brazão s.n. (RB), B.Maguire et al s.n. (NYBG), J.A.Steyermark s.n. (NYBG); *Celiantha chimantensis* (Steyerm. & Maguire) Maguire: J.J.Wurdack s.n. (NYBG), J.A.Steyermark s.n. (NYBG), O.Huber et al s.n. (NYBG); *Celiantha imthurniana* (Oliv.) Maguire: J.A.Steyermark s.n. (NYBG), Vareschi et al 4917 (NYBG), R.Cardona 2664 (US); *Chelonanthus acutangulus* (Ruiz & Pav.) Gilg: M.Nee 48250 (NYBG), E.P.Killip & A.C.Smith 22534 (NYBG), s.c. (INPA), Benson 8302 (UEC); *Chelonanthus alatus* Pulle: E.Freire et al 12 (RB), L.Zarucchi et al 3028 (RB), A.Lisboa 66 (RB); *Chelonanthus albus* (Spruce ex Progel) V.M.Badillo: A.Ducke s.n. (RB), A.Ducke s.n. (RB), s.c. (INPA); *Chelonanthus angustifolius* (Kunth) Gilg: G.T.Prance et al s.n. (SP 225885), J.A.Silva

et al 405 (INPA), &rade 70 (SJRP), Silva 929 (SJRP); *Chelonanthus gr&iflorus* (Aubl.) E.Hassl.: C.A.Todzia et al 2307 (INPA), D.W.Stevenson 952 (INPA), B.W.P.Alburquerque et al 695 (INPA), M.F.Silva et al 618 (INPA), Castellani 14098 (UEC); *Chelonanthus matogrossensis* (J.G.M.Pers. & Maas) Struwe & V.A.Albert: G. Hatschbach 31949 (MBM), O.S.Ribas & L.B.S.Pereira 2549 (MBM), D.L.Amaral s.n (RB), s.c. (INPA), Cunha et al 440 (UEC), Nave et al 1086 (UEC); *Chelonanthus pterocaulis* Lepis: J.A.Steyermark s.n. (NYBG), S.F.R.H.J.Valles s.n. (NYBG), G.T.Prance & E.Forero 4008 (INPA), W.A.Rodrigues s.n. (INPA), I.S.Mir&oa 404 (INPA); *Chelonanthus purpurascens* (Aubl.) Struwe, S.Nilsson & V.A.Albert: P.C.Porto. 2371 (RB), L.Montone 1007 (RB), W.Fonseca s.n. (RB), J.R.Pirani et al 5441 (SPF), J.R.Pirani et al 5456 (SPF), R.Mello-Silva & R.C.Forzza 2839 (SPF), R.Mello-Silva & R.C.Forzza 2845 (SPF), M.F.Calió et al 119 (SPF), M.L.O.Trovó et al 328 (SPF), V.C.Souza et al 22940 (SPF); V.C.Dalvi et al 30 (VIC), V.C.Dalvi et al 31 (VIC), V.C.Dalvi et al 32 (VIC), V.C.Dalvi et al 36 (VIC), V.C.Dalvi et al 37 (VIC), V.C.Dalvi et al 48 (VIC), V.C.Dalvi et al 52 (VIC), V.C.Dalvi et al 60 (VIC); *Chelonanthus viridiflorus* (Mart.) Gilg: G. Hatschbach et al 36058 (MBM), G. Hatschbach & F.J.Zelma 49058 (MBM), M.R.P.Silva & I.Fern&es 3089 (MBM), J.A.Lombardi et al 3879 (RB), S.R.Neto et al 615 (SP), J.R.Pirani et al 4048 (SP), M.Alves et al 2250 (SP), M.F.A.Calió et al 38 (SPF), M.F.A.Calió et al 83 (SPF), A.Rapini et al 495 (SPF), M.F.Calió et al 204 (SPF); V.C.Dalvi et al 02 (VIC), V.C.Dalvi et al 03 (VIC); *Chorisepalum ovatum* Gleason: B.Maguire & L.Politi s.n. (RB), K.D.Phelps & C.B.Hitchocock s.n. (NYBG), B.K.Holst & R.L.Liesner 3313 (NYBG); *Chorisepalum psychotrioides* Ewan: S.S.Tillett & C.L.Tillett s.n. (NYBG), S.S.Tillett & C.L.Tillett s.n. (NYBG), H.D.Clarke et al 11682 (NYBG); *Chorisepalum sipapoanum* (Maguire) Struwe & V.A.Albert: B.Maguire & L.Politi s.n.

(NYBG), B.Maguire & L.Politi s.n. (NYBG), B.Maguire & L.Politi s.n. (NYBG); *Chorisepalum rotundifolium* Ewan: R.Liesner et al 21016 (NY), O.Huber & S.Gorzula s.n. (NY), J.A.Steyermark s.c. (NY); *Helia brevifolia* Cham.: R.Kummrow & J.Cordeiro 3391 (MBM), O.S.Ribas et al 5762 (MBM), W.Hoehne s.n. (SPF), W.Hoehne s.n. (SPF), G.Hatschbach & S.R.Ziller 64500 (SPF), H.Longhi et al s.n. (SPF), Luederwaldt s.n. (SPF), F.C.Hoehne s.n. (SPF), G.Gehrt s.n. (SPF), M.Kuhlmann s.n. (SPF), L.Krieger 9810 (SPF), G.C.T.Ceccantini & V.Alves 1480 (SPF), M.F.Calió et al 167 (SPF), M.F.Calió et al 168 (SPF), M.L.O.Trovó et al 316 (SPF), J.P.Souza et al 297 (SPF), R.Simão-Bianchini et al 889 (SPF), R.J.F.Garcia et al 1451 (SPF), C.M.Izumisaw et al 80 (SPF), H.Serafim 27 (SPF), E.P.Santos et al724 (UPCB), s.c. (HUEFS); *Helia oblongifolia* Mart.: A.C.Brade s.n. (SP), s.c. (SP), R.M.Harley et al 25648 (SPF), G.Ceccantini 180 (SPF), M.F.Calió et al 156 (SPF), M.F.Santos et al 108 (SPF), M.F.Calió et al 205 (SPF); *Irlbachia cardonae* (Gleason) Maguire: P.J.Maas et al s.n. (RB), P.J.Maas et al s.n. (RB), P.J.Maas & J.Steyermark s.n. (RB); *Irlbachia nemorosa* (Willd. ex Roem. & Schult.) Merr.: A.Ducke 11952 (RB), A.Ducke s.n. (RB), A.Ducke s.n. (RB), A.Ducke 12528 (RB), A. Loureiro et al s.n. (RB), F.Barros 947 (SPF), W.Montovani & D.M.S.Rocha s.n. (SPF); *Irlbachia poeppigii* (Griseb.) L.Cobb & Maas: A.Ducke s.n. (RB); W.R.&erson 11215 (INPA); P.L.B.Lisboa 793 (INPA); *Irlbachia pratensis* (Kunth) L.Cobb & Maas: J.J.Wurdack & L.S.Adderley s.n. (NYBG); I.Cordeiro et al 120 (INPA); *Irlbachia pumila* (Benth.) Maguire: C.Farney et al 1885 (RB 281371), James L. Zarucchi et al 3080 (RB 350583), P.J.M.Maas 6867 (INPA); *Lagenanthus princips* (Lindl.) Gilg: P.J.M.Maas & S.S.Tillet 5242 (NY), H.G.Barriga & R.Jaramilho s.n. (US), J.A.Steyermark et al (US); *Lehmanniella splendens* (Hook.) Ewan: J.L.Luteyn & R.Callejas 12502 (MBM), R.Callejas et al 4191 (NYBG); R.C.Fonenegra & F.J.Roldán

2698 (NYBG); R.Romero-Castaneda 1519 (NYBG); *Macrocarpaea angelliae* J.R.Grant & Struwe: D.Neil & L.Jost 15345 (NYBG), J.R.Grant et al 02-4289 (NYBG); *Macrocarpaea arborescens* Gilg: J.Grant & L.Struwe 01-4066 (NYBG), J.E.Madsen & L.Elleman 75280 (NYBG), J.L.Luteyn et al 6669 (NYBG); *Macrocarpaea bangiana* Gilg: St.G.Back 8699 (MBM), St.G.Back 8699 (NYBG), F.F.Alfredo & H.Huaylla 13130 (NYBG); *Macrocarpaea obtusifolia* (Griseb.) Gilg: M.Barreto 8845 (MBM), I.Cordeiro & R.Mello-Silva 2785 (SPF), L.Kollmann et al 1986 (SPF), V.C.Dalvi et al 85 (VIC), V.C.Dalvi et al 86 (VIC); *Macrocarpaea valerioi* St&l.: R.W.Lent 26 (NYBG), A.Jimenez 967 (NYBG), Steven R.Hill et al 17751 (NYBG); *Neblinantha parvifolia* Maguire: C.Farney et al 876 (RB), C.Farney et al 892 (RB), J.A.Steyermark s.n.(RB); *Neblinantha neblinae* Maguire: J.A.Steyermark s.n. (NYBG), T.Plowman & W.W.Thomas s.n. (NYBG), J.A.Steyermark s.n. (US); *Prepusa alata* Porto & Brade: S.Lima & Brade 14101 (RB), C.Farley & J.M.Caruso 1195 (RB), C.G.Gomes et al 153 (SPF); *Prepusa connata* Gardner: G.Martinelli 602 (RB), G.Martinelli 6102 (RB), G.Martinelli 16216 (SPF); *Prepusa hookeriana* Gardner: C.Farrey et al795 (RB), C.G.Gomes et al 153 (MBM), C.G.Gomes et al 153 (RB), A.P.Duarte s.n. (RB), C.B.Costa et al 508 (SPF), C.B.Costa et al 508 (SP), M.Nadruz et al 1735 (SPF), J.Meireles & M.K.Caddah 433 (UPCB); *Prepusa montana* Mart.: V.C.Souza et al 26365 (SP), A.Furlan et al s.n. (MBM), G.Martinelli et al 5259 (RB), G.Martinelli et al 5425 (RB), H.C.Lima et al 3900 (RB), N.L.de Menezes et al s.n. (SPF), A.Furlan et al s.n. (SPF), G.Hatschbach & O.Guimarães 42398 (SPF), R.M.Harley 22878 (SPF), R.M.Harley 22755 (SPF), A.Freire-Fierro et al 1749 (SPF), A.Freire-Fierro et al 1767 (SPF), W.Ganev 816 (SPF), N.Hind et al 3165 (SPF), A.Oliveira et al 64 (SPF), T.B. Cavalcanti et al 3214 (SPF), M.F.Calió et al 116 (SPF), J.L.Hage et al 2330 (SPF), &rade-Lima 6118 (SPF), E.Melo

et al 4794 (SPF), C.N.Fraga et al 2691 (SPF), G.Hatschbach & R. Kummarow 47930 (UPCB); *Prepusa viridiflora* Brade: C.N.Fraga & L.Kollmann 722 (RB), Brade 14782 (RB), L.Kollmann & C.N.Fraga 3188 (SPF), P.H.Labiak et al 4209 (UPCB), C.N.Fraga et al 2233 (UPCB), R.C.Forzza et al 4955 (UPCB), L.Kollmann & A.P.Fontana 11081 (UPCB); *Purdieanthus pulcher* (Hook.) Gilg: M.L.Grant 10519 (NYBG), H.St John 20673 (NYBG), M.L.Grant 10519 (NYBG); *Rogersonanthus arboreus* (Britton) Maguire & B.M.Boom: O.Huber et al s.n. (NYBG), J.L.Luteyn & J.A.Steyermarck 9606 (NYBG), J.A.Steyermarck & J.J.Wurdack s.n. (NYBG); *Rogersonanthus quelchii* (N.E.Br.) Maguire & B.M.Boom: B.Maguire s.n. (NYBG), B.Maguire s.n. (NYBG), G.H.H.Tate 397 (NYBG), P.Fiaschi & G.M.Plunklet 3192 (SPF); *Senaea coerulea* Taub.: M.C.E.Amaral et al s.n. (SP), M.C.E.Amaral et al s.n. (SPF), Dora Ramariz 127 (RB); *Senaea janeirensis* Brade: S.Lima & Brade 14215 (RB), J.Santos Lima s.n. (RB), G.Martinelli et al 12003(RB), *Sipapoantha ostrina* Maguire & B.M.Boom: B.Maguire & L.Politi s.n. (NYBG), B.Maguire et al s.n. (NYBG), J.A. Steyermark et al s.n. (NYBG); *Symbolanthus anomalus* (Kunth) Gilg: L.A.Scobar et al. 3116 (US), J.C.Mutis 5602 (US), O.Haught 6138 (US); *Symbolanthus australis* Struwe: Yunga 339 (US), C.Davidson 4962 (US), H.W.Hodge 6032 (US); *Symbolanthus calygonus* Griseb. Ou Gilg: S.Knapp et al 2111 (MBM), G.Harling & L.&ersson s.n. (MBM), M.Y.Rimachi 3711 (MBM), P.A.Loizeau et al 713 (MBM), St.G.Beck 8740 (MBM), R.Ferreyra 6852 (US), H.A.Hallard 21095 (US); *Symbolanthus elisabethae* Gilg: S.E.Tillet et al s.n. (NYBG), J.A.Steyermark s.n. (US), E.S.S.Silva 11 (INPA); *Symbolanthus frigidus* (Sw.) Struwe & K.Gould: J.A. Steyermark et al s.n. (NYBG-D106), s.c. (US), H.Stehle 1595 (US), s.c. (US); *Symbolanthus mathewsii* (Griseb.) Ewan: F.Woitkowski 8288 (US), P.C.Hutchison & J.K.Wright 5565 (US), F.R.Fosberg & M.A.Giler 23172 (US),

A.Lopez et al s.n. (US); *Symbolanthus pterocaulis* Struwe: C.L.Core 321 (US), B. Daniel 1565 (US), W.A.Archer 1287 (US); *Symbolanthus vasculosum* (Griseb.) Gilg: E.Delgado 178 (US), J.A.Steyermarck s.n. (US), J.L.Luteyn et al 6054 (US); *Symbolanthus yaviensis* Steyermark: B.Maguire & C.K.Maguire s.n. (NYBG), B.Maguire & C.K.Maguire s.n. (NYBG), O.Huber s.n. (NYBG); *Tachia gr&iflora* Maguire & Weaver: Ducke s.n. (RB), L.A.Pereira & J.O.Cardoso 1247 (RB), L.A.Pereira et al 1186 (RB), J.E.L.S. Ribeiro et al 839 (SP), J.C.Ongley & J.F.Ramos s.n. (NYBG), A.A.Oliveira et al s.n. (NYBG), C.Sastre et al 3878 (NYBG), J.H.E.Rova et al s.n. (NYBG), A.M.Pohlit s.n. (INPA); *Tachia guianensis* Aubl.: P.A. Loizeau et al 766 (MBM), T.G.Tutin 303 (RB), B.Maguire s.n. (NYBG), Oldeman 2182 (NYBG), Lescure 236 (NYBG); *Tachia loretensis* Maguire & Weaver: M.Y.Rimachi 6243 (RB), G. Klug s.n. (NYBG), L.Hendrix 300 (NYBG), P.Acevedo et al 1624 (NYBG); *Tetrapollinia caerulescens* (Aubl.) Maguire & B.M.Boom: L.O.A.Teixeira et al 1293 (RB), H.S.Irwin et al s.n. (RB), F.Barros 862 (RB), R.M.Harley et al 25990 (SP), R.C.Forzza & R.Mello-Silva 3748 (SPF), M.F.Calió et al 154 (SPF), P.G.Windisch et al 7836 (SPF), P.G.Windisch et al 7191 (SPF); *Zonanthus cubensis* Griseb.: J.A.Shafer s.n. (US).

CAPÍTULO 6

GENTIANACEAE OF THE ESPINHAÇO MOUNTAIN RANGE, BRAZIL

Valdnéa Casagrande Dalvi, Renata Maria Strozi Alves Meira, Gilmar Edilberto Valente & Aristéa Alves Azevedo

Guia de campo publicado no The Filed Museum, Chicago, USA

GENTIANACEAE of the Espinhaço Mountain Range, Brazil. 1

Valdnéa Casagrande Dalvi, Renata Maria Strozi Alves Meira, Gilmar Edilberto Valente & Aristéa Alves Azevedo
Universidade Federal de Viçosa, Minas Gerais

Photos by Valdnéa Casagrande Dalvi, except where indicated. Produced by Valdnéa Casagrande Dalvi.

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1 ***Calolisianthus*** *pedunculatus*
HELIEAE

2 ***Calolisianthus*** *pedunculatus*
HELIEAE

3 ***Calolisianthus*** *pendulus*
HELIEAE

4 ***Calolisianthus*** *speciosus*
HELIEAE (Photo G.E.Valente)

5 ***Calolisianthus*** *speciosus*
HELIEAE

6 ***Chelonanthus*** *purpurascens*
HELIEAE

7 ***Chelonanthus*** *viridiflorus*
HELIEAE

8 ***Chelonanthus*** *viridiflorus*
HELIEAE

9 ***Curtia*** *diffusa*
SACCIFOLIEAE

10 ***Curtia*** *tenella*
SACCIFOLIEAE

11 ***Curtia*** *tenuifolia*
SACCIFOLIEAE

12 ***Curtia*** *verticillaris*
SACCIFOLIEAE

13 ***Curtia*** *verticillaris*
SACCIFOLIEAE

14 ***Deianira*** *chiquitana*
CHIRONIEAE

15 ***Deianira*** *damazioi*
CHIRONIEAE

16 ***Deianira*** *damazioi*
CHIRONIEAE

17 ***Deianira*** *nervosa*
CHIRONIEAE

18 ***Deianira*** *pallescens*
CHIRONIEAE

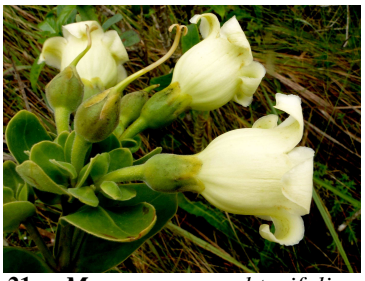
19 ***Deianira*** *pallescens*
CHIRONIEAE

20 ***Deianira*** *pallescens*
CHIRONIEAE

GENTIANACEAE of the Espinhaço Mountain Range, Brazil. **2**

Valdnéa Casagrande Dalvi, Renata Maria Strozi Alves Meira, Gilmar Edilberto Valente & Aristéa Alves Azevedo
Universidade Federal de Viçosa, Minas Gerais

Photos by Valdnéa Casagrande Dalvi, except where indicated. Produced by Valdnéa Casagrande Dalvi.
 Support: FAPEMIG, CAPES and CNPq.
471 version 1 05/2013



21 ***Macrocarpaea*** *obtusifolia*
HELIEAE

22 ***Macrocarpaea*** *obtusifolia*
HELIEAE

23 ***Macrocarpaea*** *obtusifolia*
HELIEAE

24 ***Macrocarpaea*** *obtusifolia*
HELIEAE

25 ***Prepusa*** *montana*
HELIEAE

26 ***Prepusa*** *montana*
HELIEAE

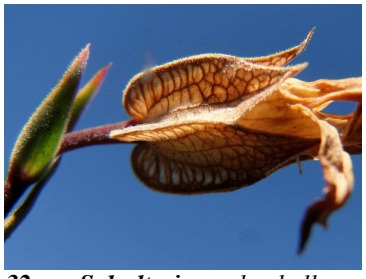
27 ***Schultesia*** *bahiensis*
CHIRONIEAE

28 ***Schultesia*** *bahiensis*
CHIRONIEAE

29 ***Schultesia*** *gracilis*
CHIRONIEAE

30 ***Schultesia*** *gracilis*
CHIRONIEAE

31 ***Schultesia*** *pachyphylla*
CHIRONIEAE



32 ***Schultesia*** *pachyphylla*
CHIRONIEAE

Morro do Pai Inácio (Chapada Diamantina)
Bahia

Serra de Ouro Branco
Minas Gerais

Photo: G.E.Valente

CONCLUSÕES GERAIS

Os resultados desta tese evidenciam a importância da anatomia de espécies de Gentianaceae em um contexto ontogenético, histoquímico, taxonômico e filogenético.

A presença de coléteres em *Macrocarpaea obtusifolia* foi comprovada pelos dados anatômicos, ontogenéticos e histoquímicos. O tipo de coléter descrito para esta espécie difere dos coléteres descritos na literatura para outras espécies de Gentianaceae. Estes resultados apontam para perspectivas de trabalhos futuros que versem sobre a investigação dos diferentes tipos de coléteres em Gentianaceae e sobre as implicações taxonômicas e filogenéticas.

Os resultados sobre a presença, tipo e padrão de distribuição de NEFs em folhas de 27 espécies neotropicais de Gentianaceae representam o primeiro registro que demonstra o quão comuns são tais estruturas para as espécies da região neotropical. Além dos novos registros de NEFs, evidenciamos a importância destas estruturas para a taxonomia da família. Este trabalho é o primeiro a apresentar um estudo sobre a diversidade e evolução de NEFs foliares da família Gentianaceae com uma abordagem filogenética. Foi demonstrado que os NEFs são uma provável sinapomorfia da família e que eventos de perdas de tais estruturas se processaram durante a evolução do grupo. Além disso, a presença ou ausência de NEFS em linhagens particulares dentro da família, bem como a diversidade quanto ao tipo e disposição de tais estruturas são informações que auxiliam na delimitação e caracterização de grupos. A presença de NEFs está relacionada ao padrão de distribuição geográfico das espécies e consequentemente com a ocorrência de formigas. Dessa forma, a importância dos NEFs na evolução e diversificação de Gentianaceae como um todo deve ser considerada.

A constatação de NEFs caulinares em cerca de 50% das espécies estudadas (17 de 38) permite concluir que estas estruturas também são comuns em caules. Correlações com o padrão de distribuição geográfico das espécies revelaram que NEFs caulinares são comuns em espécies neotropicais estando ausentes em espécies das demais regiões.

Os resultados de anatomia foliar de 60 espécies de Helieae, distribuídas em 21 gêneros, juntamente com as análises de similaridade permitiram identificar caracteres úteis para a delimitação e reconhecimento de espécies e da maioria dos gêneros da tribo. Os dados anatômicos não apenas constituem uma ferramenta, mas representam uma fonte de dados importantes em um grupo de taxonomia complicada como Helieae.

E por último, as excursões de campo ao longo do trabalho permitiram a montagem de um guia de campo com fotos ilustrativas para o reconhecimento das espécies de Gentianaceae ao longo da Cadeia do Espinhaço.