UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BOTÂNICA

Morfologia, anatomia e evolução em Tigridieae (Iridoideae: Iridaceae)

Tamara Pastori

Porto Alegre, março de 2018

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Tese apresentada ao Curso de Doutorado do Programa de Pós Graduação em Botânica da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do Título de Doutora em Ciências (Botânica).

Orientadora: Prof[a] Dr [a]. Lilian Eggers

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Tese aprovada pela banca examinadora para a obtenção do Título de Doutora em Ciências (Botânica) no Programa de Pós Graduação em Botânica da Universidade Federal do Rio Grande do Sul.

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Aos meus amados pais Adriano e Neusa,

e irmãos Mateus e Erick,

Ao meu amado companheiro Daniel,

dedico.

“aprendi com as primaveras a deixar-me cortar

e a voltar sempre inteira”

(Cecília Meireles)

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RESUMO

Morfologia, anatomia e evolução em Tigridieae (Iridoideae: Iridaceae)

Iridaceae apresenta distribuição cosmopolita e constitui uma das famílias mais diversas pertencentes à ordem Asparagales. Atualmente, estima-se que existam 2025 espécies e 66 gêneros, e a África subsaariana e a área neotropical são os prováveis centros de diversidade. Crocoideae e Iridoideae são as duas subfamílias mais diversas em Iridaceae, compreendendo 95% da riqueza de espécies. Iridoideae é formada por quatro grandes tribos e uma quinta tribo constituída exclusivamente pelo gênero australiano *Diplarrena*, grupo-irmão das demais tribos. Tigridieae compreende de 15 a 20 gêneros e 172 espécies, que ocorrem no Sul da América do Norte, América Central e América do Sul. Tigridieae foi subdividida em duas subtribos: Cipurinae e Tigridiinae, com base em caracteres citogenéticos, palinológicos e morfológicos. No entanto, a filogenia da subfamília Iridoideae envolvendo a tribo Tigridieae, mostrou que tanto Cipurinae quanto Tigridiinae não são monofiléticas e propôs a divisão de Tigridieae em dois clados (A e B). Com relação aos gêneros pertencentes ao Clado A, os dois gêneros mais representativos em número de espécies, *Cypella* e *Calydorea*, são não-monofiléticos e os caracteres tradicionalmente utilizados para a separação destes gêneros, como a fusão dos estames e a ramificação do estilete, não têm se mostrado eficientes para a separação genérica. O objetivo geral desta tese é fornecer dados para elucidar questões referentes à evolução e diversificação de Tigridieae (Iridoideae: Iridaceae) utilizando abordagens morfológicas, anatômicas, filogenéticas, químicas e evolutivas. Para este trabalho foram delimitadas diversas abordagens. A delimitação de espécies de *Cypella* foi realizada através da utilização de dados morfológicos e análises multivariadas, combinadas com análises filogenéticas de marcadores nucleares e plastidiais (Capítulo II). Posteriormente foram realizadas análises filogenéticas, anatomia da seção transversal das folhas de Tigridieae e evolução de caracteres (Capítulo III). Análises da composição química dos óleos florais foram realizadas a fim de compreender a relação dos mesmos com as estratégias de polinização (Capítulo IV). Os resultados obtidos com as diversas abordagens possibilitaram a delimitação de espécies de *Cypella*, e a sinonimização de *Cypella gloriana* em *Cypella pusilla*. Além disso, os caracteres de anatomia foliar, principalmente relacionados ao esclerênquima, possibilitaram a indicação de caracteres diagnósticos e uma nova circunscrição para Cipurinae e Tigridinae. Resultados das análises químicas dos óleos florais possibilitaram a identificação de lipídios e revelaram uma ampla gama de variações na composição de ácidos graxos livres entre espécies. Este trabalho forneceu uma caracterização importante para estudos futuros em biologia de polinização e para a

utilização deste óleo para as abelhas coletoras. O conjunto de resultados finais contribuiu para a compreensão dos processos de diversificação de Tigridieae e poderão ser utilizados em estudos futuros, principalmente para a revisão taxonômica dos principais gêneros de Cipurinae.

Palavras chave: filogenia, delimitação de espécies análises estatísticas multivariadas, anatomia foliar, caracteres diagnósticos, Cipurinae, Tigridineae, recursos florais, óleos florais.

ABSTRACT

Morphology, anatomy and evolution in Tigridieae (Iridoideae: Iridaceae)

Iridaceae presents cosmopolitan distribution and constitutes one of the most diverse families belonging to the order Asparagales. Presently, there are an estimated 2025 species and 66 genera, and sub-Saharan Africa and the Neotropical area are likely centres of diversity. Crocoideae and Iridoideae are the two most diverse subfamilies in Iridaceae, comprising 95% of species richness. Iridoideae is formed by four large tribes and a fifth tribe constituted exclusively by the Australian genus *Diplarrena*, sister group of the other tribes. Tigridieae comprises 15 to 20 genera and 172 species, occurring in southern North America, Central and South America. Tigridieae was subdivided into two subtribes: Cipurinae and Tigridiinae, based on cytogenetic, palynological and morphological characters. However, the phylogeny of the subfamily Iridoideae involving the tribe Tigridieae, showed that both Cipurinae and Tigridiinae are not monophyletic and proposed the division of Tigridieae into two clades (A and B).In relation to the genera belonging to Clade A, the two most representative genera in number of species, *Cypella* and *Calydorea*, are not monophyletic and the characters traditionally used for the separation of these genera, such as the fusion of the stamens and the branching of the style, are not efficient for generic delimitation. The aim of this thesis is to provide data to elucidate questions regarding the evolution and diversification of Tigridieae (Iridoideae: Iridaceae) using morphological, anatomical, phylogenetic, chemical and evolutionary approaches. For this study several approaches were delimited. The species delimitation of *Cypella* was performed through the use of morphological data and multivariate analyses, combined with phylogenetic data of nuclear and plastid markers (Chapter II). Later, phylogenetic analyzes, the leaf anatomy of Tigridieae and evolution of characters were performed (Chapter III). Analyses of the chemical composition of the floral oils were carried out in order to understand the relationship between them and the pollination strategies (Chapter IV). The results obtained with the different approaches allowed the delimitation of species of *Cypella*, and the synonymization of *Cypella gloriana* in *Cypella pusilla*. In addition, leaf anatomy characters, mainly related to sclerenchyma, allowed for the designation of diagnostic characters and a new circumscription for Cipurinae and Tigridinae. Results of the chemical analyses of the floral oils allowed the identification of lipids and revealed a wide range of variations in the composition of free fatty acids between species, provided an important characterization for future studies in pollination biology and for the use of this for oil collecting bees. The set of final results contributed to the

understanding of the processes of diversification of Tigridieae and could be used in future studies, mainly for the taxonomic revision of the main genera of Cipurinae.

Key words: phylogeny, species delimitation, multivariate statistical analyses, morphology, leaf anatomy, diagnostic characters, Cipurinae, Tigridineae, floral rewards, floral oils.

APRESENTAÇÃO

A presente tese está organizada em quatro capítulos: o primeiro fornece uma introdução geral sobre a família Iridaceae e os assuntos abordados na tese, bem como os objetivos gerais e específicos desta tese de doutorado. Os conteúdos dos capítulos seguintes são artigos científicos que serão ou estão submetidos a periódicos B1 ou superior. São estes: Capítulo II) "Iterative taxonomy based on morphological and molecular evidence to estimate species boundaries: a case study in *Cypella* Herb. (Tigridieae: Iridaceae)" (submetido para o periódico: Plant Systematics and Evolution), no qual o objetivo é a delimitação de cinco espécies do gênero *Cypella* utilizando análises filogenéticas e análises morfológicas multivariadas. Capítulo III) "Phylogeny, leaf anatomy and evolution of characters in Tigridieae (Iridoideae: Iridaceae)", a ser submetido para o periódico American Journal of Botany, onde o objetivo foi investigar caracteres de anatomia foliar entre espécies de Tigridieae e testar se estes podem ser utilizados como caracteres diagnósticos. O capítulo III) "Chemical of floral rewards: the role of non-volatile lipids on evolution of the Tigridieae (Iridoideae: Iridaceae)" a ser submetido para o periódico Phytochemistry, onde o objetivo do manuscrito é caracterizar quimicamente os óleos florais e identificar os lipídios presentes nas espécies de Tigridieae. O capítulo IV) Considerações finais, trata das conclusões obtidas nesta tese de doutorado. O capítulo V) consiste no o artigo "Overlooked diversity in Brazilian *Cypella* (Iridaceae, Iridoideae): four new taxa from the Río de la Plata grasslands" já publicado na revista Phytotaxa, onde estão descritas quatro táxons novos para o gênero *Cypella*, que foram utilizados nas análises desta tese.

CAPÍTULO I

INTRODUÇÃO GERAL

Iridaceae Juss.

Iridaceae é uma das famílias mais diversas pertencentes à ordem Asparagales (APG IV), com cerca de 2.025 espécies e 66 gêneros (Goldblatt e Manning, 2008). Iridaceae possui distribuição cosmopolita, a maior parte das espécies está distribuída na África subsaariana e na área neotropical, regiões indicadas como prováveis centros de diversidade (Goldblatt, 1990; Goldblatt e Manning, 2008). As espécies desta família possuem importância econômica principalmente no setor de paisagismo (por exemplo, os gêneros *Neomarica* Sprague, *Iris* L. e *Gladiolus* L.) e alimentação (*Crocus sativus* L., açafrão).

As Iridáceas são, em sua maioria, plantas herbáceas e de pequeno porte, podem possuir bulbos, rizomas, cormo ou caule lenhoso quando arbustivas (exemplo *Klattia* Baker, *Nivenia* Vent. e *Witsenia* Thunb.) (Goldblatt *et al*., 1998). São reconhecidas principalmente pela grande variedade na morfologia floral e foliar (Rudall, 1994; Goldblatt e Manning, 2006). Em Iridaceae, as folhas são alternas, dísticas, com presença de cristais prismáticos de oxalato de cálcio nas bainhas dos feixes vasculares (Prychid e Rudall, 1999; Rudall, 1995). As flores são geralmente actinomorfas, formadas por seis tépalas distribuídas em dois verticilos, apresentam três estames, grãos de pólen com exina reticulada e o ovário é ínfero (Goldblatt, 1990; Goldblatt e Manning, 2008).

Atualmente, Iridaceae está dividida em sete subfamílias: Isophysidoideae, Patersonioideae, Geosiridoideae, Aristeoideae, Nivenioideae, Crocoideae e Iridoideae (Fig. 1). Crocoideae e Iridoideae são as mais diversas, compreendendo 95% da riqueza de espécies (Goldblatt *et al*., 2008), havendo cerca de 29 gêneros e 1.032 espécies em Crocoideae e pelo menos 20 gêneros e 900 espécies em Iridoideae (Goldblatt e Manning, 2008).Os demais 5% das espécies estão distribuídas em cinco subfamílias: Isophysidoideae e Geosiridoideae são monoespecíficas, Aristeoideae e Patersonioideae são monogenéricas e representadas pelos gêneros *Aristea* Aiton e *Patersonia* R.Br., respectivamente. Nivenioideae possui cerca de 15 espécies, distribuídas em três gêneros *Klattia*, *Nivenia* e *Witsenia.*

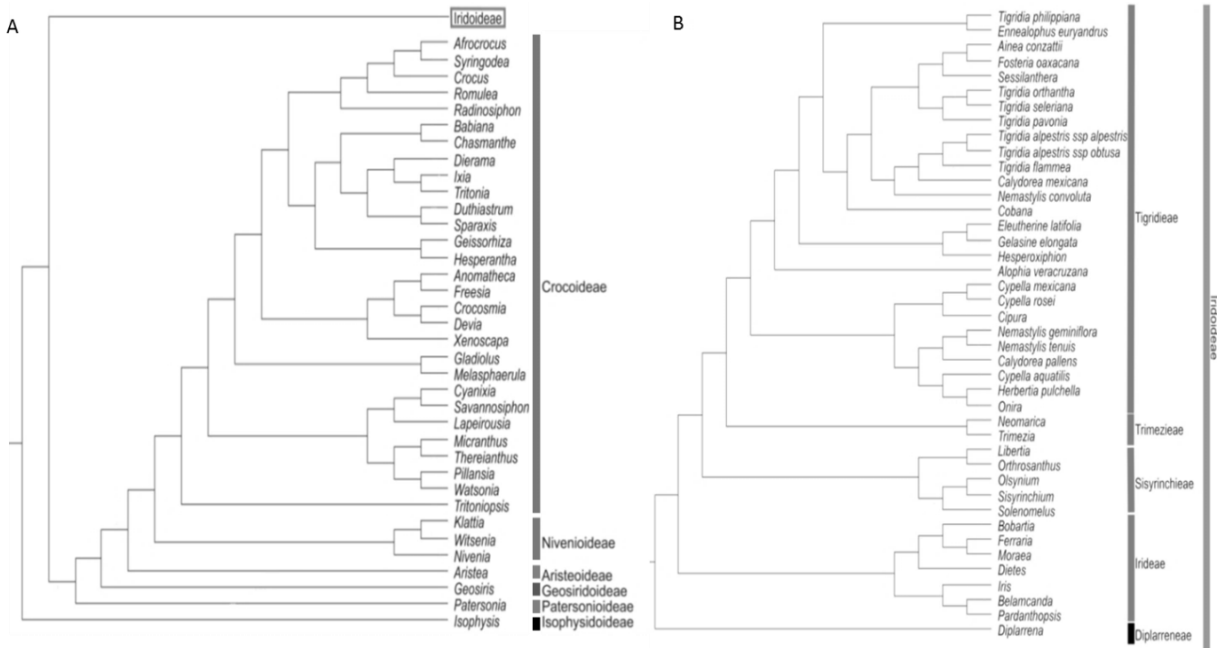

Figura 1 - Árvore filogenética das subfamílias e tribos de Iridaceae (A) e da subfamília Iridoideae (B), mostrando as cinco tribos. Adaptada de Goldblatt *et al.* (2008).

A subfamília Iridoideae é formada por cinco grandes grupos circunscritos ao nível taxonômico de tribo: Irideae, Sisyrinchieae, Trimezieae, Tigridieae e Diplarreneae, cujo gênero australiano *Diplarrena* Labill., único representante de Diplarreneae, é grupo irmão das demais tribos (Goldblatt e Manning, 2008). A tribo Irideae, predominantemente originária do Velho Mundo (com algumas espécies de *Iris* na América do Norte) e a tribo Sisyrinchieae (cujos gêneros *Libertia* Spreng. e *Orthrosanthus* Sweet apresentam ocorrência Austral-asiática e Americana) são grupos irmão das tribos Trimezieae e Tigridieae. Trimeziae e Tigridieae formam uma linhagem monofilética definida por sinapomorfias moleculares e morfológicas (Reeves *et al*., 2001; Rodriguez e Sytsma, 2006; Goldblatt *et al*., 2008; Goldblatt e Manning, 2008) e ocorrem exclusivamente no continente americano. A separação destas duas tribos ocorreu provavelmente no Eoceno tardio, há cerca de 35 milhões de anos (Goldblatt *et al*., 2008).

As espécies de Tigridieae estão distribuídas desde o sul da América do Norte, até América do Sul (Rodriguez e Sytsma, 2006; Goldblatt *et al*., 2008). Tigridieae compreende de 15 a 20 gêneros e 172 espécies, caracterizadas principalmente por possuírem bulbos, folhas plicadas ou foliadas, flores actinomorfas, tépalas geralmente livres e ramos do estilete desde achatados a petaloides, simples ou bifurcados (Goldblatt e Manning, 2008).

Tigridieae foi subdividida em 1982 em duas subtribos: Cipurinae e Tigridiinae, com base em caracteres citogenéticos, palinológicos e morfológicos. A subtribo Cipurinae foi caracterizada por possuir número cromossômico x = 7, grãos de pólen monosulcados e ramos do estilete petaloides a cilíndricos, bifurcados ou simples, enquanto a subtribo Tigridiinae foi diferenciada pelo número cromossômico x = 14, grãos de pólen bissulcados e ramos do estilete cilíndricos, profundamente bifurcados (ou simples) (Goldblatt, 1982). No entanto, a filogenia da subfamília Iridoideae, publicada por Chauveau *et al*. (2012) envolvendo a tribo Tigridieae, mostrou que tanto Cipurinae quanto Tigridiinae não são monofiléticas e propôs a divisão de Tigridieae em dois clados (A e B). O Clado A englobou alguns gêneros incluídos anteriormente na subtribo Cipurinae (*Ainea* Ravenna, *Calydorea* Herb., *Catila* Ravenna, *Cipura* Aubl., *Cypella* Herb., *Herbertia* Sweet, *Nemastylis* Nutt, *Larentia* Klatt, *Kelissa* Ravenna e *Onira* Ravenna) (Fig. 2) e o Clado B, os gêneros restantes da subtribo Cipurinae (*Cardenanthus* R.C. Foster, *Eleutherine* Herb., *Ennealophus* N.E. Br., *Gelasine* Herb. (Fig. 3j), *Hesperoxiphion* Baker, *Phalocallis* Herb. (Fig. 3k) e *Mastigostyla* I.M. Johnst.) e todos os gêneros da subtribo Tigridiinae (*Cobana* Ravenna, *Fosteria* Molseed, *Tigridia* Juss., *Alophia* Herb., *Sessilanthera* Molseed & Cruden).

Com relação aos gêneros pertencentes ao Clado A, os dois gêneros mais representativos em número de espécies, *Cypella* e *Calydorea*, são não-monofiléticos (Chauveau *et al*., 2012). Na filogenia recentemente publicada, *Kelissa* e *Onira* formam um primeiro agrupamento não-monofilético com *Cypella hauthalii* (Kuntze) R.C. Foster, sendo pouco diferenciadas de *Cypella* (Chauveau *et al*., 2012). Goldblatt e Manning (2008) consideraram que os caracteres tradicionalmente utilizados para a separação destes gêneros, como a fusão dos estames e a ramificação do estilete, não têm se mostrado eficientes para a separação genérica, conforme proposição de inclusão de *Kelissa* e *Onira* em *Cypella*, elaborada por Roitman e Castilho (2007). As variações observadas na organização dos estames e estiletes em *Catila*, *Itysa* Ravenna, *Lethia* Ravenna e *Tamia* Ravenna também foram consideradas insuficientemente discriminantes por Goldblatt e Manning (2008) para a manutenção dos gêneros, sendo incluídos em *Calydorea* por estes autores. Ravenna (2009), por sua vez, propôs a revalidação dos gêneros *Kelissa*, *Onira*, *Catila*, *Itysa* e *Tamia*, reafirmando a distinção com base na análise dos caracteres citados.

Figura 2: Espécies de *Cypella* pertencentes ao Clado A de Tigridieae (Iridaceae) a) *C. hauthalii* (Kuntze) R.C.Foster subsp. *hauthalii*; b) *C. hauthalii* subsp. *opalina* Ravenna*;* c) *C. hauthalii* subsp. *minuticristata* Chauveau & L.Eggers; d) *C. ravenniana* Deble & F.S.Alves*;* e) *C. armosa* Ravenna*;* f) *C. rivularis* Chauveau & L.Eggers*;* g) *C. luteogibbosa* Deble*;* h) *C. discolor* Ravenna*;* i) *C. osteniana* Beauverd; j) *C. fucata* Ravenna*;* k) *C. herbertii* (Lindl.) Herb.; l) *C. amplimaculata* Chauveau & L.Eggers. (a-j): Scale bar = 1 cm; (k-l) Scale bar = 5 mm.

Figura 3: Espécies de Tigridieae (Iridaceae) pertencentes aos Clados A e B a) *Calydorea approximata* R.C. Foster (foto de L.Eggers); b) *Calydorea alba* Roitman & J.A.Castillo; c) *Catila amabilis* Ravenna; d) *Cipura paludosa* Aubl.; e) *Kelissa brasiliensis* (Baker) Ravenna; f) *Herbertia zebrina* Deble; g) *Herberia pulchella* Sweet; h) *Herbertia furcata* (Klatt) Ravenna; i) *Herbertia darwinii* Roitman & J.A.Castillo; j) *Gelasine uruguaiensis* Ravenna, k) *Gelasine elongata* (Graham) Ravenna, l) *Phalocallis coelestis* (Lehm.) Ravenna (foto de M. Verdi). Scale bar = 1cm.

Com relação ao Clado B, os resultados obtidos por Goldblatt *et al*. (2008) e Chauveau *et al*. (2012) sugerem a inclusão de *Cardiostigma* Baker, *Colima* (Ravenna) Aarón Rodr. & Ortiz-Cat., *Fosteria*, *Rigidella* Lindl. e *Sessilanthera* em *Tigridia* Além destes, *Phalocallis* também apresenta divergências taxonômicas. Este gênero foi sinonimizado em *Cypella*, com base na morfologia floral (Roitman e Castillo, 2007; Goldblatt e Manning, 2008). Os caracteres utilizados para circunscrição de *Phalocallis* e *Cypella* não são distintivos e análises filogenéticas e cromossômicas recentes sugerem que estes gêneros devem ser separados (Chauveau *et al*., 2012; Moraes *et al*., 2015). Assim, a ausência de diferenças morfológicas claras entre gêneros de Tigridieae sugere que as circunscrições genéricas devem ser revistas (Chauveau *et al*., 2012).

Delimitação de espécies

O conhecimento taxonômico é complexo e peculiar, considerando que muitas espécies ainda são desconhecidas, outras foram descritas muitas vezes com nomes científicos diferentes, fazendo com que o número de espécies varie, particularmente em grupos menos estudados (Isaac *et al*., 2004). A delimitação de espécies é uma tarefa complexa, principalmente em um cenário evolutivo (Sites e Marshall, 2003). Idealmente, a caracterização das espécies deveria utilizar diferentes informações (morfológicas, anatômicas, fisiológicas, ecológicas, filogenéticas, geográficas, etc.) através de uma abordagem integrativa para a delimitação e identificação de *taxa* (Dayrat, 2005; Schlick-Steiner *et al*., 2010).

A ausência de limites claros entre as linhagens dificulta estratégias para a conservação e distribuição das espécies, principalmente para a formulação de políticas públicas e compilação de dados de diversidade atualizados. A taxonomia integrativa é capaz de fornecer melhores inferências sobre os limites das espécies (Dayrat, 2005; Padial e De la Riva, 2006; Padial *et al*., 2010). Entretanto, esta delimitação entre as linhagens envolve questões como o conceito de espécie a ser utilizado. A questão sobre "O que é uma espécie?" é muito antiga, já que muitos conceitos de espécie já foram propostos baseados em diferentes propriedades biológicas, como por exemplo, morfologia, isolamento reprodutivo, filogenia, ecologia, etc., e isso se deve provavelmente ao fato de que cada pesquisador aplica o conceito que entende como o mais adequado (De Queiroz, 2007). De fato, quanto maior o número de critérios de espécies atendidos por um grupo, mais provável é que ele seja uma linhagem distinta, sendo menos questionável o seu reconhecimento como espécie (De Queiroz, 2007). Apesar de existirem diversos conceitos de espécies, praticamente todos convergem em um elemento comum, que as

espécies são linhagens de metapopulações evoluindo separadamente, e este é o conceito de linhagem generalizada (GLC), amplamente adotado (Padial e De La Riva, 2006) e flexibilizado (Naciri e Linder, 2015; Freudenstein *et al*., 2017). No entanto, de acordo com Carstens *et al*. (2013), é um erro assumir que o GLC é um pré-requisito para a delimitação de espécies. Então, parece que o mais apropriado para a delimitação de espécies é analisar os dados com diferentes abordagens e, em seguida, delimitar as linhagens consistentes (Carstens *et al*., 2013; Naciri e Linder, 2015). Entretanto, a maioria das discordâncias entre diferentes autores sobre a ideia de espécie, não está relacionado ao conceito em si, mas sim em como reconhecer uma espécie.

Nas últimas décadas, os pesquisadores desenvolveram uma série de métodos para reconhecer novas espécies ou testar hipóteses de espécies (Wiens, 2007, Naciri e Linder, 2015). Entretanto, essas ferramentas para a delimitação de espécies envolvem muitas vezes métodos moleculares e computacionais dispendiosos e exigentes em mão de obra especializada. Além disso, nem todos os trabalhos que utilizam esses métodos propõem considerações taxonômicas, ou descrição de espécies novas, o que evidenciou a revisão realizada por Carstens *et al*. (2013): menos de 30% dos estudos avaliados propuseram recomendações taxonômicas e apenas 25% descrevem novas espécies. Segundo os autores, isso poderia indicar uma falta de confiança nos resultados, possivelmente ocasionada pela falta de treinamento taxonômico ou uma incapacidade de conciliar incongruências entre diferentes métodos. Nesse sentido, a obtenção de recursos e a formação de mão-de-obra especializada são essenciais para a compreensão dos limites entre as espécies e da diversidade como um todo.

Grupos de diversificação recente, ou grupos cientificamente pouco estudados, geralmente são os mais difíceis em taxonomia e sistemática. Iridaceae é uma das maiores famílias de monocotiledôneas e é considerado "taxonomicamente" difícil por diversos fatores, desde a duração efêmera das flores, dificuldade no processo de herborização, até processos de hibridação e evolução recente, o que dificulta a identificação e delimitação das espécies.

A taxonomia em Iridaceae, especialmente para os representantes americanos, está baseada principalmente na utilização de medidas morfológicas clássicas, principalmente de exsicatas e onde poucos indivíduos são amostrados. Nos últimos oito anos, somente para o Clado A de Tigridieae foram descritos 19 novos *taxa*: dois novos *taxa* de *Herbertia* (Deble, 2010; Deble, 2013), três de *Calydorea* (Deble, 2011, 2013, 2016) e 14 de *Cypella* (Deble *et al*., 2012; Chauveau *et al*., 2014; Deble *et al*., 2015a, b; Deble e Alves, 2017). Dentre estes novos *taxa* descritos, 100% foram baseados somente em análises morfológicas clássicas e, destes,

78,9% foram baseados na utilização de medidas de exsicatas e/ou através da utilização de uma ou duas populações.

De fato as diferenças morfológicas são úteis na delimitação de espécies, e têm sido utilizadas durante séculos. Entretanto, em alguns grupos, processos evolutivos recentes dificultam a delimitação, e muitas vezes a descrição de novos *taxa* baseada somente variação morfológica intraespecífica pode gerar uma superestimação no número de espécies (*inflated species delimitation*) (Duminil e Di Michele, 2009). No caso de Tigridieae, a diversidade pode ter sido negligenciada até poucos anos atrás, mas, por outro lado, se deve considerar a hipótese que talvez a diversidade tenha sido superestimada.

Os caracteres morfológicos permitem a identificação taxonômica, embora as inferências sobre os limites das espécies sejam melhor feitas usando uma abordagem que integre a taxonomia em diferentes tipos de dados e análises (Dayrat, 2005; Padial *et al*., 2010; Carstens *et al*., 2013).

Anatomia foliar

Iridaceae é uma das poucas famílias onde estão disponíveis diversos estudos sobre anatomia, principalmente anatomia foliar, e, além disso, muitos caracteres foram utilizados para circunscrições taxonômicas na família (Rudall, 1984; 1986; 1990; 1993; Rudall e Burns, 1989; Rudall e Goldblatt, 1991; 1993). Em Tigridieae, os caracteres vegetativos são muito semelhantes e a distinção de gêneros está basicamente indicada pela morfologia floral. A subdivisão de Tigridieae nos Clados A e B, com base em análises filogenéticas (Chauveau *et al*., 2012) evidenciou que caracteres anteriormente utilizados para a separação das duas subtribos (Cipurinae e Tigridiinae) não são homólogos e, portanto, uma investigação mais profunda é necessária, tendo em vista que outros atributos não evidentes, como a morfologia interna de estruturas vegetativas, poderia auxiliar na identificação de caracteres homólogos para os Clados A e B.

Estudos envolvendo anatomia foliar em Tigridieae foram realizados há cerca de três décadas atrás, e inexistência de filogenias moleculares bem resolvidas dificultou as considerações taxonômicas. Análises anatômicas realizadas por Rudall (1991, 1994) evidenciaram dois tipos de morfologia foliar, caracterizadas como folhas “plicadas” e “foliadas”, e os gêneros de Tigridieae foram classificados de acordo com esta. No entanto, ambos os tipos foliares ocorrem em Cipurinae e em Tigridiinae. Outras características anatômicas também foram consideradas por Rudall (1991; 1995), como o aspecto da margem

foliar e a presença de esclerênquima marginal. A presença, posição e constituição deste esclerênquima nas folhas mostra-se um caráter promissor para a separação de grupos em Tigridieae e, muito provavelmente, útil para a identificação de gêneros da tribo.

Oferta de recursos florais

A ampla variação na morfologia floral observada em Iridaceae esta relacionada com a diversidade dos sistemas de polinização, e é relatada principalmente para os gêneros africanos (Goldblatt e Manning, 2006). Na maioria das espécies de Iridaceae, o sistema de cruzamento preponderante é a fecundação cruzada, isto torna a presença do polinizador fundamental. Além disso, nas espécies africanas de Iridaceae, por exemplo, a grande maioria é polinizada por uma ou por poucas espécies de insetos, e somente 3% são visitadas por polinizadores generalistas (Goldblatt e Manning, 2006).

A oferta de recursos florais aos polinizadores é um fator chave a ser considerado para a evolução e a diversidade na morfologia floral. Neste sentido, Iridaceae é uma das poucas famílias de angiospermas a oferecer uma ampla gama de recursos florais. O néctar é o recurso mais comumente oferecido (Rudall *et al.*, 2003), em Crocoideae e Nivenioideae o néctar é produzido por células especializadas, localizadas entre as paredes ou septos no ovário e são chamados nectários septais (Rudall, 2003; Goldblatt e Manning, 2008). Em Iridoideae, os nectários septais são ausentes (exceto em Diplarreneae), o néctar é produzido em glândulas na superfície das tépalas (ex. *Tigridia*, *Moraea* Mill., *Ferraria* Burm. ex Mill.) ou dentro do tubo floral (Daumann, 1970; Rudall *et al*., 2003). Em Trimezieae, os nectários são do tipo tricomáticos, presentes nas tépalas internas do gênero *Neomarica* (Rudall, 2003) e em Sisyrinchieae o único registro de nectários é em *Olsynium* Raf. Já em Tigridieae, a presença de nectários inclui o gênero *Tigridia* (Clado B), que possui nectários tricomáticos nas tépalas internas, e *Cypella* (Clado A) que possui nectários no conectivo das anteras (Vogel, 1974; Devoto e Medan, 2008, Pastori *et al*., 2013, Pastori, 2014).

Os óleos florais constituem o terceiro tipo de recurso oferecido em Iridaceae. Este recurso é restrito a um conjunto de 11 famílias botânicas e dentre estas, Iridaceae é uma das únicas onde esse recurso surgiu independentemente diversas vezes (Orquidaceae é a outra família) (Renner e Schaefer, 2010). Em Iridaceae, os elaióforos são exclusivamente observados em representantes de Iridoideae e estão localizados geralmente nas tépalas ou no tubo estaminal (Vogel, 1974, Chauveau *et al.*, 2011, Silvério *et al.*, 2012). A única exceção é uma espécie pertencente à subfamília Crocoideae, *Tritoniopsis parviflora* (Jacq.) G.J. Lewis, que é

conhecida como produtora de óleos (Manning e Goldblatt, 2002, 2005). Segundo Buchmann (1987), possuem elaióforos os gêneros *Alophia, Cypella, Ennealophus, Sisyrinchium* L., *Sphenostigma* Baker, *Tigridia* e *Trimezia* Salisb. ex Herb. No entanto, Chauveau *et al.* (2012) mostraram, a partir de dados bibliográficos, que elaióforos estão presentes também nas espécies *Cardenanthus vargasii* R.C. Foster, *Catila amabilis* Ravenna, *Cipura paludosa* Aubl., *Ennealophus euryandrus* (Griseb.) Ravenna, *Hesperoxiphion*, *Mastigostyla* e nos gêneros monotípicos *Kelissa* e *Onira*. Apesar da existência de elaióforos ter sido relatada para estes gêneros, apenas algumas espécies de *Sisyrinchium* foram testadas através de análises histoquímicas para a detecção destas estruturas (Silvério *et al.*, 2012), os demais gêneros necessitam de testes de confirmação.

As relações entre as flores secretoras de óleo e abelhas coletoras constituem exemplo de uma especialização funcional e de uma interação incomum entre plantas e polinizadores, já que apenas polinizadores especializados são capazes de coletar estes óleos florais (Chauveau *et al*., 2011, 2012). Estudos sugerem que a seleção mediada por polinizadores constitui a principal força motriz para a diversidade floral (Valente *et al.*, 2012; Van der Niet e Johnson, 2012; Forest *et al.*, 2014). Adaptações a diferentes agentes polinizadores adicionados a fatores evolutivos seriam responsáveis por padrões de diversidade encontrados em espécies de plantas com flores (Goldblatt e Manning, 2008; Givnish, 2010; Valente *et al.*, 2012).

O padrão de diversificação floral observado em espécies de Iridaceae parece estar diretamente relacionado com a ampla oferta de recursos florais aos polinizadores. Alguns gêneros africanos da família, como *Tritoniopsis* L. Bolus e *Lapeirousia* Pourr. (Manning e Goldblatt, 2002, 2005; Goldblatt e Manning, 2008), já são reconhecidos por possuir polinização bimodal, oferecendo combinações de recursos florais aos polinizadores, e estudos recentes tem demostrado a especiação direcionada por essa interação (Valente *et al*., 2012; Forest *et al*., 2014). Os gêneros ocorrentes no continente americano são desconhecidos quanto a sua interação com os polinizadores. Estudos recentes tem demostrado que em Tigridieae ocorre uma ampla oferta de recursos florais e com diferentes combinações como, por exemplo, *Calydorea* e *Catila* oferecem somente pólen, *Herbertia* e *Kelissa* oferecem pólen e óleos florais, *Cypella* e *Onira* oferecem pólen, óleos florais e néctar (Fig. 4) (Pastori *et al*., 2013, Pastori, 2014). A ampla oferta e as diferentes combinações de recursos florais pode ter sido a força motriz para diversificação e a evolução da família na América do Sul, especialmente nas regiões Sul e Sudeste do Brasil (Pastori, 2014). A compreensão dos fatores envolvidos na diversificação das espécies é fundamental para a formulação de hipóteses sobre a evolução de um grupo

vegetal. Nesse contexto Iridaceae se destaca das demais famílias de angiospermas por ser uma das poucas que incluem ampla oferta de recursos florais e síndromes de polinização aliadas à alta diversidade de espécies (Manning e Goldblatt, 2005; Devoto e Medan, 2008; Goldblatt e Manning, 2008).

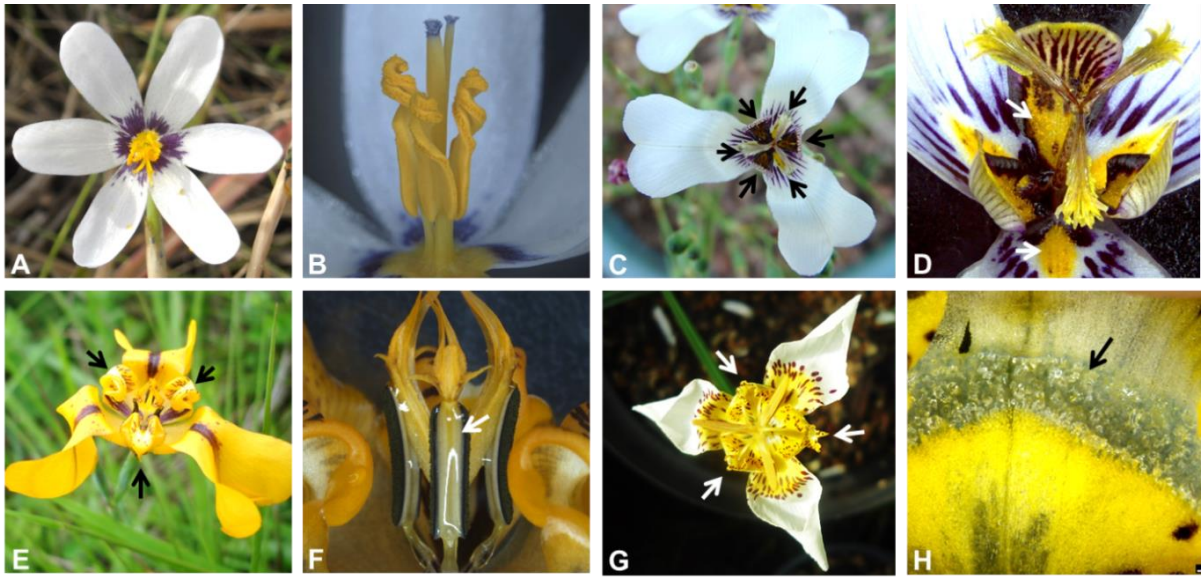

Figura 4: Espécies e diversidade de recursos florais oferecidos aos polinizadores em Tigridieae. A-B) *Calydorea alba* oferece como recurso somente pólen; C-D) *Herbertia zebrina*, pólen e óleos florais, que são produzidos em elaióforos localizados nas tépalas externas e internas (C) (localização indicada por setas); E-F) *Cypella amplimaculata*, pólen, óleos florais em elaióforos localizados somente nas tépalas internas (E), e néctar produzido no nectário localizado no conectivo das anteras (F); G-H) *Tigridia chiapensis* Molseed ex Cruden, pólen e néctar, o nectário tricomático, está localizado nas tépalas internas (H).

OBJETIVOS

O objetivo geral desta tese é fornecer dados para elucidar questões referentes à evolução e diversificação de Tigridieae (Iridoideae: Iridaceae) utilizando abordagens morfológicas, anatômicas, filogenéticas, químicas e evolutivas.

Objetivos específicos

1) Contribuir para a circunscrição e descrição de espécies de *Cypella*, através de uma abordagem iterativa, utilizando caracteres morfológicos e moleculares;
2) Gerar uma filogenia para a tribo Tigridieae baseada em marcadores plastidiais, focada especialmente no Clado A e utilizar uma ampla amostragem, a fim de testar as relações filogenéticas entre os gêneros;
3) Gerar uma matriz de dados anatômicos para os gêneros de Tigridieae;
4) Inferir a evolução dos caracteres de anatomia foliar de Tigridieae a partir da filogenia obtida com marcadores moleculares;
5) Caracterizar quimicamente os óleos florais em espécies de Tigridieae.

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CAPÍTULO II

Iterative taxonomy based on morphological and molecular evidence to estimate species boundaries: a case study in *Cypella* Herb. (Iridaceae: Iridoideae)

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Abstract *Cypella* Herb. is one of the largest genera of Tigridieae (Iridaceae: Iridoideae) with more than 30 species accepted by the World Checklist of Iridaceae (WCI). The current infrageneric taxonomy rests exclusively on morphological characters, which are more or less reliable because of their intraspecific variability, and the delimitation of various species recently described remains obscure. Therefore, multiple lines of evidence were used to assess species boundaries in a morphologically conserved group of five species, including various populations of *Cypella pusilla* and the closely related *C. gloriana*. Phylogenetic analyses based on ten genetic markers (eight plastid and two nuclear loci) and multivariate analyses of 38 qualitative and quantitative phenotypic characters were performed. Results revealed that species-level diversity was overestimated and intraspecific morphological variability of *C. pusilla* underestimated. Overall, our findings support the utility of combining genetic evidence and broad morphological sampling to validate and describe species using complementary approaches. Consequently, an updated taxonomic treatment of *C. pusilla* is presented, including an expanded description of the species.

Keywords discriminant analysis, integrative taxonomy, multiple correspondence analysis, phylogenetic delimitation, species recognition, Tigridieae.

Introduction

The discovery and description of taxa are preliminary steps for biodiversity assessments, and accurate identification of species is fundamental in almost all biological studies (Duminil et al. 2012; Su et al. 2015). Consequently, the process by which species boundaries are recognized based on operational criteria is crucial and must be robustly established (Hey 2001; Sites and Marshall 2003). There is a growing consensus that species should be circumscribed as evolutionary distinct lineages and that multiple lines of evidence should be used to test such delimitations as objectively as possible (Duminil et al. 2012; Carstens et al. 2013; Su et al. 2015). For centuries, morphology has been the major source of data used for taxonomic description and species discrimination. Even though morphological differences are useful operational criteria for delimiting species, they can fail to distinguish phenotypically similar species, whereas undersampling may inflate species richness estimate through erroneous perception of morphological variation within and between species (Duminil and Di Michele 2009). Beside qualitative characters, phenotypic differences among closely related taxa are predominantly quantitative and require multiple measurements and objective analyses (Pessoa et al. 2012). Multivariate statistical methods can combine large numbers of qualitative and quantitative data in few discriminant components to identify reliably phenotypic discontinuities among taxonomic groups (Borba et al. 2002; Sites and Marshall 2004) and are powerful estimates of species boundaries (Henderson 2006; Ezard et al. 2010). However, ample intralineage phenotypic plasticity, interlineage introgressions and morphologically cryptic speciation are frequent among plant species (Bickford et al. 2007; Adams et al. 2014; Su et al. 2015) and it is often difficult to separate them into discrete clusters. In this context, the use of traditional morphological characters evaluated in a rigorous statistical framework is increasingly improved by approaches based on molecular genetic data (e.g. Pessoa et al. 2012; Pante et al. 2015; Lu et al. 2016).

The resolution of phylogenetic relationships among any given set of taxa by tree-based methods is fundamental not only for evolutionary studies but also for the issue of species delimitation (Duminil et al. 2012; Fujita et al. 2012), and DNA sequences became the most extensively source of data used over the past decade to explore relationships among closely related taxa and to characterize species boundaries (Tautz et al. 2002; Naciri and Linder 2015). Chloroplast and nuclear genomes are the most commonly used to estimate species boundaries among plants (Duminil et al. 2012; Pessoa et al. 2012). Until recently, DNA analyses were frequently conducted by concatenating data across multiple genes to search for strongly

supported monophyletic groups that could represent species. However, discordance between species tree and gene trees caused by complex evolutionary processes, such as incomplete lineage sorting, reticulated evolution or polyploidization, is not uncommon (Padial et al. 2010; Tonini et al. 2015). Traditionally, molecular systematists increased the total number of loci included in the concatenated data matrices, assuming that the signal of multiple DNA regions from different genomes recovers the true species tree or, alternatively, allows to identify incongruences related to gene tree-species tree discordance (Tonini et al. 2015). The primacy of concatenation is now challenged and the problem of discordance has resulted in a major paradigm shift towards coalescent-based methods, which allow the user to test alternative hypotheses of lineage divergence (Fujita et al. 2012; Lambert et al. 2015). Nonetheless, concatenation methods remain useful tools for phylogenetic inference and species-delimitation assessment (Tonini et al. 2015). Recent empirical studies have shown that (1) the properties of concatenated trees can predict their concordance with species-tree estimates (McVay and Carstens 2013; Lambert et al. 2015), (2) coalescent methods are confronted by their own problems (Springer and Gatesy 2016) and are novel sources of previously under-appreciated, systematic errors (Simmons and Gatesy 2015). Since the concatenation approach of genetic data based on sequencing of multiple plastid and nuclear regions can discriminate between closely related species that have undergone dichotomous divergence, but also identify incongruences that putatively reveal discordant phylogenies among DNA regions, we explored such a case study using morphologically similar species of *Cypella* (Iridaceae).

This genus, with 36 species and five subspecies accepted by the World Checklist of Iridaceae (Barker 2017), is the largest South American member of the exclusively New World tribe of Tigridieae (Iridioideae), which is distinguished from the four remaining tribes of the subfamily Iridoideae by subterranean bulbs, plicate or foliate leaves and a basic chromosome number of x = 7 (Rudall 1991; Goldblatt and Manning 2008). Most species share a structurally complex androgynoecium apparatus and such variable floral structures contrast markedly with the high degree of vegetative uniformity observed in the tribe (Rodríguez and Sytsma 2006; Goldblatt et al. 2008). Indeed, variations in floral traits, which are often trivial differences, are commonly used for species circumscription within *Cypella* and closely related genera of Tigridieae (Goldblatt and Manning 2008). However, these taxonomic delimitations are currently challenged at both generic and species levels (Goldblatt and Manning 2008; Chauveau et al. 2012; Deble et al. 2015a; Deble 2017). During the last five years, 14 new taxa of *Cypella* exclusively distributed in the Rio de la Plata Grasslands (RPG) were described (Deble et al.

2012a; Chauveau et al. 2014; Deble et al. 2015a, b; Deble and Alves 2017). The RPG is one of the most extensive grassland biomes worldwide (Medan et al. 2011) and encompasses more than 80% of the species richness presently recognized for the genus, the highest level of diversity being encountered in a vast subarea, which extends from Southern Brazil to Uruguay, Northeast Argentina and the South East Paraguay (Chauveau et al. 2014). In this region, various members of *Cypella* are sympatric over part or most of their ranges and it is often difficult to discriminate among morphologically close species using diagnostic characters from original descriptions (e.g. Ravenna 1981a, 2005, 2009; Deble et al. 2015a). The recent description of *Cypella gloriana* Deble & F.S.Alves (Deble et al. 2015b), a new species morphologically close to *C. pusilla* (Link & Otto) Benth. & Hook. f. ex B.D. Jacks, represents a striking example of such taxonomic ambiguity. Both species are from the RPG area and more specifically from sandy and stony grasslands of Rio Grande do Sul (RS), the southernmost state of Brazil (Deble et al. 2015b). The latter species was described by Link and Otto (1828) as *Ferraria pusilla* from bulbs collected in Porto Alegre (RS) by Sello in 1826, and the new combination in *Cypella* was established by Jackson (1893), who validated the previous inclusion of *Ferraria pusilla* in *Cypella* by Bentham and Hooker (1883). The original basionym description was succinct and came with a drawing but without type specimen deposited in herbarium (Link and Otto 1828). During the 20th century, no further taxonomic information was added and, except two mentions related to the geographic distribution and infrageneric classification of the species (Ravenna 1977, 1981b), *C. pusilla* remained poorly known in the absence of new collections. Three years ago, Deble et al. (2015b) described *C. gloriana*, a new species only known from a small hill in São Vincente do Sul (RS) and separated from *C. pusilla* by tiny variations of continuous morphological characters. These differences were only related to floral traits: perigone (diameter), central concavity (diameter and depth), outer tepals claw (width), inner tepals blade (length and width), elaiophores (size), filaments (length), anthers (length and width) and anthers connective (width). Comparative measurements with *C. pusilla* were not provided for most of these traits and for the few characters truly compared, overlaps were observed or intervals between measurements did not exceed 1 mm.

In this study, we inferred framework phylogenies of *Cypella* and members of closely related genera. These preliminary findings were combined with morphological information available in the literature to select a subset of species nearly allied to *C. pusilla* (Fig. 1). We then used an iterative approach to assess species boundaries and expand on previous knowledge about diversification and morphological variation in this taxonomic subset. Because

phylogenetic relationships, species limits and taxonomy were previously incompletely resolved, we developed the most comprehensive sampling and multilocus sequencing from across the geographical distribution of this group of species to date. More specifically, molecular and morphological data were used at the population level to (1) examine species relationships based on the genetic differentiation revealed, respectively, by plastid (cpDNA) and nuclear (nuDNA) fragments; (2) establish morphological clusters based on statistical analyses of multiple variable traits; (3) evaluate the congruence between genetic and phenotypic observations; (4) test the reliability of nominal taxa currently recognized in *Cypella*, especially those delimited by morphotyping few specimens from a single locality, and (5) clarify the description and geographic distribution of *C. pusilla*.

Material and methods

Taxonomic sampling for preliminary phylogenetic analyses

Morphological species concept was used for the sampling used to infer framework phylogenies. Twenty species and three subspecies of *Cypella*, as well as six species of the closely related genera *Catila* Ravenna, *Calydorea* Herb., *Herbertia* Sweet, *Kelissa* Ravenna and *Onira* Ravenna were collected mainly from Rio Grande do Sul state of Brazil, each represented by one accession. Members of the genera allied to *Cypella* and the three species of *Cipura* Aubl. used as outgroups were selected according to previous studies (Goldblatt et al. 2008; Chauveau et al. 2012). Species list, collection data and voucher information are supplied in Online Resource 1.

Species and population sampling for taxonomic assessment

A dense population-level sampling strategy was adopted to study phylogenetic and morphological relationships among *C. pusilla* and closely related species. Ingroup species (i.e. *Cypella aquatilis* Ravenna, *C. discolor* Ravenna, *C. gloriana*, *C. pusilla* and *C. ravenniana* Deble & F.S.Alves) were selected according to topological proximities observed in the framework phylogenies and morphological affinities established by Deble et al. (2012b, 2015a, b) with *C. gloriana* and/or *C. pusilla* (see Results section). Plant material was mostly sampled from the wild in Brazil and Uruguay and, among the 23 distinct populations representing the ingroup, only one accession of *C. aquatilis* was from unknown geographic origin. Taxa and populations sampled for phylogenetic and morphological analyses, respectively, as well as

accession numbers, collection data and voucher information are given in Table 1. Based on the framework phylogenies, three different accessions of *Catila amabilis* Ravenna were selected as outgroup to root phylogenetic trees. The geographic distribution of ingroup and outgroup accessions is presented in Fig. 2.

DNA isolation, amplification and sequencing

Specimens of different populations distributed among the species studied as indicated in Online Resource 1 and Table 1 were mostly field-collected and total DNA was extracted from 15–20 mg of silica dried leaf material using a modified CTAB protocol with volume adjusted to 2 mL tubes (Doyle and Doyle 1990). A combination of ten coding and non-coding DNA regions previously used to assess phylogenetic relationships among Iridaceae (Rymer et al. 2010; Schnitzler et al. 2011; Chauveau et al. 2011, 2012) was selected: three coding cpDNA genes (*mat*K, *rbc*L and *rps*4), three cpDNA intergenic spacers (*psb*A-*trn*H, *rps*4-*trn*S and *trn*Q-*rps*16), two cpDNA introns (*mat*K-*5'trn*K and *rps*16), the nuDNA ITS (ITS1–5.8S–ITS2) and one intron within the nuclear gene RPB2. Primers used to amplify each DNA region and additional primers for sequencing are given in Online Resource 2. PCR amplifications were performed using a Verity 96–Well thermal cycler (Applied Biosystems, Foster City, CA, USA) in a 50 µL total volume reaction consisting of 15–50 ng of genomic DNA, 0.5 µM of each primer, 100 µM dNTP, 1× GoTaq flexi buffer, 2 mM $MgCl_2$, 1.25U Taq DNA polymerase GoTaq G2 Hot Start Polymerase (Promega, Madison, WI, USA), except for RPB2. PCRs of the nuclear intron were carried out in the same volume reaction but with the following components: 25–50 ng of genomic DNA, 0.4 µM of each primer and 25 µL of Qiagen HotStarTaq Master Mix (Qiagen, Valencia, CA, USA). The *rbc*L, *rps*4-*trn*S (coding and non-coding adjacent regions) and *psb*A-*trn*H loci were successfully amplified with the addition of DMSO (2 µL) to the PCR mix. Detailed PCR conditions for each DNA locus used in this study are supplied in Online Resource 3. Each DNA region was amplified as a single fragment except in a few cases where internal primers originally designed for sequencing were used to amplify the targeted region in smaller fragments. PCR products were subsequently purified and sent to the Molecular Biology and Genetic Engineering Centre of the State University of Campinas (CBMEG/UNICAMP, SP, Brazil) for sequencing in an ABI 3500 xL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Raw forward and reverse sequences for each sample were assembled with CodonCode Aligner 6.0.2 (CodonCode Corp., Dedham, MA, USA); ambiguous bases were initially checked by chromatograms examination; detected

polymorphisms were validated using a base quality threshold above 20 and consensus contigs were edited. Identifiers of GenBank sequence records are available in Appendix 1.

Alignment and phylogenetic analyses

Alignments of DNA sequences were conducted with MAFFT 7 (Katoh and Standley 2013) and manually validated with MEGA6 (Tamura et al. 2013). Unambiguously aligned gaps shared by two or more taxa were checked and coded with SeqState 1.4.1 (Müller 2005), following the Modified Complex Indel Coding approach (Simmons et al. 2007). Phylogenetic analyses were conducted using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) approaches and were initially performed on each DNA region separately (single region analyses) and on cpDNA and nuDNA markers respectively combined to detect potential incongruences among loci or genomes. Conflicts among the different datasets were explored through visual comparison of resulting tree topologies and support values. Incongruent topologies were considered significant with parsimony or likelihood bootstrap supports (PBS and LBS, respectively) ≥ 70% or Bayesian posterior probability (PP) ≥ 0.95 (Pirie 2015; Tkach et al. 2015), whereas a minimum of 85% bootstrap support (Baker et al. 2011; Tkach et al. 2015) and 0.99 posterior probability (Inácio et al. 2017) were considered as threshold values for strongly supported incongruences.

The MP analyses were conducted using PAUP* v.4.0b10 (Swofford 2002) with the following settings: heuristic search option, tree bisection-reconnection (TBR) branch swapping with 1000 random addition replicates and multrees on, all character states being unordered and equally weighted. Strict consensus trees were calculated from all most parsimonious trees and statistical supports (PBS) of internal clades were evaluated by parsimony bootstrap analysis (Felsenstein 1985) of 1000 replicates with the same heuristic search settings and multrees option off. ML and BI analyses were performed on the plastid and nuclear datasets respectively partitioned for each gene, intron or spacer region, and codon position in the regions coding for proteins (Online Resource 4) to accommodate locus-specific variations. Gap-coded characters were also partitioned by locus. Maximum likelihood (ML) analyses were carried out with RAxML 8.2.0 (Stamatakis 2014) using the GTRGAMMA model applied to each partition separately. To assess the stability of the tree topology and branch length values, 200 independent ML searches with different randomized stepwise addition parsimony starting trees were conducted to find the best-scoring ML tree and branch supports (LBS) were evaluated by 1000 pseudo-replicates of non-parametric standard bootstrap tests. BI analyses were run using MrBayes 3.2.6 (Ronquist et al. 2012). The most appropriate evolutionary model for each dataset

and data partition among the datasets (Online Resource 4) was selected with the Akaike Information Criterion (AIC) implemented by MrModeltest 2.3 (Nylander 2004). Gap-coded characters were included as additional datatype and treated using a simple model with variable rates. Two independent Markov Chain Monte Carlo (MCMC) runs, each with four Markov chains starting with a random tree, were performed simultaneously, sampling trees every 100 generations. Analyses of the different datasets were performed for 4×10^6 to 50×10^6 generations and convergence was verified by checking the Average Deviation of Split Frequencies (ADSF < 0.01), the Effective Sample Size (ESS > 200) and the Potential Scale Reduction Factor (0.99 < PSRF < 1.01) reported by MrBayes. Default value was used for the burn-in phase, a 50% majority-rule consensus tree was computed and Bayesian posterior probabilities (PP) were generated for the resulting tree.

Phylogenetic trees resulting from MP (strict consensus tree), ML (best-scoring tree) and BI (majority-rule tree) analyses were outgroup-rooted and PBS, LBS and PP were reported on the ML best-scoring topologies to summarize the results of all analyses at once. A given node was kept in the final representation of the ML trees only if one of the bootstrap values (PBS and LBS) reached at least 70% or if the PP was ≥ 0.95 and in the absence of topological conflict among MP, ML and BI trees.

Morphological measurements and multivariate analyses

Morphological data were obtained from observation and measurements performed in fresh material and in herbarium specimens of 20 populations of *Cypella* listed in Table 1 and distributed as follows: *Cypella aquatilis* (5 populations/8 specimens), *C. discolor* (3/8), *C. gloriana* (1/9), *C. pusilla* (8/64), *C.* cf. *pusilla* (1/9) and *C. ravenniana* (2/5). For morphological analyses, individuals of different populations were pooled together for all species, except for *C. pusilla*, where each population was analysed separately. Measurements were performed with a digital pachymeter and/or a stereomicroscope Leica M165. A total of 38 morphometric and categorical characters recorded in Table 2 were analysed on both vegetative and reproductive traits. Morphological terminology follows Goldblatt and Manning (2008) and Beentje (2010). Among the morphological features used by Deble et al. (2015b) to distinguish *C. gloriana* from *C. pusilla*, the diameter and depth of the central concavity of the perigone were excluded from our morphometric measurements because preliminary observations have shown that these dimensions were not stable for a given flower and varied considerably during anthesis. Matrices of morphological data collected during the current study are available on request from the corresponding author. The SPSS statistical package version 18 (SPSS Inc., Chicago, IL, USA)

was used for all data analyses. Mean and standard deviation values were calculated for all variables and are presented in Online Resource 5. Pearson's correlation coefficients were estimated among all pairs of morphometric characters and linear regressions were performed to estimate missing data when highly correlated characters ($r \geq 0.8$ or ≤ -0.8) were identified. Characters were evaluated for normality and homoscedasticity, whereas significance of variability among species and/or populations was estimated for each morphological trait using one-way ANOVA for morphometric and normalised characters and Kruskal-Wallis test for the remaining morphological traits (Online Resource 6). Characters were removed from the data matrix when insignificant variability was detected ($p > 0.05$).

Two distinct multivariate statistics were conducted to assess species limits. Both statistical analyses were run for two different taxonomic subsets using (1) all specimens of *Cypella* included in the current study or (2) exclusively specimens belonging to populations of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla*. Unless otherwise stated, the two taxonomic subsets will be called, respectively, subset A and B in the rest of the manuscript. To detect homogeneous groups among specimens of *Cypella* according to morphological character-states and to identify variables that best contribute to distinguish these groups, multiple correspondence analyses (MCA) extended to accommodate morphometric and categorical traits (Hill and Smith 1976) were performed. In addition, discriminant analyses (DA) of morphometric traits were used to discriminate among the pre-classified species of *Cypella* (subset A), *C.* cf. *pusilla* being considered a putative species, or among populations of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla* (subset B). Species assignment was performed by prior probabilities, estimated from groups size (casewise testing) and cross-validated (leave-one out classification) using a covariance matrix within groups, with a predictive model based on Fisher's linear discriminant functions of the predictor variables providing the best discrimination between species. Morphological characters used with MCA and DA are listed in Table 2 for each taxonomic subset.

Univariate analyses of morphometric variation

Morphological variation among populations of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla* was also assessed using univariate analyses of variance (ANOVA). The significance of morphometric differences among populations was tested using ten continuous parameters (i.e. perigon diameter, inner tepals length and width, connate and free filament lengths, anthers length and width, anther connectives width, style: length of filiform base and free part of style arms) considered discriminatory between *C. gloriana* and *C. pusilla* in Deble et al. (2015b).

Simple descriptive statistics (mean, standard deviation, standard error) of these characters were calculated for each population of *C. gloriana, C. pusilla* and *C.* cf. *pusilla* included in the current study and were tested for normality, skewness and homogeneity of variance. Box plots of mean, 25-75 percentiles, inner fences and outliers values were computed and post hoc comparisons using Fisher's least significant difference test (LSD, $p < 0.05$) were conducted for the ten selected characters to assess their potential to distinguish among the three putative taxa listed above.

Results

Preliminary phylogenetic framework

Properties and maximum parsimony statistics of each separate and combined dataset used in this part of the study are summarized in Online Resource 7 and alignments including coded gaps are available on request from the corresponding author. Little homoplasy was observed in most datasets (CI > 0.80; RI > 0.90), except for ITS (CI = 0.72; RI = 0.79) and the nuDNA dataset (CI = 0.78; RI = 0.83). The two most parsimony-informative DNA regions were the nuclear ITS (11.78%) and RPB2 (9.81%). Separate analyses of the different plastid and nuclear DNA regions did not reveal significantly supported incongruence among datasets of the same genomic compartment. Therefore, cpDNA and nuDNA regions were respectively combined into two independent molecular matrices for subsequent analyses. Phylogenies obtained from MP, ML and BI analyses of the combined cpDNA dataset (*rps*4-*trn*S, *rbc*L, *mat*K-*trn*K, *trn*Q-*rps*16, *rps*16, *trn*H-*psb*A) on the one hand and the combined nuDNA dataset (ITS, RPB2) on the other hand were largely identical in topology. Consequently, the best-scoring cladograms resulting from ML analysis of the combined cpDNA dataset (Online Resource 8) and the combined nuDNA dataset (Online Resource 9) are presented with support values from MP, ML and BI analyses reported.

The phylogenetic framework of *Cypella* resulting from the concatenated cpDNA dataset was largely consistent with previous studies (Goldblatt et al. 2008; Chauveau et al. 2012), corroborating the non-monophyletic status of the genus. However, the current phylogenetic analyses with increased taxonomic sampling of *Cypella* and additional plastid DNA regions yielded relevant information related to the relationships among taxa of *Cypella* and allied genera. *Catila amabilis* and *Calydorea pallens* Griseb. formed a strongly supported clade (PBS = 99.9%, LBS = 100%, PP = 1) sister with full support (PBS = LBS = 100%, PP = 1) to the

remaining species of *Cypella*, *Herbertia*, *Kelissa* and *Onira*. All sampled species of *Cypella*, except *C. hauthalii* (Kuntze) R.C.Foster, were recovered in a clade with weak to full support (PBS = 71.1%, LBS = 56%, PP = 1), whereas the three subspecies of *C. hauthalii* were nested among taxa of *Herbertia*, *Kelissa* and *Onira* into a strongly to fully supported (PBS = 99.9%, LBS = 100%, PP = 1) sister clade. Within the main clade of *Cypella*, *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla* formed with strong to full support (PBS = 99.8%, LBS = 100%, PP = 1) one of the earliest diverging clade, which was closely related to *C. aquatilis*, *C. charruana* Deble & F.S.Alves and the fully supported clade *C. discolor*+*C. ravenniana*.

The combined cpDNA matrix provided a better resolved topology than the combined nuDNA matrix, probably because of a larger number of variable sites (777 vs. 347) and/or a greater consistency (CI = 0.85 vs. CI = 0.78). In the nuDNA phylogeny, *Catila amabilis* and *Calydorea pallens* were also recovered with full support as sister to a poorly to moderately supported clade (PBS = 58.6%, LBS = 62%, PP = 0.96) where all species of *Cypella*, *Herbertia*, *Kelissa* and *Onira* were included. However relationships among *C. amabilis*, *C. pallens* and the clade formed by the remaining species of the ingroup were not resolved. Within this clade, *Cypella charruana* was the earliest diverging species, but this phylogenetic position was only moderately supported in Bayesian analysis (PP = 0.96). As observed in the cpDNA phylogeny, *Cypella* was not monophyletic since sampled species of *Herbertia*, *Kelissa* and *Onira* were deeply nested among taxa of the former genus with moderate to full support (PBS = 78.3%, LBS = 82%, PP = 1). *Cypella gloriana*, *C. pusilla* and *C.* cf. *pusilla* formed a fully supported clade closely related to *C. aquatilis* and *C. laxa* Ravenna but distantly related to *C. charruana* and the strongly supported clade (PBS = 97%, LBS = 99%, PP = 1) *C. discolor*+*C. ravenniana*.

Visual comparisons of the cpDNA and the nuDNA combined topologies disclosed significantly to strongly supported discrepancies related to the phylogenetic relationships of various species of the ingroup. The most striking incongruences were the placement of *C. charruana*, *C. discolor*+*C. ravenniana* and *C. hauthalii* in relation to the remaining species sampled. Because of the discrepancies detected between the cpDNA and the nuDNA datasets, the two matrices were not combined for further analyses.

Species sampling for taxonomic assessment

Among the taxa currently accepted in *Cypella*, *C. gloriana* and *C. pusilla* were considered morphologically more or less closely related to *C. charruana*, *C. discolor*, *C. ravenniana* and *C. suffusa* Ravenna (Deble et al. 2012b; Deble et al. 2015b). The two former

species are easily distinguished because they produce flowering spathes almost sessile (see Link and Otto 1828; Deble et al. 2015b) and the length of the peduncles does not exceed 0.8 cm in *C. gloriana* and 0.5 cm in *C. pusilla* according to Deble et al. (2015b). Data available in the literature (Ravenna 1981a; Deble et al. 2015b) showed that the measurements of peduncles length in the four closely related species listed above slightly overlap those of *C. gloriana* and *C. pusilla* only in *C. discolor* (0.6 to 1.9 cm) and *C. ravenniana* (0.5 to 1.8 cm). Descriptions of *C. charruana* and *C. suffusa* revealed that the peduncles present in both species are much longer and vary from 1.4 to 2.8 cm in *C. charruana* (Deble et al. 2015b) and from 2.5 to 5.5 cm in *C. suffusa* (Deble et al. 2015a). Moreover, the former species exhibits very distinct floral characteristics (Deble et al. 2015b) and the latter species, which is distributed in a very small area of north-eastern Argentina (Deble et al. 2015a) far from the range area of *C. gloriana* and *C. pusilla*, is also readily recognized from any other species contemplated here by the presence of two-flowered spathes (vs. one-flowered spathes), this characteristic being one of the major morphological features used to discriminate taxa of *Cypella* in identification keys (e.g. Deble et al. 2015a, b; Deble and Alves 2017).

Based on these preliminary morphological data and the results revealed by the framework phylogenies, we chose to exclude *C. charruana* and *C. suffusa* from the comparative sampling and to add *C. aquatilis*, which was never compared with *C. gloriana* and *C. pusilla* on a morphological point of view but was phylogenetically close to these species in both cpDNA and nuDNA phylogenies. Therefore, the following species were selected for taxonomic assessment: *C. aquatilis*, *C. discolor*, *C. gloriana*, *C. pusilla* and *C. ravenniana*.

Phylogenetic analyses for taxonomic assessment

The number of characters and the maximum parsimony statistics in separate and combined datasets alignments of the cpDNA and nuDNA regions, including nucleotides and coded gaps, are presented in Table 3 and data matrices are available on request from the corresponding author. There was little homoplasy in most datasets (CI > 0.80; RI > 0.90), except for *mat*K-*5'trn*K (CI = 0.76; RI = 0.80) and ITS (CI = 0.78; RI = 0.91) and the two most parsimony-informative DNA regions were the nuclear ITS (13.4%) and the plastid spacer *trnQ-rps16* (4.4%). Since no significantly supported incongruence was detected among tree topologies obtained from the five plastid datasets on the one hand and the two nuclear markers on the other hand, cpDNA and nuDNA regions were respectively combined into two independent molecular matrices for subsequent analyses.

The matrix of the combined plastid data included 6964 characters (6939 nucleotide positions and 25 coded indels) of which 162 (2.32%) were parsimony-informative. MP, ML and BI phylogenies were largely identical in topology and support values obtained from the three analyses were reported on the ML best-scoring cladogram presented in Fig. 3a. All resolved relationships were strongly to fully supported (PBS ≥ 90.1%, LBS ≥ 91%, PP ≥ 0.99). Among species of *Cypella* sampled in the present study, *C. aquatilis*, *C. discolor* and *C. ravenniana* were unambiguously monophyletic. Accessions of *C. pusilla* were distributed among two strongly-supported sister lineages, one of them including *C. gloriana* and *C.* cf. *pusilla* in a polytomy. The earliest diverging clades were formed by *C. discolor* and *C. ravenniana* on the one hand, and *C. aquatilis* resolved as sister group to the different populations of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla* on the other hand.

The alignment of the combined nuDNA regions included 1408 characters (1371 nucleotide positions and 37 coded indels) of which 144 (10.23%) were parsimony-informative. Tree topologies resulting from the MP, ML and BI approaches were largely congruent as shown by the number of clades significantly supported by PBS, LBS and PP in the ML best-scoring tree (Fig. 3b). Even though most of the relationships among populations of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla* remained poorly resolved or unresolved, these accessions formed a fully supported clade (PBS = 100%, LBS = 100%, PP = 1), sister to the remaining species of *Cypella* with full support. Unless otherwise stated, this clade will be called *gloriana+pusilla* clade in the rest of this manuscript. Moreover, both *C. gloriana* and *C.* cf. *pusilla* were nested among populations of *C. pusilla* into a sublineage with strong to full support in ML and BI analyses (LBS = 91%, PP = 1). The monophyly of *C. aquatilis* and *C. discolor* was strongly to fully supported (PBS ≥ 98.3%, LBS ≥ 99%, PP = 1) and *C. ravenniana* was strongly supported in ML and BI analyses (LBS = 82%, PP = 0.99) but only weakly supported in MP analysis (PBS = 66.2%). The sister relationships between *C. discolor* and *C. ravenniana* and the dichotomy between both species and *C. aquatilis* were also strongly supported (PBS ≥ 89.6%, LBS ≥ 85%, PP = 1).

Only two strongly supported discrepancies were observed between the cpDNA and the nuDNA combined trees (Fig. 3): (1) *C. aquatilis* was sister to the *gloriana+pusilla* clade in the cpDNA tree, while this species was sister to the clade formed by *C. discolor* and *C. ravenniana* in the nuDNA tree; (2) one population of *C. pusilla* (Pop. ID: 818) formed, with another accession of the same species (Pop. ID: 194), a small clade sister to all other populations of the *gloriana+pusilla* clade in the cpDNA tree, whereas this population was more distantly related

to pop. 194 in the nuDNA tree and included in a larger clade sister to *C. gloriana* within the *gloriana+pusilla* clade. Even though these incongruent placements could indicate potential reticulate evolution, the taxonomic sampling should be increased within the species-rich genus *Cypella* and the phylogenetic resolution should be improved within the *gloriana+pusilla* clade to test properly this hypothesis. Because of the discrepancies detected, we did not combine the cpDNA and nuDNA matrices for further analyses.

Morphology: multiple correspondence analyses (MCA)

The approach adopted showed that the matrix of distances could be summarized adequately by using the two first dimensions of the MCAs performed on taxonomic subsets A and B. The MCA conducted on the former taxonomic subset revealed four main groups (Fig. 4a), three of them being congruent with *C. aquatilis*, *C. discolor* and *C. ravenniana*, respectively, whereas the populations of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla* were gathered in another group. The first dimension (D1) accounted for 57.5% of the total variance (inertia = 0.575, λ = 15.2) and the second dimension (D2) for 31.3% (inertia = 0.313, λ = 11.7). Cronbach's alpha coefficients, provided as an indication of reliability, were 0.977 and 0.935 for D1 and D2, respectively. Sixteen of the 24 characters used in the MCA had relatively high squared factor loadings (discrimination measures (DM) > 0.7) on at least one of the first two dimensions, the DM of five characters being higher than 0.8 on both dimensions (Online Resource 10). These characters were distributed not only in different flower parts (perigon, androecium and gynoecium) but also in other reproductive (floral stem: length of the first cauline leaf or bract present after the first internode, internodes number, synflorescence: inner peduncles length, rhipidia number) and vegetative (plant height, basal cauline leaves length and width) parts. However, this set of highly discriminant characters did not separate *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla*.

This distribution pattern was corroborated by the MCA analysis conducted with 33 morphological characters on the taxonomic subset B, where only populations of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla* were included (Fig. 4b). The first dimension (D1) accounted for 36.9% of the total variance (inertia = 0.369, λ = 13.5) and the second dimension (D2) for 29.1% (inertia = 0.291, λ = 9.7). Cronbach's alpha coefficients were 0.951 and 0.930 for D1 and D2, respectively. The MCA results showed that only one population of *C. pusilla* (Pop. ID: 148) and one individual of another population (Pop. ID: 174) were separated along the second dimension from a large group formed by the rest of the taxonomic sampling. In this context,

both *C. gloriana* and *C.* cf. *pusilla* were not distinguished from most of the populations of *C. pusilla*. Among the 33 morphometric and categorical characters used to conduct this analysis, only four (i.e. plant height, length of basal cauline leaves, inner valve and ovary length) had relatively high discrimination measure on dimension 1 and/or dimension 2, but 28 characters were significantly discriminant (DM > 0.2) on one of the first two dimensions (Online Resource 10).

Morphology: discriminant analyses (DA)

Since more than 80% of the morphological variation observed in taxonomic subsets A and B was captured by the first two discriminant functions (DF1 and DF2), results were summarized using 2D ordination diagrams based on DF1 and DF2 (Fig. 5). The ordination diagram of the DA performed on the first taxonomic subset (A) with 18 morphometric characters (Online Resource 11) showed four main groups (Fig. 5a), *C. aquatilis*, *C. discolor* and *C. ravenniana* being clearly separated from each other in three different groups, whereas specimens of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla* clearly overlapped in another group. The two discriminant functions explained 82% of the total variation (DF1 = 60.7%, DF2 = 21.3%) and were strongly correlated (Canonical correlation: DF1 = 0.991, DF2 = 0.941) and highly significant (DF1 through DF2: Wilk's lambda = 1.42×10^{-5}, $\chi^2 = 909.56$, $p < 10^{-10}$; DF2: Wilk's lambda = 6.59×10^{-4}, $\chi^2 = 596.92$, $p < 10^{-10}$). Based on the correlation coefficients obtained between each character and the discriminant functions, largest absolute correlations were detected for four variables: width of basal cauline leaves, length of the first cauline leaf or bract present after the first internode of the floral stem, length of inner peduncles of the synflorescence and length of adaxial crests of the gynoecium (Online Resource 11).

The overall correct classification rate was 95% for the casewise testing (CT) method and 92% for the cross-validation (CV) method. Both CT and CV methods classified correctly all specimens of *C. aquatilis*, *C. discolor* and *C. ravenniana* into the taxonomic group to which they were a priori assigned to test the morpho-species hypothesis. Specimens not assigned to the correct taxonomic group were mainly *C. gloriana* misidentified as *C. pusilla* (CT: 22.2%, CV: 44.4%) and *C.* cf. *pusilla* misidentified as *C. pusilla* (CT and CV: 33.3%).

The first and second canonical discriminant function of the DA performed on the taxonomic subset B with 31 morphometric characters (Online Resource 11) separated two populations of *C. pusilla* (Pop. ID: 148, 194) from each other and from the remaining specimens of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla*, which formed together a distinct cluster (Fig. 5b).

The two functions explained 81.1% of total variation (DF1 = 54.8%, DF2 = 26.3%) and the samples grouping was strongly supported by both discriminant functions, with high correlation values (DF1= 0.995, DF2 = 0.971) and discriminatory power (DF1 through DF2: Wilk's lambda = 7.74×10^{-4}, $\chi^2 = 250.73$, $p < 10^{-10}$; DF2: Wilk's lambda = 1.14×10^{-2}, $\chi^2 = 156.67$, $p < 10^{-10}$). Based on the correlation coefficients obtained between each character and the discriminant functions, largest absolute correlations were detected for five floral characters: anthers width, length of the filiform base of the style, adaxial and abaxial crests length, stigmatic appendages length (Online Resource 11).

However, classification results showed that only 60.3% of the specimens were assigned to the correct population using the CT method and the classification rate fell to zero with the CV method. The CT method revealed that most individuals of *C. gloriana* (85.7%) were misidentified as *C. pusilla*, whereas all specimens of the former species fell into *C. pusilla* (42.9%) and *C.* cf. *pusilla* (57.1%) with the CV method. Moreover, the classification results based on the CT method showed that 11.1% of the individuals of *C.* cf. *pusilla* were incorrectly assigned to *C. pusilla*, while all specimens were misidentified as this latter species with the CV method.

Morphology: univariate analyses

The range of variation of ten floral characters considered useful to distinguish *C. gloriana* from *C. pusilla* by Deble et al. (2015a) are presented in box and whisker plots (Fig. 6). Generally, *C. pusilla* showed the greatest variability for all ten morphometric characters, which was expected as this species was the most widespread geographically and the most extensively sampled. For all characters, except the inner tepals width, results of descriptive statistics revealed a considerable interquartile overlap among the three taxa considered and the univariate tests of significance confirmed the absence of statistical differences at the 5% level between different populations of *C. pusilla* and both *C. gloriana* and *C.* cf. *pusilla*. Moreover, six characters (i.e. inner tepals length, connate and free filament lengths, anthers length, style: length of filiform base and free part of style arms) did not provide significant differences between *C. gloriana* and *C.* cf. *pusilla*. The population of *C. gloriana* had significantly broader inner tepals ($p < 0.05$) than the rest of the populations included in the current sampling but the former population showed a 25-75 percentile overlap with one population of *C. pusilla* (Pop. ID: 778) and a minimum-maximum overlap with *C.* cf. *pusilla* and three populations of *C. pusilla* (Pop. ID: 184, 194, 778).

Discussion

Descriptions of new species in Iridaceae are still almost exclusively based on morphological differences as the single indicator of species-level differentiation (e.g. Goldblatt and Manning 2015; Huaylla 2015; Oliveira et al. 2016; Deble and Alves 2017; Memariani and Joharchi 2017) and the intra- and interspecific character variation is never thoroughly addressed using statistical methodologies. However, a growing number of taxonomists is convinced that the use of multiple lines of evidence in a statistical context improves the quality of species hypotheses and should contribute to a better understanding of biodiversity (Pessoa et al. 2012; Pante et al. 2015). In the current study, molecular data were used to provide a phylogenetic framework where monophyletic groups and evolutionary relationships between and within these groups were identified among specimens of six pre-classified and closely related taxa of *Cypella*. Concomitantly, the morphological diversity present in the taxonomic sampling was estimated based on a large number of continuous and discrete characters, whereas univariate and multivariate statistical methods were used to test the validity of species hypotheses. Finally, results of each different approach were compared to assess for congruence before any revision of formal species delimitation and description. The combination of multiple lines of evidence to test species delimitation is often called integrative taxonomy (Dayrat 2005; Will et al. 2005) but the present work more closely conforms to the concept of iterative taxonomy (Yeates et al. 2011) as molecular and morphological data were used to iteratively assess and evaluate species hypotheses. Analyses of molecular and morphological evidence provided consistent and robust support to consider that among the six taxonomic entities sampled in the current study, only four species should be maintained (*C. aquatilis*, *C. discolor*, *C. pusilla*, *C. ravenniana*), whereas *C. gloriana* and *C.* cf. *pusilla* should be regarded as conspecific to *C. pusilla*.

Phylogenetic analyses and interspecific relationships

The respective combined datasets of plastid and nuclear DNA regions robustly distinguished four main monophyletic groups corresponding to three extant species (*C. aquatilis*, *C. discolor*, *C. ravenniana*), populations of *C. gloriana*, *C. pusilla*, *C.* cf. *pusilla* being included in the remaining clade. Within this clade, phylogenetic analyses of both combined datasets resulted in large polytomies but phylogenetic relationships recovered by cpDNA and nuDNA regions were sufficiently resolved to consistently place *C. gloriana* and *C.* cf. *pusilla* within *C. pusilla*. Based on the general lineage concept, *C.* cf. *pusilla* should not be considered

different from *C. pusilla*, and *C. gloriana* should be reduced to synonym of the latter species. In this context, our results clearly showed that for the group of closely related species included in the current study, standard plastid and nuclear markers were helpful for resolving phylogenetic relationships among species and grouped together 13 different populations of the same taxonomic species (*C. pusilla*). However, DNA markers from plastid and nuclear genomes revealed different levels of resolution among the closely related populations of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla* that could result from differential rates (1) of intra- and interspecific gene flow and/or (2) of molecular evolution between the two genomic compartments (Hollingsworth et al. 2011; Pessoa et al. 2012; Turner et al. 2016). Numerous studies claim that sequences variation from cpDNA regions could not properly distinguish closely related species, whereas nuDNA data are considered more appropriate for species delimitation (e.g. Petit and Excoffier 2009; Wang et al. 2009; Hassel et al. 2013; Lu et al. 2016). However, the combination of molecular data from different genomic compartments is essential to circumscribe species limits among complex and diverse taxa and to detect incongruences that could give insights into hybridization and/or chloroplast capture events (Naciri and Linder 2015). Even though similar patterns of species diversity were provided by cpDNA and nuDNA regions, phylogenies inferred from each dataset were particularly helpful in this study to detect discordant evolutionary relationships among or within species.

Morphological analyses: intra- and interspecific variability

The two multivariate statistical approaches used in the present work differ in the way they can assess species delimitation. MCA extended to accommodate continuous characters can combine metric and categorical variables and needs no *a priori* taxonomic information, while DA is only based on continuous characters and each individual must be pre-assigned to a specific taxonomic group. Both methods yielded similar results and were useful to distinguish morphologically consistent groups and identify substantial variation within these groups. The taxonomic distinctness of *C. aquatilis*, *C. discolor* and *C. ravenniana* was strongly supported, each species forming a morphologically differentiated group. However, the large intraspecific variation observed, especially among specimens of *C. aquatilis* and *C. ravenniana* (Figs. 4a, 5a) suggests that future study should explore more thoroughly the intra- and interpopulational variability using more individuals from different populations distributed across the whole geographic range of each species considered. Theoretically, multivariate analyses require a sampling strategy that covers as much as possible the overall morphological variation observed across the distribution area of the studied species (Duminil and Di Michele 2009). Because the

main goal of the current study was to test species delimitations between *C. gloriana* and *C. pusilla*, a special effort was put into sampling of both species, including numerous specimens collected across more than 10 different populations of *C. pusilla* to conduct robust interspecific comparisons with individuals of *C. gloriana*, a species only known from a small hill in central-western Rio Grande do Sul State, Brazil (Deble et al. 2015b), and to test the species delimitation hypothesis with a white-flowered population of *C. pusilla* called *C.* cf. *pusilla* in the present work. Populations of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla* were recovered together by MCA and DA in a group clearly distinct from *C. aquatilis*, *C. discolor* and *C. ravenniana*. Both multivariate statistical methods provided evidence for extensive morphological variation among populations of *C. pusilla* and did not reveal significant differentiation with *C. gloriana* and *C.* cf. *pusilla* since considerable and consistent overlap was observed among specimens of the three pre-classified taxa (Figs. 4, 5). Moreover, the high level of misidentification observed among *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla* in classificatory discriminant analyses reinforced the single polymorphic species hypothesis, the recognition of *C. gloriana* and *C.* cf. *pusilla* as distinct species from *C. pusilla* being persistently not supported by multivariate analyses. Morphological univariate tests of significance were also performed for ten characters considered as potentially diagnostic to distinguish *C. gloriana* and *C. pusilla* and the results obtained did not demonstrate any clear separation between the two species or between them and *C.* cf. *pusilla*. As additional evidence, the distribution map (Fig. 2) clearly shows that there are no geographic distinction between these three pre-classified taxa since the range area of *C. pusilla* encompasses the localities where *C. gloriana* and *C.* cf. *pusilla* were found.

Our results suggest that the sampling method used for *C. gloriana* and *C. pusilla* should be generalized among species of *Cypella* to provide more robust taxonomic proposals. Indeed, we found that out of 14 taxa descriptions published within the last seven years (Deble et al. 2012a; Chauveau et al. 2014; Deble et al. 2015a, b; Deble and Alves 2017), more than 40% were based on measurements of five or fewer type specimens and none of the 14 descriptions used more than 20 type specimens, without any clear mention of additional measurements conducted in the field. Furthermore, numerous taxa previously described for the genus were based on only one to three type specimens (e.g. Ravenna 1965, 1981, 2005, 2009) and the intraspecific morphological variation was never properly re-evaluated. Because most of the taxa recently published are often closely related to each other or to species anteriorly described, it should be essential to combine larger sample size and statistical analyses to provide more reliable taxonomic decisions. Another relevant outcome of the present study is that distinctions

among morphologically close species of *Cypella* are traditionally and mainly based on floral characters, but MCA and to a lesser extent DA revealed that vegetative (i.e. plant height, basal cauline leaf length and width) and other reproductive characters (i.e. floral stem: length of first cauline leaf or bract after first internode, internodes number; synflorescence: length of inner peduncles, rhipidia number) were useful to distinguish nearly related taxa and should be more widely used for interspecific comparisons.

Comparison of molecular and morphological data

Overall, this study contribute to a growing consensus that taxonomic interpretations should involve multiple lines of molecular and morphological evidence obtained from broad geographic samplings (e.g. Carstens et al. 2013; Bagley et al. 2015; Su et al. 2015). In this context, it is generally recommended to compare results of several species-delimitation methods to assess for congruence before formal taxonomic decisions (Carstens et al. 2013; Rocha et al. 2017). In the present study, consistent results were provided under different priors, algorithms and data for the recognition of four species (*C. aquatilis*, *C. discolor*, *C. pusilla*, *C. ravenniana*) and the reduction of *C. gloriana* to *C. pusilla*. Valuable information about the species diversity and geographic distribution of *C. pusilla* was obtained, which highlights the need for further investigations among other species of *Cypella* to better circumscribe intraspecific variation and improve future taxonomic comparisons. Another relevant finding provided by the current phylogenetic analyses was the discovery of discordant evolutionary relationships revealed by plastid and nuclear data. However, additional and broader taxonomic sampling among different populations of related species of *Cypella* is needed to achieve coalescent-based analyses with more complete ingroup representativeness and to detect and characterise patterns of reticulate evolution on multilocus species trees.

According to the results obtained in the present study, an expanded description of *C. pusilla* based on individuals used in the present work and completed by additional specimens listed hereafter is presented and *C. gloriana* is synonymised with *C. pusilla*.

Taxonomic treatment

Cypella pusilla (Link & Otto) Benth. & Hook.f. ex B.D.Jacks. Index Kew. 1: 689 (1893). ≡ *Ferraria pusilla* Link & Otto, Icon. Pl. Select. 10: 125 (1828) TYPE: Brazil, Rio Grande do Sul, Porto Alegre, 1826, *Sello s.n.* (holotype, presumed lost; lectotype **designated here**: tab. 59

in Link & Otto's Icones Plantarum Selectarum Horti Regii Botanici Berolinensis cum descriptionibus et colendi ratione, 10: 125. 1828). EPITYPE: BRAZIL: Brésil Province de Rio-Grande, *Sellow 2599* (P01846365!), designated by Deble and Alves (2017); ≡ *Herbertia pusilla* (Link & Otto) Sweet, Hort. Brit. 2: 497. 1830; ≡ *Polia pusilla* (Link & Otto) Klatt, Linnaea 31: 545. 1862; ≡ *Hesperoxiphion pusillum* (Link & Otto) Baker, J. Linn. Soc., Bot. 16: 127. 1877; ≡ *Phalocallis pusilla* (Link & Otto) Kuntze, Revis. Gen. Pl. 2: 702. 1891.
= *Cypella gloriana* Deble & F.S.Alves, Darwiniana, 2: 235. 2015. TYPE: BRAZIL. Rio Grande do Sul, São Vicente do Sul, 25 Oct 2014 (fl., fr.), *L.P. Deble, F.S.Alves & M.I.P. Deble 15034* (holotype, SI; isotypes, MVFA, PACA!). [**syn. n.**]

Description: Perennial herb, 8.7–29(–36.5) cm tall, underground stem 3–10(–12.5) cm long. *Bulb* ovoid, outer cataphylls dark brown, 13.3–28(–31.6) × (9–)10.2–22.6(–27) mm, prolonged in a pseudocollar (15–)19–69(–104.7) mm. *Basal leaves* green at anthesis (0–)1–4, linear, plicate, (1.4–)3.5–19.2(–22.3) cm × 0.4–2.8(–3.2) mm. *Basal cauline leaf* linear, plicate, (1.1–)5.9–18(–21.7) cm × 0.6–2.4(–3.4) mm. *Flowering stem* cylindrical, (5.7–)6.5–25.6(–32.5) cm long, first internode (5.5–)6–25(–31.5) cm long; first cauline leaf one and reduced, bractiform, (1.4–)1.9–4.6(–6.5) cm. *Synflorescence* fasciculiform, (1–)3–5(–9) rhipidia, one-flowered. *Spathes* papyraceous, bivalved, sessile or shortly pedunculate; peduncle (0.4–)0.9–8(–10) mm long; outer valve (6.4–)10–20(–21.6) mm long, inner valve 15.8–28.7 mm long. *Pedicel* filiform, ovary partly included, the top of the ovary 1–4.9 mm above the top of the upper valve, occasionally enclosed for 0.3–1.5(–2.3) mm. *Flowers* pale to sulphur yellow, occasionally white, 19–41 mm diam. *Tepals* unequal, shortly fused proximally for 0.2–1.8(–2.2) mm. *Outer tepals* pandurate, (16.3–)19–30(–32.5) × (9.5–)10.8–16.5(–18) mm; proximal part concave with scattered brown dots and trichomes; distal part patent or reflex, shortly apiculate. *Inner tepals* reduced, (12.2–)13.5–19 × 5.2–8.3(–9) mm, the proximal two-thirds erecto-patent, then curved upward, narrowly cuneate and slightly constricted lastly, shortly unguiculate, with scattered purple-brown dots; the distal one-third incurved and abruptly reclinate, longitudinally depressed, except at the distal end, with a dense lanceolate yellow area of oil-producing trichomes (elaiophores), 1.5–4 mm wide, the lateral sides firmly revolute, each spotted with purple-brown, acute. *Filaments* 3.8–4.3(–6.6) mm, connate at the base for 0–1(–1.7) mm, erect to porrect, white to pale yellow, sometimes marked with red-brown dots, filiform. *Anthers* narrowly oblong, (3.8–)4.1–6.1 × (0.6–)0.8–1.6(–1.8) mm, adnate to the style arms for two-thirds to three-fourths of the length (2.4–4.7 mm); connective oblong, apex acute to acuminate, truncate-apiculate or retuse-apiculate, base truncate to reniform, whitish yellow, 0.3–1(–1.3)

mm wide, usually covered with a viscous and transparent secretion; pollen whitish to greenish-yellow. *Ovary* narrowly oblong, glabrous, (2.6–)3.2–6.8(–7) × (1.2–)1.5–2.6(–2.9) mm. *Style* whitish to pale yellow, 9.2–13.4(–15.8) mm total length. *Style arms* whitish to pale yellow usually with sparse to dense purple or brownish stripes from the stalk towards the distal end, and brownish or purplish yellow apically, conduplicate, (3.9–)4.5–7.2(–8.4) mm long; crests pale yellow to yellow at the apex, frequently striated with purple or brown; adaxial crests 2, erect, falcate inwards, **(**1.6–)2.1–4.1(–4.6) mm long; abaxial crest ovate, obtuse, 0.7–2.6 mm long; stigmatic appendages, 2, on each side at the base of the abaxial crest, transverse, dull yellow to purple, 0.2–0.8(–1) mm long. *Capsule* obovoid, 5.8–11.1(–16.8) × 2.8–5.9(–7) mm. Seeds irregularly obovate to conical, sharply angulate, brown, 1.5–2.3 mm long.

Phenology: The species presents two flowering periods, from October to December and from March to April.

Distribution area and habitat: Endemic species of *Campos* eco-region of Southern Brazil. A plant collected by Riedel in "Serra da Lapa, Prov. of Minas Geraes" (presently known as Serra do Cipó, Minas Gerais state, Brazil) was assigned to *P. pusilla* in Flora Brasiliensis (Klatt 1871). However, a careful analysis of the image of the exsiccate deposited at the Komarov Botanical Institute of the Russian Academy of Sciences, acronym LE (Thiers 2018) revealed that this specimen does not belong to *Cypella* and is probably the type of *Lansbergia setacea* Klatt (Klatt 1882) [synonym of a dubious *Pseudotrimezia subtilis* Ravenna (Ravenna 1988), as reported by Lovo (2010)]. Hence, *C. pusilla* is restricted to central south and eastern part of the state of Rio Grande do Sul (Fig. 2). Populations usually consist of scattered individuals in dry grassland vegetation, over rocky substrate or outcrops of granitic or arenitic origin. The elevation records range from 107 to 431 m a.s.l.

Additional specimens examined: Brasil. Rio Grande do Sul, Alegrete, Pedras Mouras, 06 Mar 2011, *L.P. Deble, A.S. de Oliveira-Deble & F.S. Alves 12833* (ICN!); Amaral Ferrador, Fazenda da Pinheira, 228 m, 27 Nov 2014, *T. Pastori & O. Chauveau 174* (ICN!); id., 18 Mar 2015, *T. Pastori & O. Chauveau 186* (ICN!); Bagé, Estrada para o Rincão do Inferno, 318 m, 28 Nov 2014, *T. Pastori & O. Chauveau 184* (ICN!); id., 18 Mar 2015, *T. Pastori & O. Chauveau 188* (ICN!); Caçapava do Sul, BR 392, Km 271, 116 m, 12 Nov 2014, *T. Pastori et al. 170* (ICN!); id., BR 392, após BR 290, Km 271.4, 107 m, 18 Apr 2014, *L. Eggers & O. Chauveau 913*

(ICN!); id., estrada para a Pedra do Segredo, camping Galpão de Pedra, 12 Nov 2014, *T. Pastori et al. 168* (ICN!); id., Guaritas, BR 625, após à Associação de Moradores das Guaritas, 254 m, 19 Apr 2014, *L. Eggers & O. Chauveau 914* (ICN!); id., Guaritas, 17 Apr 2011, *L.P. Deble, A.S. de Oliveira-Deble & F.S. Alves 13116* (ICN!); Canguçu, estrada para Guarda Velha, 300 m, 02 May 2006, *A.A. Schneider 1260* (ICN!); Guaíba, estrada para Barra do Ribeiro, 16 Oct 2008, *L.P. Deble & A.S. de Oliveira-Deble 10088* (ICN!); Lavras do Sul, entrada secundária para Bagé, 431 m, 19 Mar 2015, *T. Pastori & O. Chauveau 190* (ICN!); id., estrada para Cerro do Ouro, entre Lavras do Sul e São Gabriel, 309 m, 27 Oct 2014, *T. Pastori et al., 147* (ICN!); id., estrada secundária para Ibaré, 431 m, 20 Nov 2012, *L. Eggers et al. 783* (ICN!); id., RS 357, direção a Lavras do Sul, 396 m, 20 Nov 2012, *L. Eggers et al. 778* (ICN!); id., RS 357, direção a Lavras do Sul, 393 m, 19 Mar 2015, *T. Pastori & O. Chauveau 189* (ICN!); Pantano Grande, propriedade particular Paulo Rolim, 117 m, 14 May 2015, *T. Pastori & O. Chauveau 194* (ICN!); Pinheiro Machado, estrada secundária, 283 m, 18 Mar 2015, *T. Pastori & O. Chauveau 187* (ICN!); Porto Alegre, Mont Serrat, 12 Apr 1946, *K. Emrich s.n.* (PACA 33756!); 15 Apr. 1944, *K. Emrich s.n.* (PACA 27470!); id., Morro Santa Teresa, 101 m, 08 May 2013, *C. Vogel-Ely et al. 389* (ICN!); id., Morro Santana, 19 Mar 2013, *L. Eggers et al. 818* (ICN!); Santana da Boa Vista, Fazenda Santo Antônio, 30 Apr 1975, *A. Sehnem s.n.* (PACA 14589!); São Gabriel, Cerro do Ouro, propriedade da família de Renato M. Backes, 249 m, 27 Oct 2014, *T. Pastori et al. 148* (ICN!); id., 19 Mar 2015, *T. Pastori & O. Chauveau 192* (ICN!); id., Estanzia Cerro Alegre, 8 Feb 1996, *R.R. Brooks et al. MS408* (MO!); id., estrada para o Cerro do Ouro, 420 m, 19 Mar 2015, *T. Pastori & O. Chauveau 191* (ICN!); São Pedro do Sul, 08 Dec. 2014, *P.J.S. Silva Filho 2199* (ICN!); São Vicente do Sul, Cerro da Glória, 16 Nov 2010, *L.P. Deble, A.S. de Oliveira-Deble & F.S. Alves 12744* (ICN!); id., 13 Oct 2014, *T. Pastori et al. 119* (ICN!); id., 20 Mar 2015, *T. Pastori & O. Chauveau 193* (ICN!).

Conservation status: *Cypella pusilla* was considered Critically Endangered (CR) subcriteria B2ab(iii) according to Brazilian regulations related to threatened flora species of Rio Grande do Sul state (Decreto No. 52.109 2014) and based on IUCN criteria (IUCN 2001). This evaluation estimated an area of occupancy (AOO) of < 10 km^2 and considered the species as severely fragmented by continue decline in area of extension (EOO) and/or habitat quality. In the present study, sampling of *C. pusilla* was increased and a new evaluation showed AOO < 52 km^2. Threatened category is now established as EN (endangered), with subcriteria B2ab(iii),

with specific threats of conversion of grassland areas in soybean and/or *Pinus* L. and *Eucalyptus* L'Hér. plantations or environmental degradation related to mining activities.

*Notes***:** *Ferraria pusilla* was described based on one plant obtained from a bulb sent from Porto Alegre (Brazil) to Berlin by Sello in 1826. The sample used for *F. pusilla* description was probably deposited in Herbarium B, lost during World War II, and the drawing associated to the original description was designated as type of the species. All combinations made posteriorly by Sweet (1830), Klatt (1861–1862), Baker (1877) and Kuntze (1891) were based on Link and Otto's protologue (1828), without additional collections. Recently, a plant collected by Sello, from Herbarium P, was designated as epitype (Deble and Alves 2017).
Similarly to other species of *Cypella*, *C. pusilla* was poorly known due to the irregular and ephemeral bloom and also because morphological variation among populations was mostly disregarded until now. Moreover, specimens of this species were often misidentified because flower's colour and marking patterns are notably variable (Fig. 1). The species and its taxonomic synonyms were successively compared with *C. discolor* (Deble et al. 2012b), *C. charruana*, *C. ravenniana*, *C. suffusa* (Deble et al. 2015b) and *C. laeta* Ravenna (Deble and Alves 2017). However, *C. pusilla* presents a remarkably distinctive fasciculiform synflorescence, which facilitates species identification, but other characters were also often applied to differentiate pairs of related taxa (Table 4). Although these features are relevant for species identification and frequently used in descriptions and comparative tables, they were never statistically evaluated. We emphasize the need to conduct statistical assessments of the characters selected for species identification and to include more characters related to vegetative organs, but also to floral stem and synflorescence, which are potentially valuable for taxonomic analyses in *Cypella*, as it was shown in the current study.

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Information on Electronic Supplementary Material

Online Resource 1. Species list, collection data and voucher information used for preliminary phylogenetic analyses.
Online Resource 2. Primers used for PCR amplification and DNA sequencing.
Online Resource 3. PCR profiles for DNA amplification.
Online Resource 4. Dataset partitions for Maximum Likelihood (ML) and Bayesian Inference (BI) analyses and evolutionary models used in BI.
Online Resource 5. Mean ± standard deviation values for each morphometric character and character states for each categorical character used in multivariate analyses of morphological traits among *Cypella* species.
Online Resource 6. Tests for differences in morphological characters among two taxonomic subsets of *Cypella* species.
Online Resource 7. Properties and maximum parsimony statistics of each separate and combined dataset used for preliminary phylogenetic analyses.
Online Resource 8. Preliminary phylogenetic analyses: ML best-scoring cladogram and phylogram obtained from the analyses of the combined chloroplast dataset.
Online Resource 9. Preliminary phylogenetic analyses: ML best-scoring cladogram and phylogram obtained from the analyses of the combined nuclear dataset.
Online Resource 10. Discrimination measures for the first and second dimension of Multiple Correspondence Analyses (MCA) performed on two taxonomic subsets of morphological characters.
Online Resource 11. Structure matrix with correlation coefficients between each character and the first two standardised discriminant functions (DF1 and DF2) of canonical and classificatory discriminant analyses (DA) performed on two taxonomic subsets of morphological characters.

Conflict of interest

The authors declare that they have no conflict of interest.

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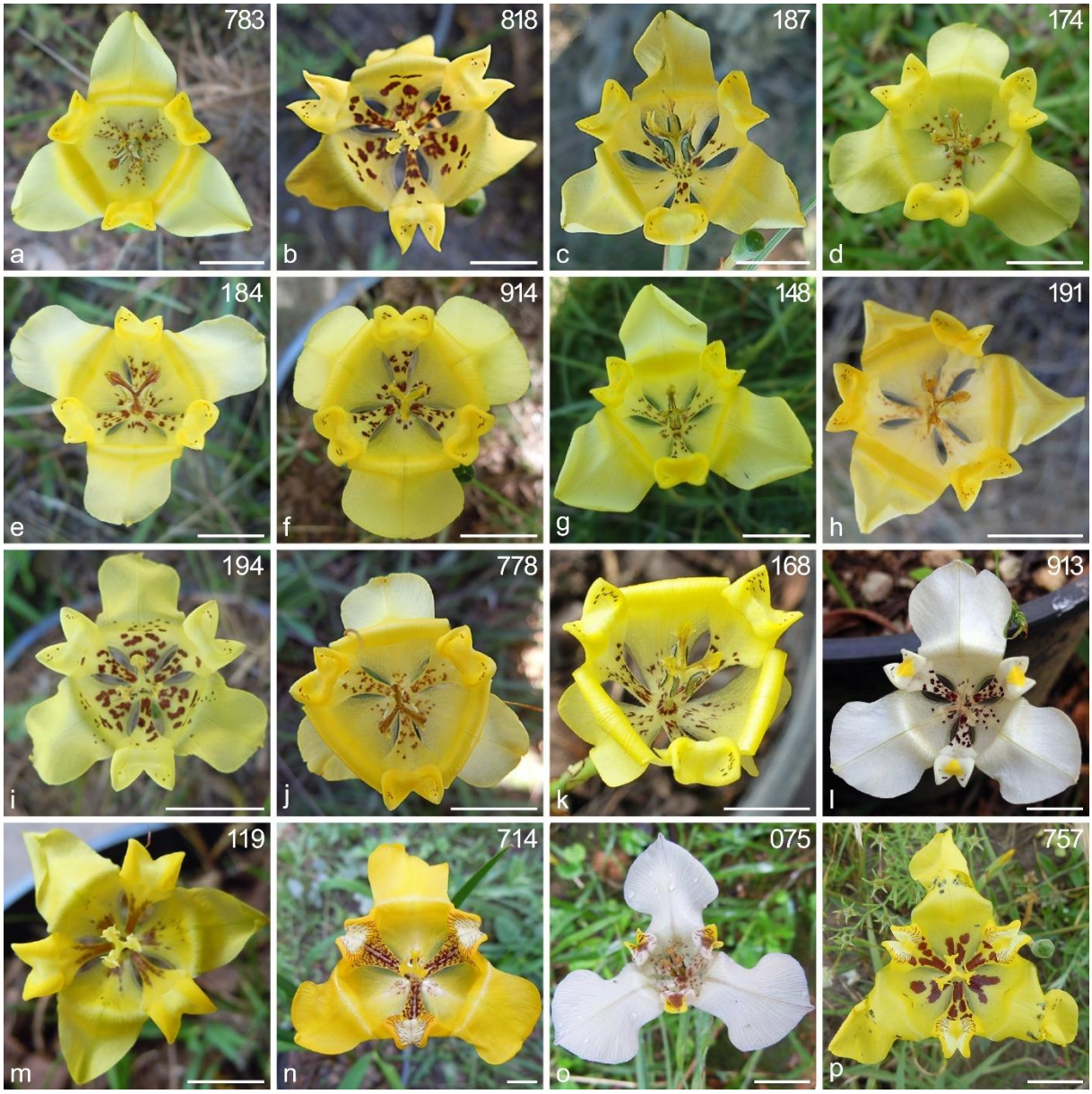

Fig. 1 Flowers in apical view of various populations of *Cypella pusilla* and allied species sampled for the current study**. a–k *C. pusilla***, **l** *C.* cf. *pusilla*, **m** *C. gloriana*, **n** *C. aquatilis*, **o** *C. discolor*, **p** *C. ravenniana*. Populations are identified by their Pop. ID as listed in Table 1. Scale bar = 20 mm.

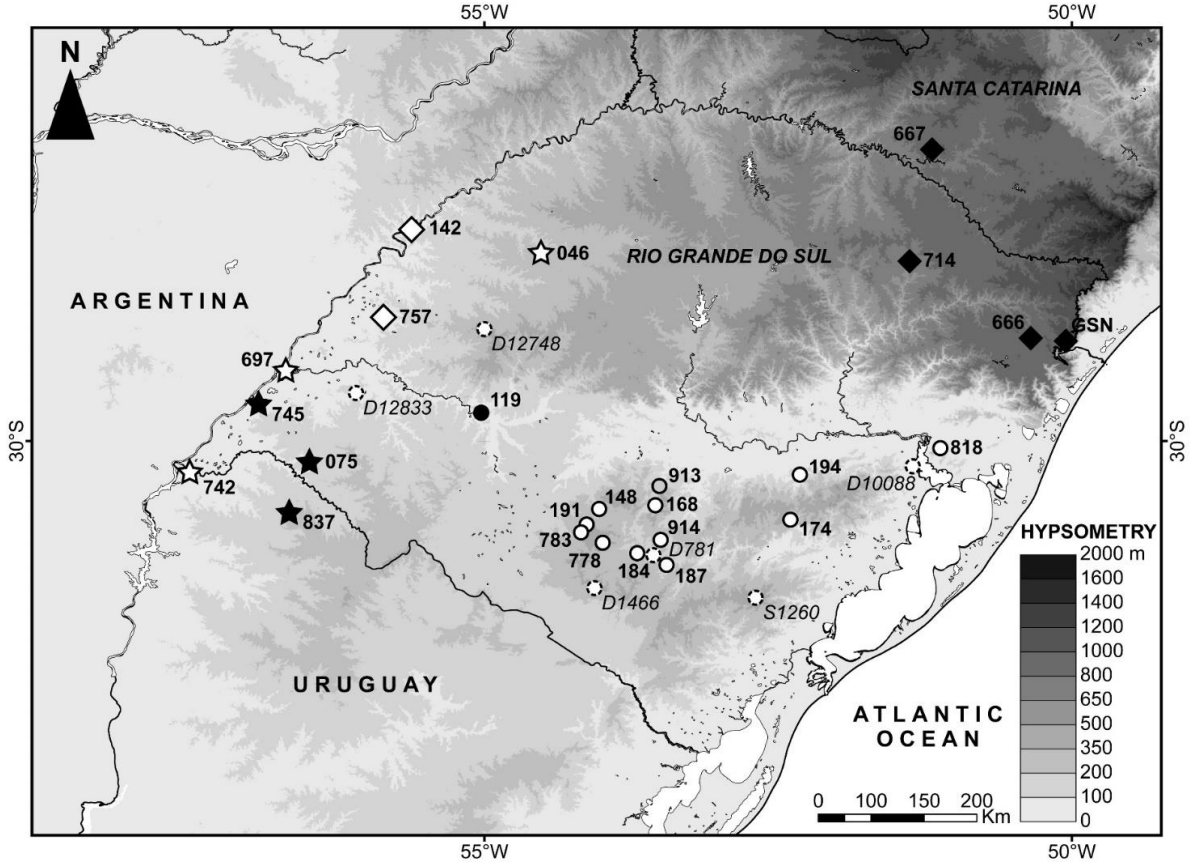

Fig. 2 2 Taxonomic assessment: distribution map of each population of Tigridieae sampled in Southern Brazil and Uruguay for the current study: ☆ = *Catila amabilis*, ◆ = *Cypella aquatilis*, ★ = *Cypella discolor*, ● = *Cypella gloriana*, ○ = *Cypella pusilla*, ◎ = *Cypella* cf. *pusilla* and ◇ = *Cypella ravenniana*. Populations are identified by their Pop. ID as listed in Table 1. ◌ indicates populations of *C. pusilla* not included in the sampling of the present study: *S1260 = Schneider 1260* (ICN), *D781 = Deble & Oliveira 781* (CNPO), *D1466 = Deble & Oliveira 1466* (CNPO), *D10088 = Deble et al. 10088* (PACA), *D12748 = Deble & Oliveira 12748* (SI), *D12833 = Deble et al. 12833* (PACA, SI). Herbarium codes between brackets follow Thiers (2017).

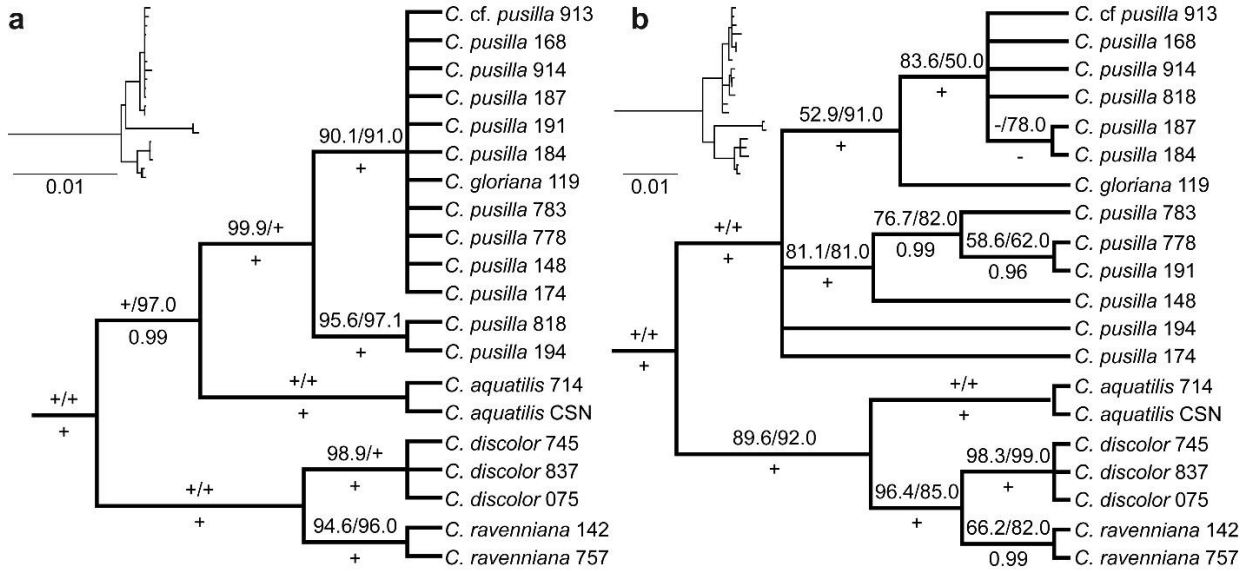

Fig. 3 Taxonomic assessment: ML best-scoring phylograms and cladograms obtained from the analyses of the combined (**a**) chloroplast and (**b**) nuclear data sets, respectively. Trees were rooted using *Catila amabilis* as outgroup. Support values indicated above branches follow the order parsimony bootstrap (PBS)/likelihood bootstrap (LBS), whereas Bayesian posterior probabilities (PP) are reported below branches. Bootstrap supports and posterior probabilities for a given node are provided only if one of the values reached the following thresholds: PBS ≥70% or LPB ≥70% or PP ≥0.95 (other nodes were collapsed). A plus sign (+) means full support, whereas a dash (-) indicates support value of less than 50% for PBS and LBS or less than 0.95 for PP.

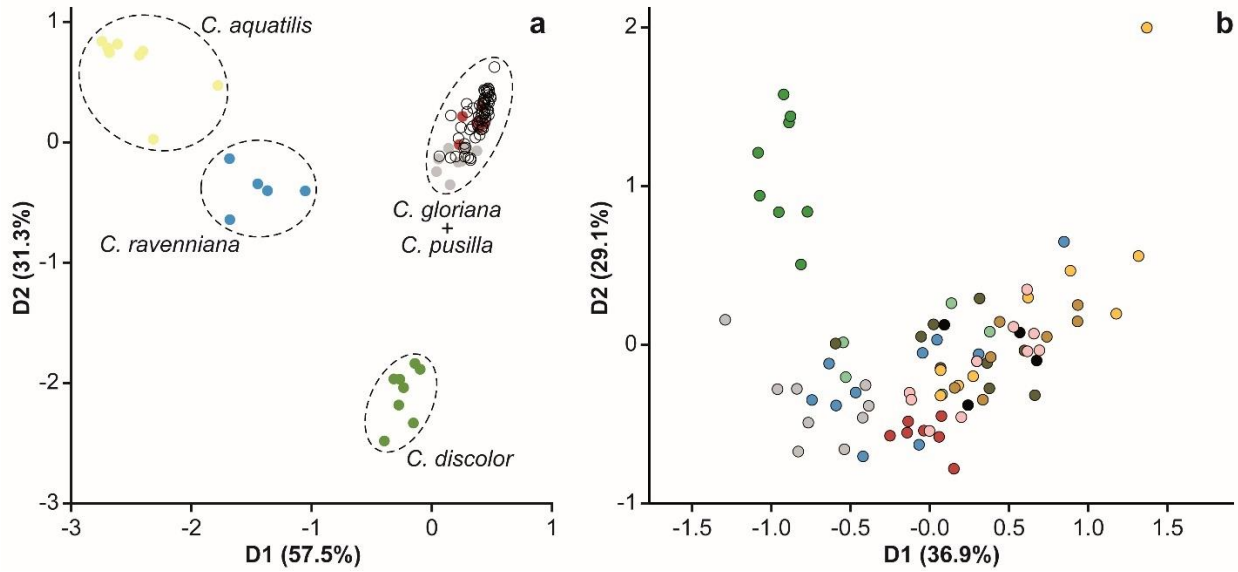

Fig. 4 Taxonomic assessment: two dimensional scatter plots obtained from Multiple Correspondence Analyses (MCA) based on: **a** 24 morphological characters tested on 100 specimens distributed among the five species of *Cypella* included in the study (● = *C. aquatilis*, ● = *C. discolor*, ● = *C. gloriana*, ○ = *C. pusilla*, ● = *C.* cf. *pusilla* and ● = *C. ravenniana*), **b** 33 morphological characters tested on 79 specimens distributed among *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla* (individuals are identified by their Pop. ID as listed in Table 1: ● = 119, ● = 818, ● = 914, ● = 778, ● = 783, ● = 174, ● = 184, ● = 148, ● = 194, ● = 913). Proportions of morphological variation captured by each of the two major dimensions are indicated between brackets.

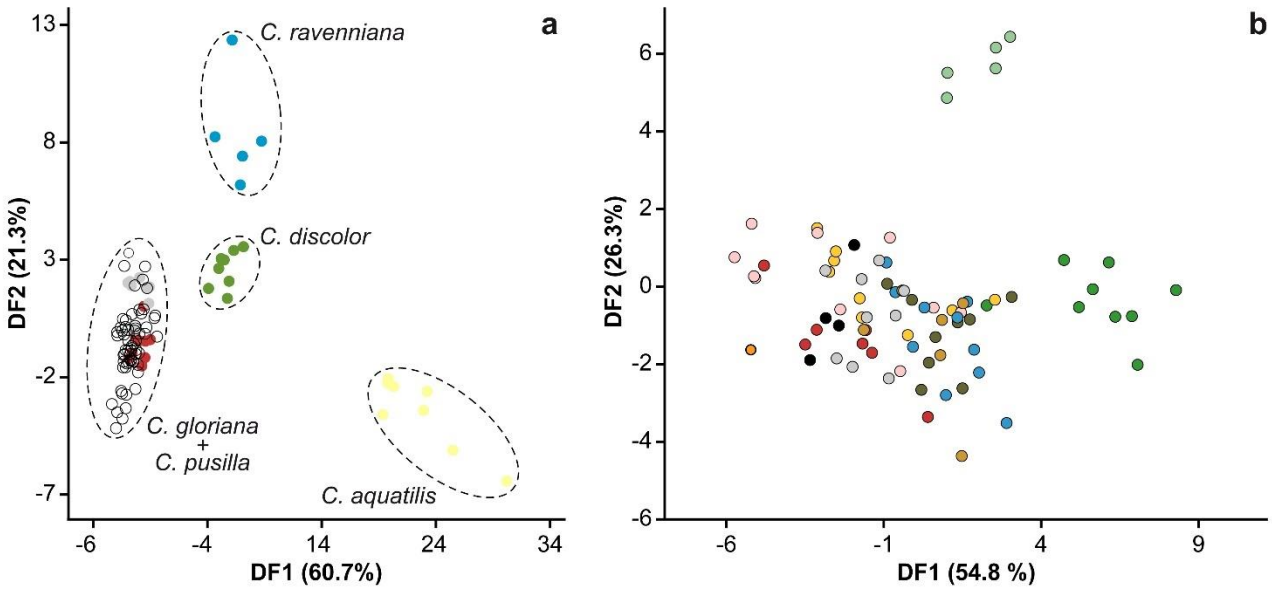

Fig. 5 Taxonomic assessment: two dimensional scatter plots obtained from Discriminant Analyses (DA) based on: **a** 18 morphological characters tested on 100 specimens distributed among the five species of *Cypella* included in the study (● = *C. aquatilis*, ● = *C. discolor*, ● = *C. gloriana*, ○ = *C. pusilla*, ● = *C.* cf. *pusilla* and ● = *C. ravenniana*), **b** 31 morphological characters tested on 79 specimens distributed among *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla* (individuals are identified by their Pop. ID as listed in Table 1: ● = 119, ● = 818, ● = 914, ● = 778, ● = 783, ● = 174, ● = 184, ● = 148, ● = 194, ● = 913). Proportions of morphological variation captured by each of the two major discriminant functions are indicated between brackets.

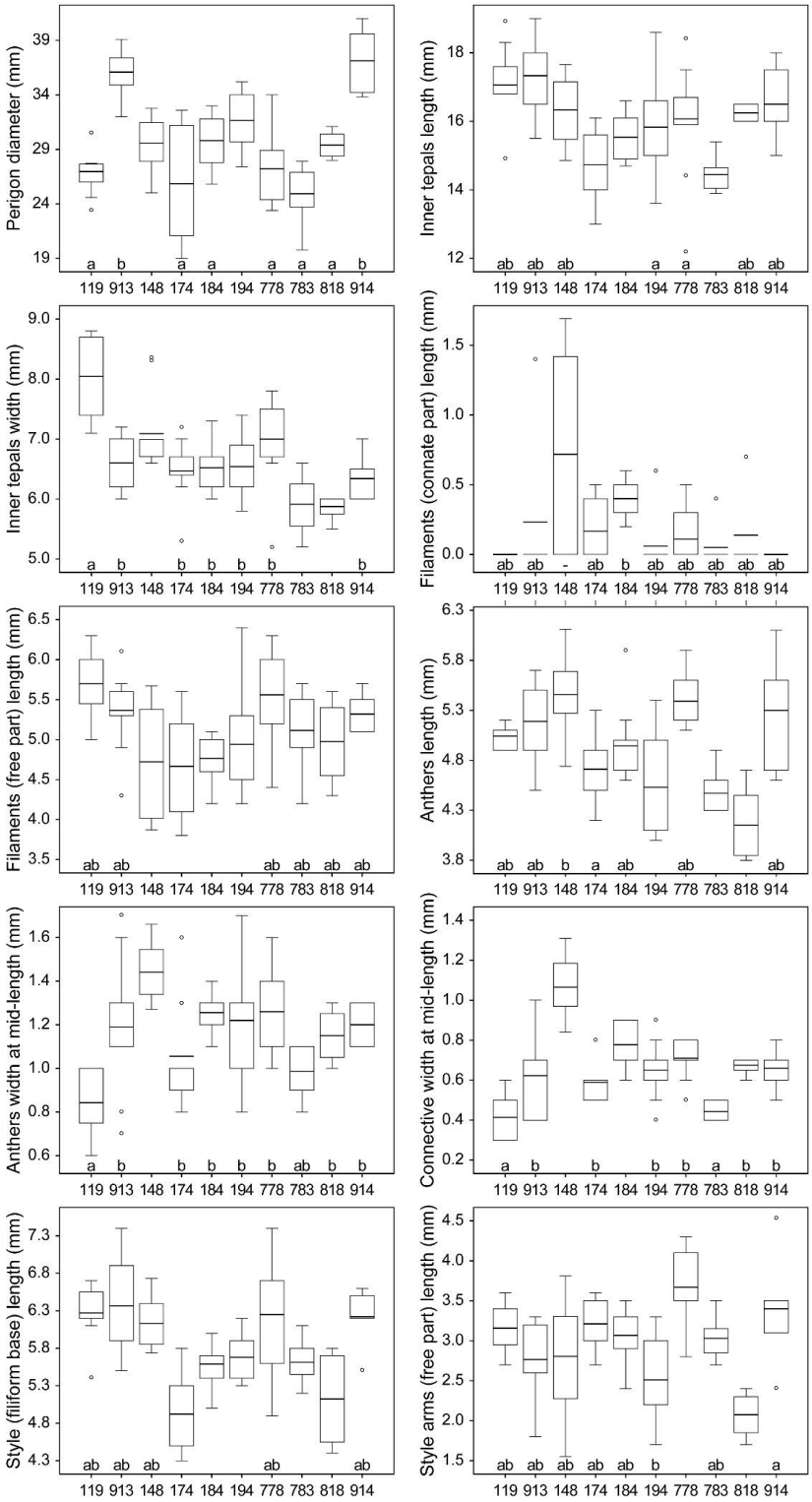

Fig. 6 Taxonomic assessment: box and whisker plots of simple statistics (*line* = mean, *bottom-top box* = 25-75th percentiles, *whiskers* = minimum and maximum values, *circles* = outliers) of ten selected morphometric characters considered useful to distinguish *C. gloriana* and *C. pusilla*. Populations of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla* are identified by their Pop. ID as listed in Table 1. The same letters below box and whisker plots indicate no significant differences (Fisher's LSD tests, $p < 0.05$) between *C. gloriana* (a) or *C.* cf. *pusilla* (b) and the other populations sampled.

Table 1 Voucher information, population ID (Pop. ID) and geographical origin (country: first-level administrative division, specific locality) of species sampled to infer phylogenetic relationships (Phyl.) and conduct multivariate analyses based on morphological characters (Morph.).

Species	Voucher	Pop. ID	Geographical origin	Phyl.	Morph.
Outgroup					
Catila amabilis	*Eggers et al. 742* (ICN)	742	BR: RS, Barra do Quaraí	X	
	IRI046 (living collection PABG)	046	BR: RS, São Miguel das Missões	X	
	Eggers 697 (ICN)	697	BR: RS, Uruguaiana	X	
Ingroup					
Cypella aquatilis	*Eggers & Chauveau 714* (ICN)	714	BR: RS, Muitos Capões	X	X
	Castillo s.n. (MO)	CSN	Unknown (from RBGK: I-202)	X	X
	Irgang s.n. (ICN 190666)	666	BR: RS, São Francisco de Paula		X
	Irgang s.n. (ICN 190667)	667	BR: SC, Campos Novos		X
	Goergem s.n. (ICN 50006)	GSN	BR: RS, Cambará do Sul		X
Cypella discolor	*Aita 75* (ICN)	075	BR: RS, Quaraí	X	X
	Eggers et al. 745 (ICN)	745	BR: RS, Uruguaiana	X	X
	Eggers et al. 837 (ICN)	837	UR: Artigas, Cuaro	X	X
Cypella gloriana	*Pastori et al. 119* (ICN)	119	BR: RS, São Vicente do Sul	X	X
Cypella pusilla	*Eggers et al. 818* (ICN)	818	BR: RS, Viamão	X	X
	Eggers & Chauveau 914 (ICN)	914	BR: RS, Caçapava do Sul	X	X
	Eggers et al. 778 (ICN)	778	BR: RS, Lavras do Sul	X	X
	Eggers et al. 783 (ICN)	783	BR: RS, Lavras do Sul	X	X
	Pastori et al. 168 (ICN)	168	BR: RS, Caçapava do Sul	X	
	Pastori & Chauveau 174 (ICN)	174	BR: RS, Amaral Ferrador	X	X
	Pastori & Chauveau 184 (ICN)	184	BR: RS, Bagé	X	X
	Pastori & Chauveau 187 (ICN)	187	BR: RS, Pinheiro Machado	X	
	Pastori & Chauveau 191 (ICN)	191	BR: RS, São Gabriel	X	
	Pastori et al. 148 (ICN)	148	BR: RS, São Gabriel	X	X
	Pastori & Chauveau 194 (ICN)	194	BR: RS, Pantano Grande	X	X
Cypella cf. *pusilla*	*Eggers & Chauveau 913* (ICN)	913	BR: RS, Caçapava do Sul	X	X
Cypella ravenniana	*Pastori et al. 142* (ICN)	142	BR: RS, Garruchos	X	X
	Eggers et al. 757 (ICN)	757	BR: RS, São Borja	X	X

Notes: ICN = Herbarium of the Federal University of Rio Grande do Sul (Brazil); MO = Herbarium of the Missouri Botanical Garden (U.S.A.); PABG = Botanical Garden of Porto Alegre (Brazil); RBGK = DNA bank of the Royal Botanic Gardens Kew; BR = Brazil; RS = Rio Grande do Sul; SC = Santa Catarina; UR = Uruguay.

Table 2 List of morphometric and categorical characters selected for multivariate analyses of morphological traits among *Cypella* species.

	Characters	MCA[(A)]	MCA[(B)]	DA[(A)]	DA[(B)]
Vegetative part					
	Plant height (cm)	X	X	X	X
Leaves	Longest basal leaf: length (mm)	-	-	-	-
	Longest basal leaf: width at mid-length (mm)	-	X	-	X
	Basal cauline leaf: length (mm)	X	X	X	X
	Basal cauline leaf: width at mid-length (mm)	X	X	X	X
Reproductive part					
Floral stem	Total length above ground level (cm)	-	X	-	X
	First internode above ground level: length (cm)	NS	X	NS	X
	First cauline leaf or bract after first internode: length (mm)	X	X	X	X
	Internodes: number	X	NS	NA	NA
Synflorescence	Inner peduncles: length (mm, mean)	X	X	X	X
	Rhipidia: number	X	NS	NA	NA
	Spathe: outer valve length (mm)	X	X	X	X
	Spathe: inner valve length (mm)	X	X	X	X
Flower perigon	Diameter (mm)	X	X	X	X
	Tepals base fusion: length (mm)	-	X	-	X
	Dominant colour	X	X	NA	NA
	Outer tepal: length (mm)	X	X	X	X
	Outer tepal: maximum width (mm)	X	X	X	X
	Inner tepal: length (mm)	X	X	X	X
	Inner tepal: maximum width (mm)	X	X	X	X
	Elaiophore: dominant colour	X	NS	NA	NA
Androecium	Filaments connate part: length (mm)	-	X	-	X
	Filaments free part: length (mm)	-	X	-	X
	Anthers: length (mm)	X	X	X	X
	Anthers: width at mid-length (mm)	-	X	-	X
	Anthers adnate to the style: length (mm)	-	X	-	X
	Connective: width at mid-length (mm)	-	X	-	X
	Connective: apex shape	X	X	NA	NA
	Connective: base shape	X	NS	NA	NA
Gynoecium	Ovary: length (mm)	X	X	X	X
	Ovary: width at distal end (mm)	X	X	X	X
	Style: filiform base length (mm)	-	X	-	X
	Style arms: total length (mm)	-	X	-	X
	Style arms: concrescent part length (mm)	-	X	-	X
	Style arms: free part length (mm)	X	X	X	X
	Adaxial crests: length (mm, mean of two measurements)	X	X	X	X
	Abaxial crest: length (mm)	X	X	X	X
	Stigmatic appendages: length (mm, mean of two measurements)	-	X	-	X

Notes: MCA = Multiple Correspondence Analysis; DA = Discriminant Analysis; [(A)] = all specimens of *Cypella* included in the current study; [(B)] = specimens belonging to populations of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla*; dash (-) = character excluded from final analyses because of insufficient data; NA = not applicable (categorical characters); NS = variation not significant or constant character state.

Table 3 Lengths and indices for the resulting most parsimonious trees in parsimony analyses of separated and combined data sets. MP trees is the number of most parsimonious trees obtained from MP analyses, whereas CI and RI are respectively the consistency and retention indices of most parsimonious topologies.

DNA data set	Number of characters			MP trees	Tree length	CI	RI
	Total	Variable (%)	Parsimony informative (%)				
rps4-trnS + [coded indels]	870 + [3]	23 + [3] (2.97%)	15 + [3] (2.06%)	1	22	1.00	1.00
rbcL	1359	16 (1.18%)	13 (0.95%)	1	16	1.00	1.00
matK-5'trnK + [coded indels]	1817 + [5]	36 + [5] (2.25%)	25 + [4] (1.59%)	45	54	0.76	0.80
trnQ-rps16 + [coded indels]	1467 + [10]	58 + [10] (4.60%)	55 + [10] (4.40%)	6	64	0.94	0.97
rps16 + [coded indels]	847 +[5]	13 + [5] (2.11%)	10 + [5] (1.76%)	1	13	1.00	1.00
psbA-trnH + [coded indels]	579 + [2]	20 + [2] (3.79%)	20 + [2] (3.79%)	1	20	1.00	1.00
cpDNA + [coded indels]	6939 + [25]	166 + [25] (2.74%)	138 + [24] (2.32%)	56	185	0.96	0.98
ITS + [coded indels]	671 + [23]	93 + [23] (16.71%)	76 + [17] (13.40%)	21	127	0.78	0.91
RPB2 + [coded indels]	700 + [14]	54 + [14] (9.52%)	41 + [10] (4.14%)	62	62	0.98	0.99
nrDNA + [coded indels]	1371 + [37]	147 + [37] (13.07%)	117 + [27] (10.23%)	63	197	0.81	0.92

Notes: numbers into brackets are numbers of characters resulting from indel-coding.

Table 4 Comparison of morphological and biogeographical characters between *C. pusilla* and related species.

		Species of *Cypella* considered morphologically and geographically closely related* to *C. gloriana* and/or *C. pusilla*				
		used in the current study		more distantly related species		
Character/species**	*C. pusilla*	*C. discolor*	*C. ravenniana*	*C. charruana*	*C. laeta*	*C. suffusa*
Plant height (cm)	8.7–29(–36.5)	15–25(–29)	8–27	20–40	20–35	25–50
Bulb length (mm)	13.3–28 (–31.6)	20–30	30–40	25–35	15–28	25–35
Bulb width (mm)	(9–)10.2–22.6(–27)	14–17	25–35	20–35	15–22	25–35
Basal leaf length (cm)	(1.4–)3.5–19.2(–22.3)	13–15(–29)	10–22	14–26	15–20	16–25
Basal leaf width (mm)	0.4–2.8(–3.2)	1.5–6(–7)	2–4	2–5	2–5	1–2
Synflorescence	fasciculiform	**branched**	**branched**	**branched**	**branched**	**branched**
Outer valve length (cm)	(0.6–)1–2(–2.2)	1.3–1.9	**2.2–3.1**	1.7–2.5	1.4–2.2	1.4–2.2
Inner valve length (cm)	1.6–2.9	2.4–3.3	**3.5–4.7**	**3–3.5**	**3.4–3.8**	2.8–3.5
Flowers number/spathe	1	1	1	1	**2**	**2**
Flower dominant colour	pale to sulphur yellow, occasionally white	whitish to yellowish	**shiny yellow**	**golden–yellow**	**bright yellow**	**bright yellow**
Perigon diameter (mm)	19–41	32–50(–53)	22–34	40–55	**45–51**	32–44
Outer tepals length (mm)	(16.3–)19–30(–32.5)	24–37(–38.5)	**32–40**	**34–40**	**34–37**	26–34
Inner tepals length (mm)	(12.2–)13.5–19	14–18	**23–27**	**22–28**	18–23	16–20
Elaiophore: dominant colour	yellow	**brownish**	yellow–cream	yellow–cream	yellow or brownish	yellow–cream
Filaments length (mm)	3.8–4.3(–6.6), connate for 0.0–1.0(–1.7)	(5.3–)5.5–6.1,entirely free	5.9–6.5, entirely free	**8.4–10.5,** connate for 0.5–0.8	near to 6, entirely free	5–6, entirely free
Anthers length (mm)	(3.8–)4.1–6.1	5.2–6.2	**6.5–7.8**	**8–9.5**	**near to 7**	6–7
Style arms length (mm)	(3.9–)4.5–7.2(–8.4)	**(2.5–)3–3.5**	6–7	**9–10**	**near to 3.6**	4–5
Adaxial crests length (mm)	(1.6–)2.1–4.1(–4.6)	2–3(–3.3)	**4.5–7**	**4.5–6.5**	3.5–4	2.5–4
Abaxial crest length (mm)	0.7–2.6	1–2.1	2–3	1–1.5	up to 2	1.5–2
Habitat	grassland, stony soils	grassland, stony soils	grassland, stony soils	**grasslands, moist soils**	grassland, well drained soils	grasslands, stony soils
Geographical distribution	Southern Brazil (central south RS)	Southern Brazil (south west RS), Uruguay (north west)	Northeast Argentina (ER, MN), Southern Brazil (western and northwestern south RS)	Southern Brazil (southwest south RS), Uruguay (northwestern)	**Northeast Argentina (ER), Western Uruguay (P)**	**Northeast Argentina (MN)**

Notes: * = according to Deble et al. (2012b, 2015b) and Deble and Alves (2017); ** = data obtained from Ravenna (1981, 2009), Deble et al. (2012b, 2015a, b), Deble and Alves (2017), the specimens used in the present study and the following measured material for *C. discolor* [BRAZIL. Rio Grande do Sul: *Stiehl-Alves 11* (ICN!); *Stiehl-Alves et al. 94* (ICN!)] and *C. laeta* [URUGUAY. Paysandú: *Eggers et al. 843* (ICN!); id., *Roitman & Tourn s.n.* (BAA24930!); id., *Roitman & Tourn s.n.* (BAA24796!)]; Provinces of Argentina: ER = Entre Ríos, MN = Misiones; State of Brazil: RS = Rio Grande do Sul; Department of Uruguay: P = Paysandú. Bold text indicates character states discriminant between a given species and *C. pusilla*.

Appendix 1 Specimens from *Cypella* and allies studied for DNA sequences. For each specimen, the species name and taxonomic authority is given, followed by the population ID used in the current study (see Table 1) and GenBanK accession numbers for *rps*4-*trn*S, *rbc*L, *mat*K-*5'trn*K, *trn*Q-*rps*16, *rps*16, *psb*A-*trn*H, ITS and RPB2, respectively. An asterisk after GenBank accession numbers indicates sequences previously published and NA is used when sequences are not available.

SAMPLING FOR FRAMEWORK PHYLOGENIES

Outgroup species: *Cipura formosa* Ravenna, 902, —, —, —, —, —, —, —, —; *Cipura paludosa* Aubl., 205, —, —, —, —, —, —, —, —; *Cipura paradisiaca* Ravenna, —, —, —, —, —, —, —, —.

Ingroup species: *Calydorea pallens* Griseb., 9579, JQ670279*, AJ309682*, JQ670442*, –, JQ670366*, –, –, –; *Catila amabilis* Ravenna, 697, JQ670284*, JQ670525*, JQ670447*, MG648215, JQ670371*, MG648259, MG648282, MG648305; *Cypella altouruguaya* Chauveau & L.Eggers, 884, –, –, –, –, –, –, –, –; *Cypella amplimaculata* Chauveau & L.Eggers, 049, –, –, –, –, –, –, –, –; *Cypella aquatilis* Ravenna, 714, MG648153, MG648174, MG648195, MG648218, MG648239, MG648262, MG648285, MG648308; *Cypella armosa* Ravenna, 761, –, –, –, –, –, –, –, –; *Cypella charruana* Deble & F.S.Alves, 203, –, –, –, –, –, –, –, –; *Cypella discolor* Ravenna, 075, MG648154, MG648175, MG648196, MG648219, MG648240, MG648263, MG648286, MG648309; *Cypella fucata* Ravenna, 746, –, –, –, –, –, –, –, –; *Cypella gloriana* Deble & F.S.Alves, 119, MG648157, MG648178, MG648199, MG648222, MG648243, MG648266, MG648289, MG648312; *Cypella guttata* Deble & F.S.Alves, 204, –, –, –, –, –, –, –, –; *Cypella hauthalii* (Kuntze) R.C.Foster ssp. *hauthalii*, 820, –, –, –, –, –, –, –, –; *Cypella hauthalii* ssp. *minuticristata* Chauveau & L.Eggers, 833, –, –, –, –, –, –, –, –; *Cypella hauthalii* ssp. *opalina* Ravenna, 764, –, –, –, –, –, –, –, –; *Cypella herbertii* ssp. *brevicristata* Ravenna, 841, –, –, –, –, –, –, –, –; *Cypella herbertii* (Lindl.) Herb. ssp. *herbertii*, 077, –, –, –, –, –, –, –, –; *Cypella laeta* Ravenna, 843, NA, –, –, –, –, –, –, –; *Cypella laxa* Ravenna, 766, –, –, –, –, –, –, –, –; *Cypella luteogibbosa* Deble, 791, –, –, –, –, –, –, –, –; *Cypella magnicristata* Deble, 076, –, –, –, –, –, –, –, –; *Cypella osteniana* Beauverd, 851, –, –, –, –, –, –, –, –; *Cypella pabstiana* Ravenna, 741, –, –, –, –, –, –, –, –; *Cypella pusilla* (Link & Otto) Benth. & Hook.f. ex B.D.Jacks., 818, MG648158, MG648179, MG648200, MG648223, MG648244, MG648267, MG648290, MG648313; *Cypella* cf. *pusilla* (Link & Otto) Benth. & Hook.f. ex B.D.Jacks., 913, MG648169, MG648190, MG648211, MG648234, MG648255, MG648278, MG648301, MG648324; *Cypella ravenniana* Deble & F.S.Alves, 757, MG648171, MG648192, MG648213, MG648236, MG648257, MG648280, MG648303, MG648326; *Cypella rivularis* Chauveau & L.Eggers, 873, –, –, –, –, –, –, –, –; *Herbertia darwinii* Roitman & J.A.Castillo, 502, JQ670296*, JQ670535*, JQ670458*, –, JQ670380*, –, –, –; *Herbertia quareimana* Ravenna, 513, –, –, –, –, –, –, –, –; *Kelissa brasiliensis* (Baker) Ravenna, 701, JQ670303*, JQ670543*, JQ670466*, –, JQ670388*, –, –, –; *Onira unguiculata* (Baker) Ravenna, 273, JQ670319*, JQ670554*, JQ670477*, –, JQ670399*, –, –, –.

SAMPLING FOR TAXONOMIC ASSESSMENT

Outgroup species: *Catila amabilis* Ravenna, 742, MG648151, MG648172, MG648193, MG648214, MG648238, MG648258, MG648281, MG648304; *Catila amabilis* Ravenna, 046, MG648152, MG648173, MG648194, MG648216, MG648237, MG648260, MG648283, MG648306; *Catila amabilis* Ravenna, 697, JQ670284*, JQ670525*, JQ670447*, MG648215, JQ670371*, MG648259, MG648282, MG648305.

Ingroup species: *Cypella aquatilis* Ravenna, 714, MG648153, MG648174, MG648195, MG648218, MG648239, MG648262, MG648285, MG648308; *Cypella aquatilis* Ravenna, CSN, JQ670287*, AJ309683*, JQ670449*, MG648217, AJ578775*, MG648261, MG648284, MG648307; *Cypella discolor* Ravenna, 075, MG648154, MG648175, MG648196, MG648219, MG648240, MG648263, MG648286, MG648309; *Cypella discolor* Ravenna, 745, MG648156, MG648177, MG648198, MG648221, MG648242, MG648265, MG648288, MG648311; *Cypella discolor* Ravenna, 837, MG648155, MG648176, MG648197, MG648220, MG648241, MG648264, MG648287, MG648310; *Cypella gloriana* Deble & F.S.Alves, 119, MG648157, MG648178, MG648199, MG648222, MG648243, MG648266, MG648289, MG648312; *Cypella pusilla* (Link & Otto) Benth. & Hook.f. ex B.D.Jacks., 818, MG648158, MG648179, MG648200, MG648223, MG648244, MG648267, MG648290, MG648313; *Cypella pusilla* (Link & Otto) Benth. & Hook.f. ex B.D.Jacks., 914, MG648159, MG648180, MG648201, MG648224, MG648245, MG648268, MG648291, MG648314; *Cypella pusilla* (Link & Otto) Benth. & Hook.f. ex B.D.Jacks., 778, MG648160, MG648181, MG648202, MG648225, MG648246, MG648269, MG648292, MG648315; *Cypella pusilla* (Link & Otto) Benth. & Hook.f. ex B.D.Jacks., 783, MG648161, MG648182, MG648203, MG648226, MG648247, MG648270, MG648293, MG648316; *Cypella pusilla* (Link & Otto) Benth. & Hook.f. ex B.D.Jacks., 168, MG648162, MG648183, MG648204, MG648227, MG648248, MG648271, MG648294, MG648317; *Cypella pusilla* (Link & Otto) Benth. & Hook.f. ex B.D.Jacks., 174, MG648163, MG648184, MG648205, MG648228, MG648249, MG648272, MG648295, MG648318; *Cypella pusilla* (Link & Otto) Benth. & Hook.f. ex B.D.Jacks., 184, MG648164, MG648185, MG648206, MG648229, MG648250, MG648273, MG648296, MG648319; *Cypella pusilla* (Link & Otto) Benth. & Hook.f. ex B.D.Jacks., 187, MG648165, MG648186, MG648207, MG648230, MG648251, MG648274, MG648297, MG648320; *Cypella pusilla* (Link & Otto) Benth. & Hook.f. ex B.D.Jacks., 191, MG648166, MG648187, MG648208, MG648231, MG648252, MG648275, MG648298, MG648321; *Cypella pusilla* (Link & Otto) Benth. & Hook.f. ex B.D.Jacks., 148, MG648167, MG648188, MG648209, MG648232, MG648253, MG648276, MG648299, MG648322; *Cypella pusilla* (Link & Otto) Benth. & Hook.f. ex B.D.Jacks., 194, MG648168, MG648189, MG648210, MG648233, MG648254, MG648277, MG648300, MG648323; *Cypella* cf. *pusilla* (Link & Otto) Benth. & Hook.f. ex B.D.Jacks., 913, MG648169, MG648190, MG648211, MG648234, MG648255, MG648278, MG648301, MG648324; *Cypella ravenniana* Deble & F.S.Alves, 142, MG648170, MG648191, MG648212, MG648235, MG648256, MG648279, MG648302, MG648325; *Cypella ravenniana* Deble & F.S.Alves, 757, MG648171, MG648192, MG648213, MG648236, MG648257, MG648280, MG648303, MG648326.

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Online Resource 1 Voucher information, population ID (Pop. ID) and geographical origin (country: first-level administrative division, specific locality) of species sampled to infer preliminary framework phylogenies.

Species	Voucher	Pop. ID	Geographical origin
Outgroup			
Cipura formosa Ravenna	*Chauveau et al. 902* (ICN)	902	Brazil: Goiás, Cavalcante
Cipura paludosa Aubl.	*Báez 205* (COL)	205	Colombia: Meta, Puerto Gaitán
Cipura paradisiaca Ravenna	*Chauveau et al. 900* (ICN)	900	Brazil: Goiás, Teresina de Goiás
Ingroup			
Calydorea pallens Griseb.	*Goldblatt 9579* (MO)	9579	Argentina: Córdoba, Cerro Colorado
Catila amabilis Ravenna	*Eggers 697* (ICN)	697	Brazil: Rio Grande do Sul, Uruguaiana
Cypella altouruguaya Chauveau & L.Eggers	*Eggers & Chauveau 884* (ICN)	884	Brazil: Rio Grande do Sul, Trindade do Sul
Cypella amplimaculata Chauveau & L.Eggers	*Aita 49* (ICN)	049	Brazil: Rio Grande do Sul, Pelotas
Cypella aquatilis Ravenna	*Eggers & Chauveau 714* (ICN)	714	Brazil: Rio Grande do Sul, Muitos Capões
Cypella armosa Ravenna	*Eggers et al. 761* (ICN)	761	Brazil: Rio Grande do Sul, São Borja
Cypella charruana Deble & F.S.Alves	*Pastori et al. 203* (ICN)	203	Brazil: Rio Grande do Sul, Santana de Livramento
Cypella discolor Ravenna	*Aita 75* (ICN)	075	Brazil: Rio Grande do Sul, Quaraí
Cypella fucata Ravenna	*Eggers et al. 746* (ICN)	746	Brazil: Rio Grande do Sul, Uruguaiana
Cypella gloriana Deble & F.S.Alves	*Pastori et al. 119* (ICN)	119	Brazil: Rio Grande do Sul, São Vicente do Sul
Cypella guttata Deble & F.S.Alves	*Pastori et al. 204*(ICN)	204	Brazil: Rio Grande do Sul, Santana de Livramento
Cypella hauthalii (Kuntze) R.C.Foster ssp. *hauthalii*	*Eggers et al. 820* (ICN)	820	Argentina: Misiones, Posadas
Cypella hauthalii ssp. *minuticristata* Chauveau & L.Eggers	*Eggers et al. 833* (ICN)	833	Brazil: Rio Grande do Sul, Soledade
Cypella hauthalii ssp. *opalina* Ravenna	*Eggers et al. 764* (ICN)	764	Brazil: Rio Grande do Sul, São Borja
Cypella herbertii ssp. *brevicristata* Ravenna	*Eggers et al. 841* (ICN)	841	Uruguay: Salto, Salto
Cypella herbertii (Lindl.) Herb. ssp. *herbertii*	*Aita 77* (ICN)	077	Brazil: Rio Grande do Sul, Quaraí
Cypella laeta Ravenna	*Eggers et al. 843* (ICN)	843	Uruguay: Paysandú, Chapicuy
Cypella laxa Ravenna	*Eggers et al. 766* (ICN)	766	Brazil: Rio Grande do Sul, Santo Antônio das Missões
Cypella luteogibbosa Deble	*Eggers et al. 791* (ICN)	791	Brazil: Rio Grande do Sul, Quaraí
Cypella magnicristata Deble	*Aita 76* (ICN)	076	Brazil: Rio Grande do Sul, Quaraí
Cypella osteniana Beauverd	*Eggers et al. 851* (ICN)	851	Uruguay: Lavalleja, Minas
Cypella pabstiana Ravenna	*Eggers et al. 741* (ICN)	741	Brazil: Rio Grande do Sul, Barra do Quaraí

Species	Voucher	Pop. ID	Geographical origin
Cypella pusilla (Link & Otto) Benth. & Hook.f. ex B.D.Jacks.	*Eggers et al. 818* (ICN)	818	Brazil: Rio Grande do Sul, Viamão
Cypella cf. *pusilla* (Link & Otto) Benth. & Hook.f. ex B.D.Jacks.	*Eggers & Chauveau 913* (ICN)	913	Brazil: Rio Grande do Sul, Caçapava do Sul
Cypella ravenniana Deble & F.S.Alves	*Eggers et al. 757* (ICN)	757	Brazil: Rio Grande do Sul, São Borja
Cypella rivularis Chauveau & L.Eggers	*Eggers et al. 873* (ICN)	873	Brazil: Rio Grande do Sul, Alegrete
Herbertia darwinii Roitman & J.A.Castillo	*Eggers & Souza-Chies 502* (ICN)	502	Brazil: Rio Grande do Sul, Livramento
Herbertia quareimana Ravenna	*Eggers & Souza-Chies 513* (ICN)	513	Brazil: Rio Grande do Sul, Quaraí
Kelissa brasiliensis (Baker) Ravenna	*Eggers 701* (ICN)	701	Brazil: Rio Grande do Sul, Caçapava do Sul
Onira unguiculata (Baker) Ravenna	*Eggers & Souza-Chies 273* (ICN)	273	Brazil: Rio Grande do Sul, Rio Grande

Notes: COL = Herbarium of the National University of Colombia (Colombia); ICN = Herbarium of the Federal University of Rio Grande do Sul (Brazil); MO = Herbarium of the Missouri Botanical Garden (U.S.A.).

Online Resource 2 Primers used for PCR amplification and DNA sequencing.

Primer name	Direction	PCR, sequencing	Primer sequence 5'–3'	Source
rps4 + spacer *rps4-trnS*				
rps4-f	Forward	PCR, Seq.	ATGTCCCGTTATCGAGGACCT	Souza-Chies et al. 1997
t trnS-r	Reverse	PCR, Seq.	TACCGAGGGTTCGAATC	Souza-Chies et al. 1997
rbcL				
rbcL-1f	Forward	PCR, Seq.	ATGAGTTGTAGGGAGGGACT	Reeves et al. 2001
rbcL-1360r	Reverse	PCR, Seq.	CTTCACAAGCAGCAGCTAGTTC	Reeves et al. 2001
rbcL-656f	Forward	Seq.	TGCGTTGGAGAGACCGTTTC	Chauveau et al. 2012
rbcL-675r	Reverse	Seq.	GAAACGGTCTCTCCAACGC	Chauveau et al. 2012
matK + *matK*-5'*trnK* intron				
matK-f1	Forward	PCR, Seq.	ATGGAAGAATTACAAGGATAT	Chauveau et al. 2012
rnK-2r	Reverse	PCR, Seq.	AACTAGTCGGATGGAGTAG	Johnston and Soltis 1995
matK-f4	Forward	Seq.	GGTATCAATCGTACAGGAT	This study
matK-r2	Reverse	Seq.	AGTTTGATAATTGGTTTATATG	Chauveau et al. 2012
trnQ-rps16				
trnQ-S1f	Forward	PCR, Seq.	GCGTGGCCAAGTGGTAAGGC	Shaw et al. 2007
rps16-S1r	Reverse	PCR, Seq.	GTTGCTTTCTACCACATCGTTT	Shaw et al. 2007
trnQ-f6	Forward	Seq.	AACTCTTGATACTCGAGAAGAAGTG	This study
trnQ-r5	Reverse	Seq.	TACGCCCGTTATTTGGACTTTC	This study
rps16 intron				
rps16-f	Forward	PCR, Seq.	GTGGTAGAAAGCAACGTGCGACT	Oxelman et al., 1997
rps16-r2	Reverse	PCR, Seq.	TCGGGATCGAACATCAATTGCAA	Oxelman et al., 1997
psbA-trnH				
psbA	Forward	PCR, Seq.	GTTATGCATGAACGTAATGCTC	Shaw et al. 2005
$trnH^{GUG}$	Reverse	PCR, Seq.	CGCGCATGGTGGATTCACAATCC	Shaw et al. 2005
ITS				
AB101	Forward	PCR, Seq.	ACGAATTCATGGTCCGGTGAAGTGTTCG	Douzery et al. 1999
AB102	Reverse	PCR, Seq.	TAGAATTCCCCGGTTCGCTCGCCGTTAC	Douzery et al. 1999
RPB2				
IRID-f	Forward	PCR, Seq.	GCACATATGGGGAAAGAAGG	Schnitzler et al. 2011
E24-r	Reverse	PCR, Seq.	CTGATATCCACATTTGTGGAGAGC	This study

Notes: Seq. = sequencing

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Online Resource 3 PCR profiles for DNA amplification**.** (1) Initial denaturation; (2) Number of cycles; (3) Denaturation, annealing, and elongation steps for each cycle; (4) final elongation step. Temperature and duration are indicated for each step.

Locus	PCR profile
rps4 + spacer *rps4-trnS*	(1) 95°C–2 mn; (2) 40; (3) 94°C–1 mn, 50°C–40 s, 72°C–2 mn; (4) 72°C, 5 mn
rbcL	(1) 95°C–2 mn; (2) 40; (3) 94°C–1 mn, 56°C–40 s, 72°C–2 mn; (4) 72°C, 5 mn
matK + *matK*-5'*trnK* intron	(1) 95°C–2 mn; (2) 40; (3) 94°C–1 mn, 53°C–40 s, 72°C–2 mn; (4) 72°C–5 mn
trnQ-rps16	(1) 95°C–2 mn; (2) 40; (3) 94°C–45 s, 62°C–40 s, 72°C–1:30 mn; (4) 72°C, 5 mn
rps16 intron	(1) 95°C–2 mn; (2) 40; (3) 94°C–1 mn, 50°C–40 s, 72°C–1:30 mn; (4) 72°C, 5 mn
psbA-trnH	(1) 95°C–2 mn; (2) 40; (3) 94°C–1 mn, 53°C–40 s, 72°C–1:30 mn; (4) 72°C, 5 mn
ITS	(1) 95°C–2 mn; (2) 40; (3) 94°C–45 s, 66°C–1 mn, 72°C–2 mn; (4) 72°C, 5 mn
RPB2	(1) 95°C–15 mn; (2) 45; (3) 94°C–45 s, 50°C–45 s, 72°C–1:30 mn; (4) 72°C, 10 mn

Online Resource 4 Dataset partitions for Maximum Likelihood (ML) and Bayesian Inference (BI) analyses and evolutionary models used in BI.

Data partition	Data partition	Partition by codon position	Model
FRAMEWORK PHYLOGENIES			
cpDNA partitions			
*rps*4 (partial sequence)	600	X	HKY+Γ
*rps*4-*trn*S spacer (complete sequence)	290		HKY
*rbc*L (partial sequence)	1359	X	HKY+I
*mat*K (partial sequence)	1569	X	GTR+Γ
*mat*K-5'*trn*K intron (partial sequence)	273		HKY
*trn*Q (partial sequence)	48		K80
*trn*Q-*rps*16 spacer (complete sequence)	1888		GTR+Γ
*rps*16 exon 1 (partial sequence)	18		JC
*rps*16 intron (complete sequence)	868		GTR+Γ
*psb*A (partial sequence)	53	X	K80
*psb*A-*rps*19 spacer (complete sequence)	154		SYM
*rps*19 (complete sequence - negative strand)	369	X	GTR+Γ
*rps*19-*trn*H spacer (complete sequence)	589		GTR+I
*trn*H (partial sequence)	30		JC
nrDNA partitions			
Internal Transcribed Spacer 1 (complete sequence)	347		GTR+Γ
5.8S ribosomal RNA (complete sequence)	164		K80
Internal Transcribed Spacer 2 (complete sequence)	292		GTR+Γ
28S ribosomal RNA (partial sequence)	75		K80
RPB2 exon 23 (partial sequence)	27		JC
RPB2 intron 23 (complete sequence)	700		HKY
RPB2 exon 24 (partial sequence)	18		K80
TAXONOMIC ASSESSMENT			
cpDNA partitions			
*rps*4 (partial sequence)	585	X	HKY
*rps*4-*trn*S spacer (complete sequence)	285		HKY
*rbc*L (partial sequence)	1359	X	GTR
*mat*K (partial sequence)	1548	X	HKY
*mat*K-5'*trn*K intron (partial sequence)	269		F81
*trn*Q-*rps*16 spacer (complete sequence)	1467		GTR
*rps*16 intron (complete sequence)	847		HKY
*psb*A (partial sequence)	53	X	K80
*psb*A-*rps*19 spacer (complete sequence)	110		JC
*rps*19 (complete sequence - negative strand)	279	X	F81
*rps*19-*trn*H spacer (complete sequence)	137		HKY
nrDNA partitions			
Internal Transcribed Spacer 1 (complete sequence)	271		GTR+Γ

Data partition	Data partition	Partition by codon position	Model
5.8S ribosomal RNA (complete sequence)	164		JC
Internal Transcribed Spacer 2 (complete sequence)	236		GTR+Γ
RPB2 intron 23 (complete sequence)	700		HKY

Notes: partition by codon position = partition coding for protein.

Online Resource 5 Mean ± standard deviation values for each morphometric character and character states for each categorical character used in multivariate analyses of morphological traits among *Cypella* species. Populations of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla* are identified by their Pop. ID as listed in Table 1. The number of specimen measured for each species or population are given between brackets.

	Characters	*C. a.*[1]	*C. d.*[2]	*C. r.*[3]	*C. g.*[4]	*C. p.*[5]								*C.* cf. *p.*[6]
		[8]	[8]	[5]	119 [9]	818 [5]	914 [5]	778 [10]	783 [8]	174 [9]	184 [9]	148 [8]	194 [10]	913 [9]
	Plant height (cm)	48.6±8.0	22.1±4.2	15.4±7.0	20.8±2.6	26.6±3.7	17.6±5.0	19.0±4.0	17.5±4.8	13.6±2.9	17.2±3.1	16.8±4.7	18.5±6.2	16±2.3
Leaves	Longest basal leaf: L (mm)	-	-	-	-	-	-	-	-	-	-	-	-	-
	Longest basal leaf: W (mm)	-	-	-	1.2±0.5	1.2±0.3	0.9±0.2	1.5±0.4	1.1±0.4	0.9±0.3	1.0±0.2	1.5±0.4	1.3±0.3	2.4±0.5
	Basal cauline leaf: L (mm)	242.5 ±66.7	172.5 ±46.5	105.0 ±20.5	130.0 ±60.9	107.5 ±42	126.0 ±19.3	103.0 ±45.1	123.0 ±48.8	113.0 ±33.5	123.0 ±42.8	132.5 ±50.6	111.5 ±50.8	127.0 ±31.0
	Basal cauline leaf: W (mm)	17.5±4.5	5.1±1.9	2.8±0.7	1.4±0.5	1.3±0.3	0.8±0.3	1.1±0.4	1.2±0.4	0.7±0.2	0.8±0.2	1.3±0.3	1.3±0.3	2.5±0.5
Floral stem	Total L (cm)	-	-	-	14.2±2.2	22.9±4.5	16.0±5.2	13.8±3.8	13.4±4.6	9.9±2.6	12.6±2.6	13.1±4.2	14.4±6.0	11.7±2.2
	First internode: L (cm)	23.0±3.5	16.2±2.1	15.5±1.5	13.9±2.2	22.7±3.8	13.1±5.3	13.5±3.8	13.1±4.7	9.5±2.4	12.7±2.7	12.1±4.3	14.1±6.1	11.3±2.3
	First cauline leaf or bract: L (mm)	251.0±77.7	64.5±6.7	74.6±18.3	25.8±3.2	26.2±5.0	39.2±6.5	31.1±7.9	31.3±8.8	29.5±8.0	38.5±6.7	34.7±6.8	27.6±4.2	35.7±5.9
	Internodes: number	>3	2 to 3	2 to 3	1	1	1	1	1	1	1	1	1	1
Synflorescence	Inner peduncles: L (mm)	55.2±9.6	12.8±2.3	27.6±4.4	4.0±0.5	3.9±1.4	3.6±1.0	3.9±1.2	3.7±1.1	3.6±1.1	3.4±0.7	3.1±1.0	3.6±0.6	3.9±0.6
	Rhipidia: number	>10	5 to 10	5 to 10	≤5	≤5	≤5	≤5	≤5	≤5	≤5	≤5	≤5	≤5
	Spathe: outer valve L (mm)	19.5±1.3	21.3±5.3	15.3±2.4	13.2±1.4	13.9±2.1	13.8±1.0	16.0±1.8	14.1±0.9	13.2±1.1	15.9±1.1	18.2±1.6	14.4±1.8	17.4±1.5
	Spathe: inner valve L (mm)	30.3±6.2	28.1±3.2	36.2±6.2	20.8±1.4	20.3±0.9	22.7±1.9	23.8±2.3	20.1±2.3	19.1±2.0	22.4±1.3	27±1.3	20.1±2.2	25.1±1.9
Flower perigon	Diameter (mm)	42.5±4.0	41.8±7.1	43.2±6.7	26.9±2.3	29.4±1.3	37.1±3.1	27.2±3.3	24.9±2.8	25.8±5.2	29.8±2.6	29.5±2.5	31.6±2.5	36.0±2.2
	Tepals base fusion: L (mm)	-	-	-	0.4±0.09	0.4±0.2	1.6±0.4	0.6±0.2	0.5±0.2	0.5±0.1	0.4±0.2	1.4±0.2	0.5±0.2	0.6±0.1
	Dominant colour	yellow	white / yellowish	yellow	pale yellow	pale yellow	pale yellow	pale yellow	pale yellow	pale yellow	pale yellow	pale yellow	pale yellow	white
	Outer tepal: length (mm)	35.0±2.6	29.1±5.4	32.0±4.6	25.1±1.8	23.0±0.8	27.7±3.1	25.0±3.5	22.1±1.8	20.6±3.2	23.0±1.6	23.3±1.4	21.9±1.6	27.8±1.7
	Outer tepal: max. W (mm)	19.5±1.3	21.3±5.3	15.3±2.4	13.5±1.4	13.2±0.9	14.7±1.4	14.0±1.4	11.9±0.8	12.5±1.6	12.1±0.8	13.6±0.4	13.0±1.0	14.9±1.4
	Inner tepal: L (mm)	20.0±1.0	16.5±1.4	21.6±3.4	17.0±1.2	16.2±0.3	16.5±1.2	16.0±1.7	14.4±0.5	14.7±1.1	15.5±0.7	16.3±1.0	15.8±1.4	17.3±1.0
	Inner tepal: max. W (mm)	12.5±0.8	8.2±1.4	10.2±0.8	8.1±0.7	5.9±0.2	6.3±0.4	7.0±0.7	5.9±0.5	6.4±0.5	6.5±0.4	7.1±0.6	6.3±0.4	6.6±0.4
	Elaiophore: dominant colour	white	brownish yellow	yellow	yellow	yellow	yellow	yellow	yellow	yellow	yellow	yellow	yellow	yellow
Androecium	Filaments connate part: L (mm)	-	-	-	0.0	0.1±0.3	0.0	0.1±0.1	0.06±0.1	0.1±0.1	0.4±0.1	0.7±0.6	0.06±0.02	0.2±0.5
	Filaments free part: L (mm)	-	-	-	5.7±0.4	4.9±0.6	5.3±0.2	5.5±0.6	5.1±0.5	4.6±0.6	4.7±0.2	4.7±0.7	4.9±0.6	5.3±0.5
	Anthers: L (mm)	7.7±1.1	5.6±0.5	7.1±0.5	5.0±0.2	4.1±0.4	5.3±0.6	5.3±0.3	4.4±0.3	4.7±0.3	4.9±0.4	5.4±0.4	4.5±0.4	5.2±0.4
	Anthers: W (mm)	-	-	-	0.8±0.1	1.15±0.1	1.2±0.1	1.2±0.2	1.0±0.1	1.0±0.2	1.2±0.1	1.4±0.1	1.2±0.2	1.1±0.3
	Anthers adnate to the style: L (mm)	-	-	-	3.2±0.3	3.0±0.4	3.4±0.7	4.0±0.6	3.0±0.6	3.6±0.4	3.3±0.6	3.9±0.4	3.0±0.3	3.3±0.2
	Connective: W (mm)	-	-	-	0.4±0.1	0.6±0.05	0.6±0.1	0.7±0.1	0.4±0.01	0.5±0.09	0.7±0.1	1.0±0.1	0.6±0.1	0.6±0.2

	Characters	*C. a.*[(1)]	*C. d.*[(2)]	*C. r.*[(3)]	*C. g.*[(4)]	*C. p.*[(5)]								*C.* cf. *p.*[(6)]
		[8]	[8]	[5]	119 [9]	818 [5]	914 [5]	778 [10]	783 [8]	174 [9]	184 [9]	148 [8]	194 [10]	913 [9]
	Connective: apex shape	truncate acute	truncate	truncate acuminate	retuse apiculate	acute	truncate apiculate	apiculate	apiculate	apiculate	apiculate	truncate apiculate	apiculate	acute
	Connective: base shape	truncate reniform	truncate	sagittate	truncate	truncate	truncate	truncate	truncate	truncate	truncate	truncate	truncate	truncate
Gynoecium	Ovary: L (mm)	6.7±2.2	6.5±2.5	6.2±2.3	3.7±0.5	3.3±0.2	4.0±0.4	4.4±0.5	3.3±0.46	3.5±0.5	4.2±0.4	6.2±0.5	4.2±0.4	4.8±0.7
	Ovary: W (mm)	0.6±0.3	0.7±0.3	0.8±0.4	1.9±0.1	1.9±0.3	1.6±0.4	1.8±0.2	1.6±0.1	1.5±0.3	1.8±0.2	2.4±0.3	1.9±0.2	2.1±0.2
	Style: filiform base L (mm)	-	-	-	6.2±0.4	5.1±0.6	6.2±0.4	6.2±0.7	5.6±0.3	4.9±0.5	5.6±0.3	6.1±0.3	5.6±0.3	6.3±0.6
	Style arms: total L (mm)	-	-	-	5.9±0.4	5.1±0.3	6.0±0.7	6.1±0.5	5.6±1.2	5.3±0.5	5.9±0.7	6.1±0.5	5.0±0.5	5.7±0.5
	Style arms: concrescent part L (mm)	-	-	-	2.8±0.5	3.0±0.2	2.5±0.4	2.4±0.5	2.6±1.2	2.2±0.5	2.8±0.5	3.3±0.5	2.4±0.3	2.9±0.5
	Style arms: free part length (mm)	5.2±0.1	3.1±0.3	5.0±0.7	3.1±0.3	2.0±0.3	3.4±0.8	3.6±0.5	3.0±0.2	3.2±0.3	3.0±0.3	2.8±0.7	2.5±0.5	2.7±0.5
	Adaxial crests: L (mm)	2.8±0.5	2.7±0.3	5.7±0.9	3.0±0.3	2.2±0.4	2.7±0.2	3.2±0.4	2.7±0.3	2.5±0.5	3.1±0.3	4.0±0.4	2.7±0.4	3.6±0.5
	Abaxial crest: L (mm)	0.5±0.07	1.5±0.4	2.2±0.4	1.5±0.3	1.0±0.3	1.1±0.3	1.7±0.3	1.4±0.3	1.2±0.3	1.6±0.4	2.1±0.4	1.4±0.3	1.9±0.4
	Stigmatic appendages: L (mm)	-	-	-	0.7±0.1	0.5±0.08	0.62±0.2	0.6±0.08	0.5±0.1	0.4±0.1	0.5±0.1	0.5±0.2	0.4±0.1	0.5±0.1

Notes: L = length; W = width; [(1)] = *Cypella aquatilis*; [(2)] = *C. discolor*; [(3)] = *C. ravenniana*; [(4)] = *C. gloriana*; [(5)] = *C. pusilla*; [(6)] = *C.* cf. *pusilla*; dash (-) = character excluded from analyses because of incomplete data.

Online Resource 6 Tests for differences in morphological characters among two taxonomic subsets of *Cypella* species.

	Characters*	Taxonomic subsets			
		(1)		(2)	
		F/H-ratio	*P* value**	*F/H-ratio*	*P* value**
Vegetative part					
	Plant height (cm)	9.56	0.003	3.68	<0.001
Leaves	Longest basal leaf: L (mm)	-	-	-	-
	Longest basal leaf: W at mid-length (mm)	-	-	10.38	<0.0001
	Basal cauline leaf: L (mm)	1.91	0.02	3.03	<0.001
	Basal cauline leaf: W at mid-length (mm)	1.63	<0.001	13.12	<0.0001
Reproductive part					
Floral stem	Total length above ground L (cm)	-	-	3.86	<0.001
	First internode above ground level: L (cm)	3.39	0.236	3.82	<0.001
	First cauline leaf or bract after first internode: L (mm)	18.22	<0.001	2.39	<0.001
	Internodes: number	3.98	0.047	NA	NA
Synflorescence	Inner peduncles: L (mm, mean)	5.20	0.001	2.14	<0.001
	Rhipidia: number	3.73	0.049	NA	NA
	Spathe: outer valve L (mm)	9.45	0.008	10.85	<0.0001
	Spathe: inner valve L (mm)	5.06	0.003	14.64	<0.0001
Flower perigon	Diameter (mm)	4.76	0.05	12.82	<0.0001
	Tepals base fusion: L (mm)	-	-	23.82	<0.0001
	Dominant colour	3.26	0.048	7.62	0.043
	Outer tepal: L (mm)	16.69	<0.001	8.43	<0.0001
	Outer tepal: maximum W (mm)	5.85	0.05	5.51	<0.0001
	Inner tepal: L (mm)	2.56	0.003	4.97	<0.0001
	Inner tepal: maximum W (mm)	6.53	0.01	11.23	<0.0001
	Elaiophore: dominant colour	4.82	0.049	NA	NA
Androecium	Filaments connate part: L (mm)	-	-	4.49	<0.0001
	Filaments free part: L (mm)	-	-	3.42	0.002
	Anthers: L (mm)	4.13	<0.001	8.42	<0.0001
	Anthers: W at mid-length (mm)	-	-	5.06	<0.0001
	Anthers adnate to the style: L (mm)	-	-	3.51	0.001
	Connective: W at mid-length (mm)	-	-	14.35	<0.0001
	Connective: apex shape	2.03	0.018	2.36	0.019
	Connective: base shape	3.12	0.038	NA	NA
Gynoecium	Ovary: L (mm)	1.82	0.002	18.49	<0.0001
	Ovary: W at distal end (mm)	1.73	0.001	6.26	<0.0001
	Style: filiform base L (mm)	-	-	7.52	<0.0001
	Style arms: total L (mm)	-	-	3.13	0.003
	Style arms: concrescent part L (mm)	-	-	2.50	0.016
	Style arms: free part L (mm)	8.95	0.01	5.96	<0.0001
	Adaxial crests: L (mm, mean of two measurements)	2.11	0.05	11.50	<0.0001
	Abaxial crest: L (mm)	3.87	0.036	5.34	<0.0001
	Stigmatic appendages: L (mm, mean of two measurements)	-	-	2.42	0.019

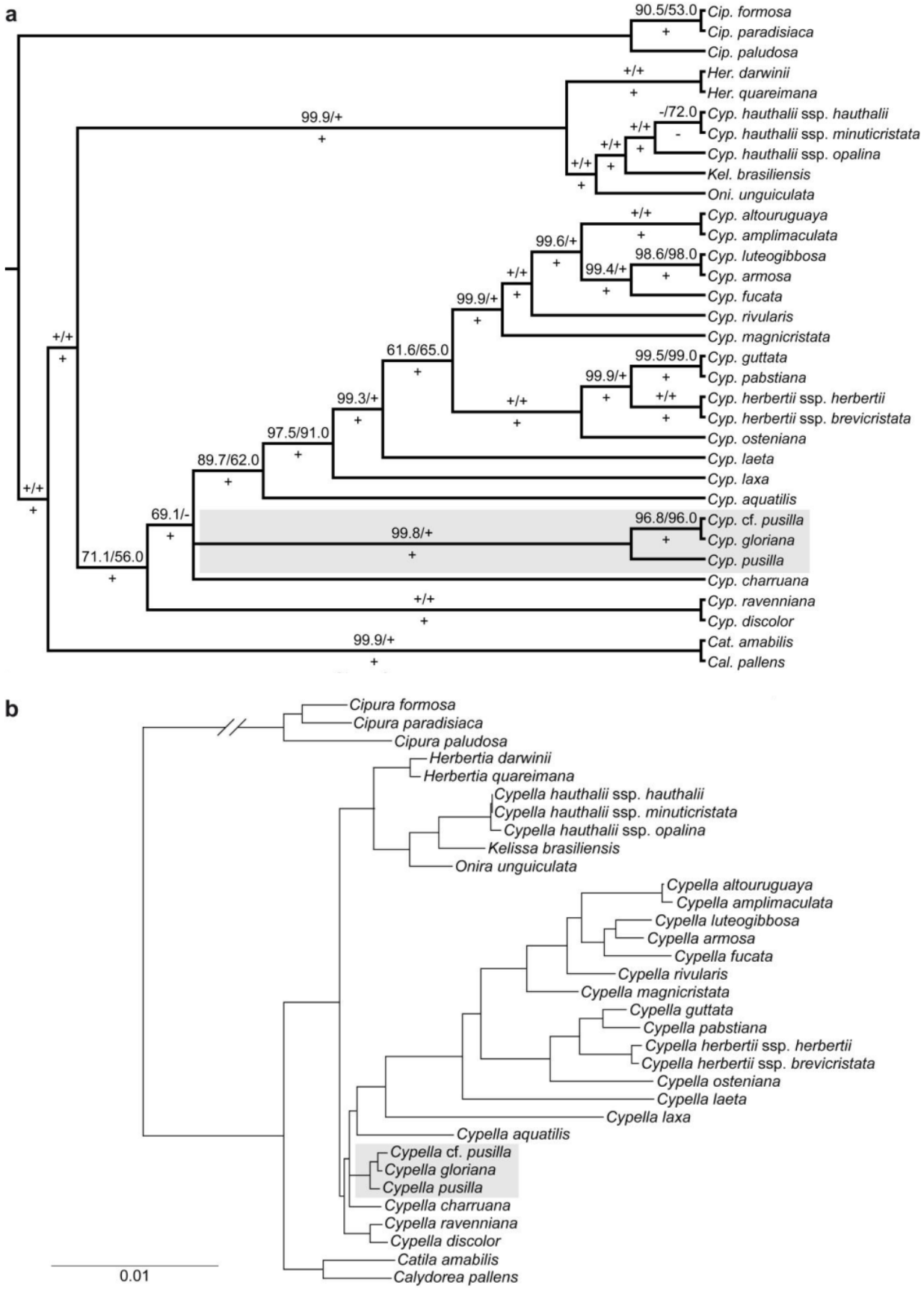
Notes: * = morphometric characters were analysed using one-way ANOVA (F), whereas categorical characters were analysed using Kruskal-Wallis test (H-ratio); (1) = all *Cypella* species; (2) = populations of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla*; ** = significant differences between species and/or populations were estimated using Fisher's (LSD) test; L = length; W = width; dash (-) = character excluded from final analyses because of insufficient data; NA = not applicable (constant character state).

Online Resource 7 Framework phylogenies: lengths and indices for the resulting most parsimonious trees in parsimony analyses of separated and combined data sets. MP trees is the number of most parsimonious trees obtained from MP analyses, whereas CI and RI are respectively the consistency and retention indices of most parsimonious topologies.

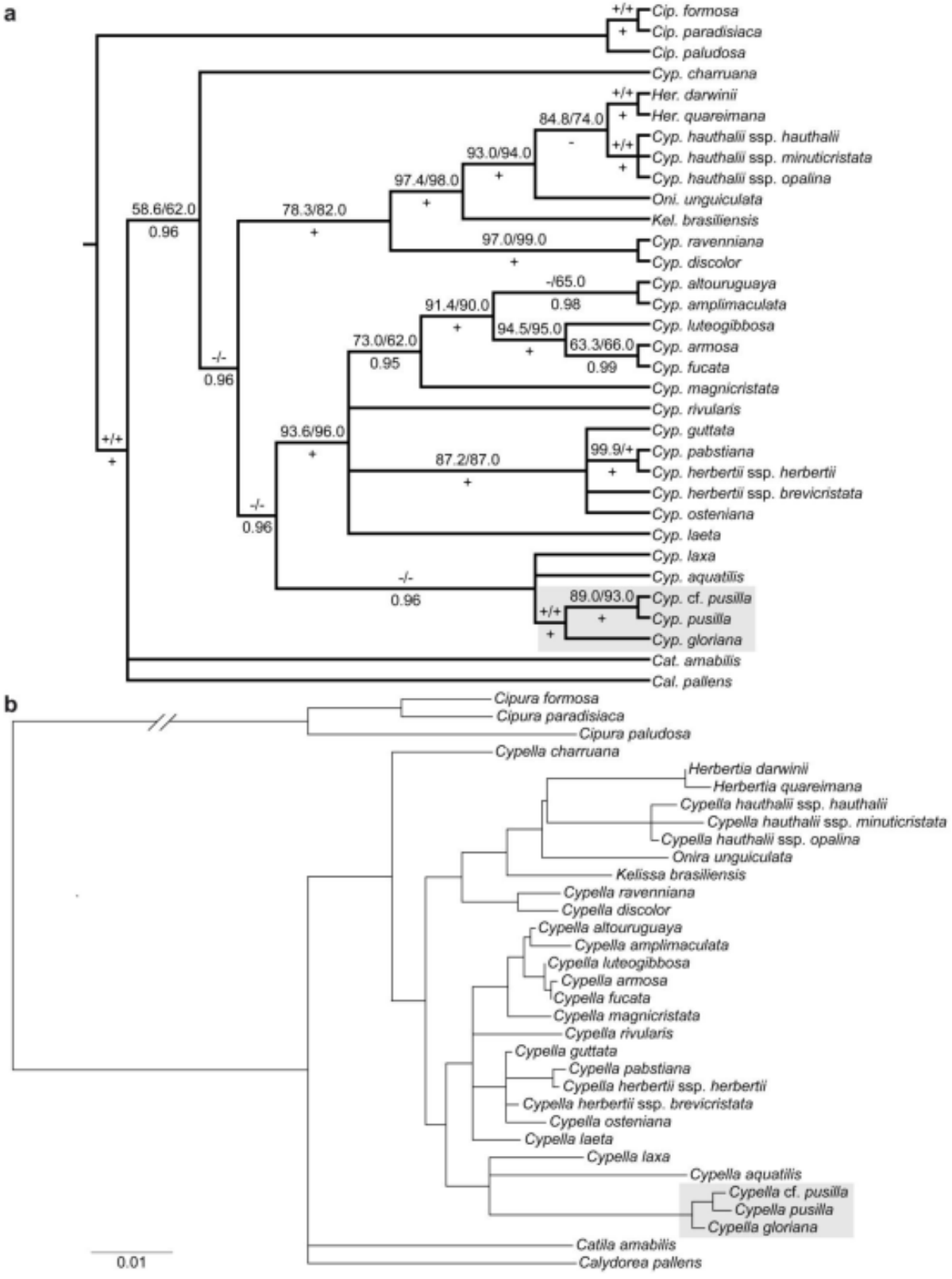
DNA data set	Number of characters			MP trees	Tree length	CI	RI
	Total	Variable (%)	Parsimony informative (%)				
rps4-trnS + [coded indels]	890 + [17]	85 + [17] (11.24%)	43 + [5] (5.29%)	104	122	0.88	0.90
rbcL	1359	64 (4.70%)	37 (2.72%)	36	77	0.88	0.93
matK-5'trnK + [coded indels]	1842 + [12]	187 + [12] (10.73%)	105 + [6] (5.99%)	40	245	0.87	0.93
trnQ-rps16 + [coded indels]	1954 + [51]	182 + [51] (11.62%)	96 + [23] (5.93%)	38	279	0.87	0.91
rps16 + [coded indels]	868 +[9]	64 + [9] (8.33%)	29 + [4] (3.76%)	156	79	0.95	0.95
psbA-trnH + [coded indels]	1192 + [17]	89 + [17] (8.77%)	48 + [11] (4.88%)	2490	152	0.85	0.91
cpDNA + [coded indels]	8105 + [106]	671 + [106] (9.46%)	358 + [49] (4.96%)	2	979	0.85	0.90
ITS + [coded indels]	879 + [55]	125 + [55] (19.27%)	75 + [35] (11.78%)	18477	319	0.72	0.79
RPB2 + [coded indels]	886 + [31]	136 + [31] (18.21%)	72 + [18] (9.81%)	1	178	0.98	0.98
nrDNA + [coded indels]	1765 + [86]	261 + [86] (18.75%)	147 + [53] (10.80%)	208	521	0.78	0.83

Notes: numbers into brackets are numbers of characters resulting from indel-coding.

Online Resource 8 Framework phylogenies: ML best-scoring cladogram (a) and phylogram (b) obtained from the analyses of the combined chloroplast data set. Trees were rooted using Cipura formosa, C. paludosa and C. paradisiaca as outgroup. Support values indicated above branches follow the order parsimony bootstrap (PBS)/likelihood bootstrap (LBS), whereas Bayesian posterior probabilities (PP) are reported below branches. Bootstrap supports and posterior probabilities for a given node are provided only if one of the values reached the following thresholds: PBS ≥70% or LPB ≥70% or PP ≥0.95 (other nodes were collapsed). A plus sign (+) means full support, whereas a dash (-) indicates support value of less than 50% for PBS and LBS or less than 0.95 for PP. Cal. = *Calydorea*, Cat. = *Catila*, Cip. = *Cipura*, Cyp. = *Cypella*, Her. = *Herbertia*, Kel. = *Kelissa*, Oni. = *Onira*.


a
90.5/53.0
Cip. formosa
Cip. paradisiaca
Cip. paludosa
Her. darwinii
Her. quareimana
99.9/+
-/72.0
Cyp. hauthalii ssp. hauthalii
Cyp. hauthalii ssp. minuticristata
Cyp. hauthalii ssp. opalina
Kel. brasiliensis
Oni. unguiculata
Cyp. altouruguaya
Cyp. amplimaculata
99.6/+
98.6/98.0
Cyp. luteogibbosa
99.4/+
Cyp. armosa
Cyp. fucata
99.9/+
Cyp. rivularis
Cyp. magnicristata
61.6/65.0
99.5/99.0
Cyp. guttata
99.9/+
Cyp. pabstiana
99.3/+
Cyp. herbertii ssp. herbertii
Cyp. herbertii ssp. brevicristata
97.5/91.0
Cyp. osteniana
89.7/62.0
Cyp. laeta
Cyp. laxa
Cyp. aquatilis
69.1/-
96.8/96.0
Cyp. cf. pusilla
99.8/+
Cyp. gloriana
Cyp. pusilla
71.1/56.0
Cyp. charruana
Cyp. ravenniana
Cyp. discolor
99.9/+
Cat. amabilis
Cal. pallens
b
Cipura formosa
Cipura paradisiaca
Cipura paludosa
Herbertia darwinii
Herbertia quareimana
Cypella hauthalii ssp. hauthalii
Cypella hauthalii ssp. minuticristata
Cypella hauthalii ssp. opalina
Kelissa brasiliensis
Onira unguiculata
Cypella altouruguaya
Cypella amplimaculata
Cypella luteogibbosa
Cypella armosa
Cypella fucata
Cypella rivularis
Cypella magnicristata
Cypella guttata
Cypella pabstiana
Cypella herbertii ssp. herbertii
Cypella herbertii ssp. brevicristata
Cypella osteniana
Cypella laeta
Cypella laxa
Cypella aquatilis
Cypella cf. pusilla
Cypella gloriana
Cypella pusilla
Cypella charruana
Cypella ravenniana
Cypella discolor
Catila amabilis
Calydorea pallens
0.01

Online Resource 9 Framework phylogenies: ML best-scoring cladogram (a) and phylogram (b) obtained from the analyses of the combined nuclear data set. Trees were rooted using *Cipura formosa, C. paludosa* and *C. paradisiaca* as outgroup. Support values indicated above branches follow the order parsimony bootstrap (PBS)/likelihood bootstrap (LBS), whereas Bayesian posterior probabilities (PP) are reported below branches. Bootstrap supports and posterior probabilities for a given node are provided only if one of the values reached the following thresholds: PBS ≥70% or LPB ≥70% or PP ≥0.95 (other nodes were collapsed). A plus sign (+) means full support, whereas a dash (-) indicates support value of less than 50% for PBS and LBS or less than 0.95 for PP. Cal. = *Calydorea*, Cat. = *Catila*, Cip. = *Cipura*, Cyp. = *Cypella*, Her. = *Herbertia*, Kel. = *Kelissa*, Oni. = *Onira*.


a
Cip. formosa
Cip. paradisiaca
Cip. paludosa
Cyp. charruana
Her. darwinii
Her. quareimana
Cyp. hauthalii ssp. hauthalii
Cyp. hauthalii ssp. minuticristata
Cyp. hauthalii ssp. opalina
Oni. unguiculata
Kel. brasiliensis
Cyp. ravenniana
Cyp. discolor
Cyp. altouruguaya
Cyp. amplimaculata
Cyp. luteogibbosa
Cyp. armosa
Cyp. fucata
Cyp. magnicristata
Cyp. rivularis
Cyp. guttata
Cyp. pabstiana
Cyp. herbertii ssp. herbertii
Cyp. herbertii ssp. brevicristata
Cyp. osteniana
Cyp. laeta
Cyp. laxa
Cyp. aquatilis
Cyp. cf. pusilla
Cyp. pusilla
Cyp. gloriana
Cat. amabilis
Cal. pallens
84.8/74.0
93.0/94.0
97.4/98.0
58.6/62.0
0.96
78.3/82.0
97.0/99.0
-/65.0
0.98
91.4/90.0
94.5/95.0
63.3/66.0
0.99
73.0/62.0
0.95
-/-
0.96
93.6/96.0
99.9/+
87.2/87.0
-/-
0.96
89.0/93.0
b
Cipura formosa
Cipura paradisiaca
Cipura paludosa
Cypella charruana
Herbertia darwinii
Herbertia quareimana
Cypella hauthalii ssp. hauthalii
Cypella hauthalii ssp. minuticristata
Cypella hauthalii ssp. opalina
Onira unguiculata
Kelissa brasiliensis
Cypella ravenniana
Cypella discolor
Cypella altouruguaya
Cypella amplimaculata
Cypella luteogibbosa
Cypella armosa
Cypella fucata
Cypella magnicristata
Cypella rivularis
Cypella guttata
Cypella pabstiana
Cypella herbertii ssp. herbertii
Cypella herbertii ssp. brevicristata
Cypella osteniana
Cypella laeta
Cypella laxa
Cypella aquatilis
Cypella cf. pusilla
Cypella pusilla
Cypella gloriana
Catila amabilis
Calydorea pallens
0.01

Online Resource 10 Discrimination measures for the first and second dimension of Multiple Correspondence Analyses (MCA) performed on two taxonomic subsets of morphological characters.

	Characters	MCA[(A)]		MCA[(B)]	
		D1 (57.5%)*	D2 (31.3%)*	D1 (36.9%)*	D2 (29.1%)*
Vegetative part					
	Plant height (cm)	0.910	0.543	0.882	0.966
Leaves	Longest basal leaf: L (mm)	-	-	-	-
	Longest basal leaf: W at mid-length (mm)	-	-	0.438	0.109
	Basal cauline leaf: L (mm)	0.937	0.860	0.572	0.841
	Basal cauline leaf: W at mid-length (mm)	0.864	0.651	0.352	0.084
Reproductive part					
Floral stem	Total length above ground L (cm)	-	-	0.175	0.171
	First internode above ground level: L (cm)	NS	NS	0.103	0.218
	First cauline leaf or bract after first internode: L (mm)	0.981	0.932	0.353	0.622
	Internodes: number	0.952	0.698	NA	NA
Synflorescence	Inner peduncles: L (mm, mean)	0.870	0.492	0.113	0.097
	Rhipidia: number	0.832	0.870	NS	NS
	Spathe: outer valve L (mm)	0.538	0.142	0.554	0.049
	Spathe: inner valve L (mm)	0.428	0.182	0.730	0.325
Flower perigon	Diameter (mm)	0.603	0.254	0.279	0.246
	Tepals base fusion: L (mm)	-	-	0.233	0.447
	Dominant colour	0.867	0.550	0.170	0.043
	Outer tepal: L (mm)	0.747	0.104	0.409	0.271
	Outer tepal: maximum W (mm)	0.721	0.619	0.547	0.187
	Inner tepal: L (mm)	0.574	0.167	0.529	0.200
	Inner tepal: maximum W (mm)	0.848	0.225	0.397	0.321
	Elaiophore: dominant colour	0.832	0.870	NA	NA
Androecium	Filaments connate part: L (mm)	-	-	0.235	0.243
	Filaments free part: L (mm)	-	-	0.226	0.402
	Anthers: L (mm)	0.806	0.302	0.504	0.198
	Anthers: W at mid-length (mm)	-	-	0.130	0.305
	Anthers adnate to the style: L (mm)	-	-	0.195	0.089
	Connective: W at mid-length (mm)	-	-	0.284	0.434
	Connective: apex shape	0.886	0.835	0.364	0.394
	Connective: base shape	0.024	0.803	NA	NA
Gynoecium	Ovary: L (mm)	0.562	0.199	0.723	0.594
	Ovary: W at distal end (mm)	0.279	0.321	0.589	0.411
	Style: filiform base L (mm)	-	-	0.426	0.180
	Style arms: total L (mm)	-	-	0.486	0.073
	Style arms: concrescent part L (mm)	-	-	0.301	0.131
	Style arms: free part L (mm)	0.824	0.081	0.095	0.088
	Adaxial crests: L (mm, mean of two measurements)	0.120	0.028	0.544	0.400
	Abaxial crest: L (mm)	0.698	0.150	0.489	0.157
	Stigmatic appendages: L (mm, mean of two measurements)	-	-	0.148	0.344

Notes: L = length; W = width; [(A)] = all specimens of *Cypella* included in the current study; [(B)] = specimens belonging to populations of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla*; * = proportions of morphological variation captured by each dimension; dash (-) = character excluded from final analyses because of insufficient data; NA = not applicable (constant character state); NS = variation not significant.

Online Resource 11 Structure matrix with correlation coefficients between each character and the first two standardised discriminant functions (DF1 and DF2) of canonical and classificatory discriminant analyses (DA) performed on two taxonomic subsets of morphological characters.

	Characters	DA[(A)]		DA[(B)]	
		DF1 (60.7%)*	DF2 (21.3%)*	DF1 (54.8%)*	DF2 (26.3%)*
Vegetative part					
	Plant height (cm)	0.224	-0.346	0.066	-0.020
Leaves	Longest basal leaf: L (mm)	-	-	-	-
	Longest basal leaf: W at mid-length (mm)	-	-	-0.052	0.194
	Basal cauline leaf: L (mm)	0.024	-0.010	0.120	-0.259
	Basal cauline leaf: W at mid-length (mm)	0.367**	-0.063	0.127	-0.220
Reproductive part					
Floral stem	Total length above ground L (cm)	-	-	0.032	-0.066
	First internode above ground level: L (cm)	NS	NS	0.025	-0.008
	First cauline leaf or bract after first internode: L (mm)	0.371**	-0.177	-0.099	0.061
	Internodes: number	NA	NA	NA	NA
Synflorescence	Inner peduncles: L (mm, mean)	0.559**	0.010	-0.024	0.076
	Rhipidia: number	NA	NA	NA	NA
	Spathe: outer valve L (mm)	0.154	-0.015	0.322	-0.219
	Spathe: inner valve L (mm)	0.161	0.391	0.370	-0.166
Flower perigon	Diameter (mm)	0.176	0.305	-0.086	0.395
	Tepals base fusion: L (mm)	-	-	0.362	0.532
	Dominant colour	NA	NA	NA	NA
	Outer tepal: L (mm)	0.177	0.175	0.188	0.058
	Outer tepal: maximum W (mm)	0.150	0.151	0.004	0.100
	Inner tepal: L (mm)	0.133	0.180	-0.007	0.066
	Inner tepal: maximum W (mm)	0.191	0.116	0.198	-0.064
	Elaiophore: dominant colour	NA	NA	NA	NA
Androecium	Filaments connate part: L (mm)	-	-	0.118	0.034
	Filaments free part: L (mm)	-	-	0.057	0.079
	Anthers: L (mm)	0.110	-0.005	0.043	-0.010
	Anthers: W at mid-length (mm)	-	-	-0.276**	0.122
	Anthers adnate to the style: L (mm)	-	-	0.312	-0.072
	Connective: W at mid-length (mm)	-	-	-0.141	0.033
	Connective: apex shape	NA	NA	NA	NA
	Connective: base shape	NA	NA	NA	NA
Gynoecium	Ovary: L (mm)	0.208	0.252	0.002	-0.332
	Ovary: W at distal end (mm)	0.068	0.157	0.048	-0.141
	Style: filiform base L (mm)	-	-	0.209**	0.034
	Style arms: total L (mm)	-	-	0.228	0.115
	Style arms: concrescent part L (mm)	-	-	0.062	-0.152
	Style arms: free part L (mm)	0.203	0.127	0.207	0.205
	Adaxial crests: L (mm, mean of two measurements)	0.032	0.538**	-0.186**	0.154
	Abaxial crest: L (mm)	0.003	0.281	0.355**	-0.096
	Stigmatic appendages: L (mm, mean of two measurements)	-	-	0.146	0.183**

Notes: L = length; W = width; [(A)] = all specimens of *Cypella* included in the current study; [(B)] = specimens belonging to populations of *C. gloriana*, *C. pusilla* and *C.* cf. *pusilla*; * = proportions of morphological variation captured by each discriminant function; ** = largest absolute correlation between a given character and any discriminant function; dash (-) = character excluded from final analyses because of insufficient data; NA = not applicable (categorical characters); NS = variation not significant.

CAPÍTULO III

Phylogeny, leaf anatomy and evolution of characters in Tigridieae (Iridoideae: Iridaceae)

Premise of the study: Tigridieae (Iridoideae: Iridaceae) is a monophyletic lineage defined by molecular and morphological synapomorphies, exclusive of the New World. Tigridieae was subdivided into Cipurinae and Tigridiinae based on cytogenetic, palynological and morphological characters. Recent phylogenetic studies evidenced that these subtribes are not monophyletic and Tigridieae was informally divided into two clades (A and B). The goals of this study were to elucidate phylogenetic relationships in Tigridieae, to perform a detailed analysis of transverse leaf anatomy of Tigridieae and to evaluate the evolution of anatomical characters.

Methods: Sequences were obtained from 76 accessions corresponding to 16 genera and 60 species. A combination of eight coding and non-coding DNA regions was analysed. Leaf anatomical analyses were performed in 81 accessions of 55 species distributed among 16 genera. Ancestral state optimizations were compiled for all species analysed using MP and ML reconstruction.

Key results: Phylogenetic analyses revealed that Cipurinae and Tigridiinae are not monophyletic and the genera *Alophia*, *Gelasine*, *Cypella* and *Calydorea* do not correspond to monophyletic groups. Structural observations evidenced differences mainly in leaf outline, in position and type of sclerenchyma, and in marginal vascular bundles.

Conclusions: Phylogenetic framework and leaf anatomy revealed diagnostic characters for Tigridieae, especially for subtribes Cipurinae and Tigridiinae and could sustain a new circumscription where Cipurinae would include all genera present in Clade A and Tigridiinae would include all genera present in Clade B.

Key words: anatomical characters; leaf anatomy; lignified sclerenchyma, pectin-rich sclerenchyma, subepidermal sclerenchyma.

Este capítulo será preparado para publicação e contou com a participação dos seguintes pesquisadores: Lilian Eggers, Tatiana Teixeira de Souza-Chies, Jorge Ernesto de Araujo Mariath, Olivier Chauveau

INTRODUCTION

Angiosperms present a great diversity of external morphological characters used in the classical taxonomy. The leaf morphology, a central theme in taxonomy and also systematics, is one of the most obvious characters that distinguish monocotyledons, for exemple (Kaplan, 1973). In addition, leaf anatomy can be an important approach for the circumscription of the groups. Leaf anatomy was been applied in different families of angiosperms, as example the Iridaceae, and vegetative characters are essential in studies of phylogenetic relationships in Iridaceae, especially those related to stems and leaves (Rudall, 1994). Studies on leaf anatomy in Iridaceae indicate that some characters can provide important traits for taxonomy and systematics (Rudall, 1994, Rudall, 2003; Goldblatt and Manning, 2008). Characters related to leaf margin, thickening of epidermis cell wall and characteristics of mesophyll cells provide important evidences for Iridaceae systematics (Rudall, 1990, Goldblatt et al. 1990; Rudall, 1994; Rudall, 1995). In Tigridieae, leaf anatomy proved to have many important characteristics for circumscription of genera as leaf outline and sclerenchyma position (Rudall, 1994).

Tigridieae (Iridaceae: Iridoideae) comprises 15 to 20 genera and about 170 species, occurring in southern North America, Central and South America (Rodriguez and Sytsma, 2006; Goldblatt et al., 2008). Tigridieae is a monophyletic lineage defined by molecular and morphological synapomorphies (Reeves et al., 2001; Rodriguez and Sytsma, 2006; Goldblatt et al., 2008; Goldblatt and Manning, 2008, Chauveau et al., 2012). Tigridieae was subdivided into two subtribes: Cipurinae and Tigridiinae, based on cytogenetic, palynological and morphological characters (Goldblatt, 1982). Cipurinae was characterized by having a chromosome number x = 7, monosulcated pollen grains and style arms petaloid to cylindrical, bifurcated or simple, while the Tigridiinae subtribe was differentiated by the chromosome number x = 14, bisected pollen grains and style branches cylindrical, deeply bifurcated (or simple) (Goldblatt, 1982; Rudall and Wheeler, 1988). However, phylogenetic reconstruction of the subfamily Iridoideae (Chauveau et al., 2012) showed that both Cipurinae and Tigridiinae are not monophyletic and proposed the subdivision of Tigridieae into two clades (A and B). The clade A encompassed some genera previously included in Cipurinae (*Ainea* Ravenna, *Calydorea* Herb., *Catila* Ravenna, *Cipura* Aubl., *Cypella* Herb., *Herbertia* Sweet, *Nemastylis* Nutt, *Larentia* Klatt, *Kelissa* Ravenna and *Onira* Ravenna) and clade B, the remaining genera of the Cipurinae (*Cardenanthus* R.C. Foster, *Eleutherine* Herb., *Ennealophus* N.E. Br., *Gelasine* Herb., *Hesperoxiphion* Baker, *Phalocallis* Herb. and *Mastigostyla* I.M. Johnst.) and all genera of Tigridiinae (*Cobana* Ravenna, *Tigridia* Juss., *Alophia* Herb., *Sessilanthera* Molseed & Cruden).

In relation to genera belonging to clade A, the two most representative genera in number of species, *Cypella* and *Calydorea*, are non-monophyletic (Chauveau et al., 2012). In this clade, vegetative characters are very homogeneous and taxonomy is basically indicated by floral morphology. Some authors have been discussing the relevance of the characters used in the circumscription of the genera. Goldblatt and Manning (2008) considered that the characters traditionally used for the separation of *Cypella*, and the monospecific genera *Kelissa* and *Onira*, as the fusion of the stamens and the branches of the style, have not been efficient for the generic separation, supporting the synonymization of *Kelissa* and *Onira* in *Cypella,* elaborated by Roitman and Castilhos (2007). However, the inclusion of *Kelissa* and *Onira* in *Cypella* was not accepted by Ravenna (2009) and the discussion remains open. The variations observed in the organization of stamens and style in *Catila*, *Itysa* Ravenna, *Lethia* Ravenna and *Tamia* Ravenna were also insufficiently discriminated by Goldblatt and Manning (2008) for the maintenance of the genera, being included in *Calydorea* by these authors.

Relationships between *Cypella* and *Phalocallis* also have divergences in taxonomy studies. Roitman and Castillo (2007) and Goldblatt and Manning (2008) synonymized *Phalocallis* in *Cypella* based on floral morphology. However, among the species accepted by World Checklist of Iridaceae (Barker, 2018) *Cypella*, three species are morphological distinct of *Phalocallis* showing good distinctive characteristics. *Cypella boliviana* Huaylla is considered strictly related to *P. coelestis* (Lehm.) Ravenna and distinguished by small variations of floral traits (Huaylla and Wood, 2012). *Cypella geniculata* (Klatt) Ravenna and *Cypella oreophila* Speg. were included by Ravenna (2009) in *Phalocallis* based on the same distinctive floral traits as the type species of the genus. Recent phylogenetic relationships of *P. coelestis* (Chauveau et al., 2012) suggest that *Phalocallis* should be considered as a separate genus of *Cypella*. In addition, *P. coelestis* appears closely related to *Gelasine elongata* (Graham) Ravenna in the B clade of Tigridieae, which further supports this separation (Chauveau et al., 2012). Additionally, chromosomal analysis performed by Moraes et al. (2015) support the taxonomic separation of *Phalocallis* from *Cypella*. These evidences indicate the necessity of a detailed investigation in order to recognize and establish the precise circumscription of these genera.

In Tigridieae, phylogenetic analyses (Chauveau et al., 2012) showed that previously used characters for the separation of the two subtribes (Cipurinae and Tigridiinae) are not homologous and, therefore, a deeper investigation is necessary, since other non-obvious attributes such as the structural study of vegetative organs could help in the identification of

homologous characters for clades A and B. Leaf anatomy analyses performed by Rudall (1991) evidenced two types of leaf morphology, characterized as "plicate" and "foliate" leaves, and the genera of Tigridieae were classified according to this one. However, both foliar types occur in Cipurinae and Tigridiinae. Other anatomical features were also considered by Rudall (1991, 1995), as leaf margin aspect and presence of marginal sclerenchyma. Presence, position and constitution of this sclerenchyma in the leaves could represent promising character for the separation of groups in Tigridieae and, most probably, useful for the identification of genera of the tribe.

The goal of this study was: (1) to elucidate phylogenetic relationships in Tigridieae based on eight plastidial markers and broad sampling, (2) to perform a detailed analysis of transverse leaf anatomy of Tigridieae, (3) to evaluate the evolution of anatomical characters with an emphasis on those that are more congruent with phylogenetic relationships and (4) address whether anatomical characters can be useful for the identification of diagnostic characters for Cipurinae and Tigridiinae.

MATERIAL AND METHODS

Taxonomic sampling for phylogenetic analysis—Taxa sampled, sources of plant material and voucher information are listed in Appendix S1. A total of 76 Tigridieae accessions, representing 16 genera and 60 species, were sampled from Argentina, Brazil, Bolivia, Chile, Peru and Uruguay. Genera sampled from Tigridieae: *Calydorea* (5 species- Fig.1A), *Catila* (1 sp.), *Cipura* (11 spp. Fig.1B-1C), *Cypella* (21 spp. Fig.1D-1F), *Kelissa* (1 sp. Fig.1G), *Onira* (1 sp. Fig.1H), *Herbertia* (6 spp. Fig.1I), *Larentia* (1 sp.), *Lethia* (1 sp.) *Alophia* (2 spp.), *Eleutherine* (2 spp.), *Gelasine* (4 spp. Fig.1J), *Hesperoxiphion* (1 sp.) and *Phalocallis* (1 sp. Fig.1K). *Trimezia* Salisb. ex Herb. (1 sp. Fig.1L) and *Neomarica* Sprague (1 sp.) from Trimezieae were used as outgroup.

DNA isolation, amplification and sequencing–Total DNA was extracted from 15–20 mg of silica dried leaf material using a modified CTAB protocol with volume adjusted to 2 mL tubes (Doyle and Doyle 1990). A combination of eight coding and non-coding DNA regions Iridaceae was selected: three coding cpDNA genes *(matK, rbcL* and *rps4*), three cpDNA intergenic spacers (*psbA-trnH, rps4-trnS* and *trnQ-rps16*), two cpDNA introns (*matK-5'trnK* and *rps1*6) (Rymer et al. 2010; Schnitzler et al. 2011; Chauveau et al. 2011, 2012). Primers used to amplify each DNA region are given in Appendix S2. PCR amplifications (Appendix S3) purification and sequencing follow the methodology described in chapter II (pag. 38).

Alignment and phylogenetic analyses–DNA sequences were aligned firstly with MAAFT 7 (Katoh and Standley 2013) and manually with MEGA6 (Tamura et al. 2013) (Appendix S4). Gaps and phylogenetic analysis of maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) are performed as described in chapter II (pag. 38). Analyses of BI the different data sets were performed for 6×10^6 to 20×10^6 generations and convergence was described in in chapter II.

Phylogenetic trees resulting from MP (strict consensus tree), ML (best-scoring tree) and BI (majority-rule tree) analyses were rooted using *Neomarica candida* (Hassl.) Sprague and *Trimezia spathata* (Klatt) Baker and PBS, LBS and PP were reported on the ML best-scoring tree to summarize the results of all analyses at once. A given node was kept in the final representation of the ML tree only if one of the bootstrap values (PBS and LBS) reached at least 60% or if the PP was ≥0.95 and in the absence of topological conflict among MP, ML and BI trees.

Taxonomic sampling from structural analysis —Taxa sampled, sources of plant material and voucher information are listed in Appendix S1. A total of 81 Tigridieae accessions, representing 16 genera and 55 species, were sampled from Brazil, Bolivia, Chile, Peru and Uruguay. Genera sampled from Tigridieae: *Calydorea* (5 spp.), *Catila* (1 sp.), *Cipura* (10 spp.), *Cypella* (15 spp.), *Herbertia* (5 spp.), *Kelissa* (1 sp.), *Lethia* (1 sp.), *Onira* (1 sp.), *Alophia* (2 spp.), *Eleutherine* (2 spp.), *Gelasine* (4 spp.), *Hesperoxiphion* (1 sp.) and *Phalocallis* (1 sp.). *Trimezia* (2 spp.) and *Neomarica* (1 sp.) from Trimezieae were selected for comparisons. Fresh leaves were collected in plants from field specimen and from bulbs collected in the wild and grown in the living collection of the Universidade Federal do Rio Grande do Sul (Brazil). The central portion of the third mature leaf blade was cut into small segments (approximately 1 cm long) and fixed at room temperature with 4% formaldehyde and 1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2 (McDowell and Trump, 1976).

Processing for inclusion and light microscopy— Samples were washed three times in 0.1 M sodium phosphate buffer for 30 min at room temperature, dehydrated in a graded series of ethanol solutions for 30 min. In the next step the material was washed in chloroform: alcohol (1: 3; 1: 1; 3: 1; chloroform 12 hrs; 3: 1; 1: 1; 1: 3) to improve resin penetration. After this, the material was embedded initially in a 1:1 mixture of 2-hydroxyethyl methacrylate resin Technovit 7100 (Heraeus Kulzer, São Paulo, Brazil) and absolute ethanol (pre-embedding step) for 48 hours, and finally embedded in 2-hydroxyethyl methacrylate resin, kept overnight at

room temperature and agitated gently with a rotating mixer (Gerrit and Smid, 1983).Transverse and longitudinal semi-thin sections (5 µm) obtained with a Leica RM 2245 microtome were stained for structural observations with 0.05 % Toluidine Blue O, in benzoate buffer at pH 4.4 (O'Brien and McCully, 1981). Observations were carried out using a bright field microscope Leica DMR-HC equipped with a digital camera Leica DFC 500.

Histochemistry— Free-hand and semi-thin sections obtained with microtome were submitted to different histochemical tests to detect the different metabolic groups: Phloroglucinol-HCl (Adler et al., 1948; Geiger and Fuggerer, 1979) solution to detect lignin (Johansen, 1940), periodic acid-Schiff (PAS reaction) for total polysaccharides (O'Brien and McCully, 1981), Ruthenium red for pectins (Johansen, 1940; Colombo and Rascio, 1977), Sudan Black B and Sudan III for lipids (Lison, 1960; Pearse, 1980), Coomassie Blue for protein (Fisher, 1968). Appropriate blank were performed and all images were captured using the bright field microscope.

Structural data and analysis– Analysis of leaf anatomy have a total of 33 potential informative anatomical characters (Table 1). Characters analysed in this study include a matrix with morphological and anatomical characterization based in the principal structures as follow: epidermis, vascular bundles, sclerenchyma and mesophyll. Character related to the distribution of subepidermal sclerenchyma was coded, especially according of the distribution and the position in the leaf outline. These characters have been cited as variable and potentially informative in Rudall (1994; 1995). The outline of leaves varies from flat, plicate, foliate (one side or both sides) and terete. We considered "foliate in one side" all species that had foliose extension in one side of the leaf surface, abaxial or adaxial, and "foliate in both sides" all species that had foliose extension in both sides of the leaf surface, abaxial and adaxial (Arber, 1921, Rudall, 1990, Rudall, 1991). Foliose extension definition was adopted according to Goldblatt et al. (2015): "foliose extensions or flanges along the keels, identifiable as such by their bifacial nature with a single row of vascular bundles". Trichomes were classified in papillae and non-branched unicellular hairs, according to Uphof (1962). In addition we estimated the ratio between the total diameter in leaf outline and the portion with subepidermal sclerenchyma, this proportion was representing in a scale between zero and one, at where zero is the absence (Table 1).The biogeographic distribution of the analysed species was performed using the region, province, dominions and sub region established by Morrone (2014) and a shapefile for biogeographical regionalization established by Löwenberg-Neto (2014) in *ArcGis*.

Ancestral state optimizations – For ancestral character optimization the character states were compiled from all species analysed. These data and the character states are summarized in Table 1. The characters were optimized on a ML best-scoring tree. The tree was obtained from phylogenetic reconstruction cited previously and the species that were not analysed anatomically were removed from the phylogenetic tree in Mesquite. Analyses of ancestral state were performed using the software Mesquite 3.40 (Maddison and Maddison, 2018). Characters were optimized on the tree using maximum parsimony (MP) and maximum likelihood (ML) methods. With MP, character states were treated as unordered, allowing any transition among states. ML optimization was conducted using the MK1 model of evolution (Pagel, 1999), which gives equal probability for changes among all character states.

RESULTS

Phylogenetic analysis— The topology of the phylogenetic trees inferred from each marker did not present incongruities, allowing combination of the datasets. The trees obtained from the analyses of combined region, including indels, resolved more clades and with more support values than trees resulting from the analyses of the each marker separately or without coded indels. Final trees were obtained through the set of cpDNA, including indels. Results of MP analysis of the eight markers including total length of all regions and indels, as well consistency and retention indices, are shown in Appendix S5. There was little homoplasy in most datasets (CI > 0.80; RI > 0.85), except for *matK-5'trnK* (CI = 0.756), *trnH-psbA* (CI = 0.671) and *rbcL* (CI = 0.716).

The plastid combined data matrix had a total length of 6871 bp, with 1243 variable characters (762 parsimony informative characters). The coded indels increased the number of variable characters (133 indels), and potentially parsimony informative characters, with 79 informative characters. The three most variable plastid regions were *trn*Q-*rps*16 (26.7%), *matK-5'trnK* (20.8%) and *trnH-psbA* (20.5%) (Appendix S5). ML searches produced a best-scoring ML tree with - ln L = -23.082 (Appendix S6). The MP, ML, and BI analyses based on combined plastid datasets generated almost identical phylogenetic trees with several resolved nodes (Appendix S7 for MP and Appendix S8 for BI). The nodes with BS values lower than 50% in ML and MP and lower than 0.95 in BI were collapsed. Maximum-likelihood best-scoring topologies are presented in Fig. 2, with bootstrap values and posterior probability above and below branches, respectively. The phylogenetic tree reconstructed from the datasets evidenced an early diverging clade of *Trimezia spathata* and *Neomarica candida* (outgroup),

fully supported in all analysis (PBS = 100/LBS = 100/PP = 1.00). Phylogenetic trees showed that the two clades (A and B) were fully supported in Tigridieae (100/ 100/1.00) as monophyletic.

Clade A is composed of genera exclusively from the subtribe Cipurinae. *Cipura* and *Larentia* constitute a sister group for the rest of the highly supported clade. At the base of Clade A, *Cipura* was recovered as monophyletic in all analyses (100/100/1.00). In the first maximum-likelihood best-scoring topology (Fig. 2), two clades encompassing *Cipura* species were observed. The first one is moderately supported in MP and BI and strongly supported in BI (79.7/75/1.00), grouping the species *Cipura formosa* Ravenna, *C. paradisiaca* Ravenna and *C. xanthomelas* Maxim. ex Klatt. *Cipura xanthomelas* is clearly not monophyletic, with a specimen in a group strongly supported with *C*. cf. *formosa* and *C*. aff. *xanthomelas* (CAM909) (95.7/95.0/1.00), and in a second group weakly supported with the taxon *C*. aff. *xanthomelas* (CA906B).

The two major genera within the clade A of Tigridieae, *Calydorea* and *Cypella*, are not monophyletic. *Calydorea* is divided into three unrelated strongly supported clades. *Calydorea alba* Roitman & A. Castillo and the monotypic genus *Catila* are in the base of the clade formed by *Cypella*, *Herbertia*, *Kelissa* and *Onira*. A strongly supported cluster is formed by the *Calydorea* species: *C. basaltica* Ravenna, *C. campestris* (Klatt) Baker and *C. crocoides* Ravenna (100/100/1.00). However, none of the groups is related to the type species of the genus (*C. xiphioides* (Poepp.) Espinosa), which is at the base of the clade formed by *Cypella hauthalii* (Kuntze) R.C. Foster, *Kelissa, Onira* and *Herbertia* (99.8/100/1.00). *Herbertia* was recovered as a moderately supported genus (85.8/89.2/1.00), with *H. pulchella* Sweet sister to the rest of the genus (59.3/64.5/1.00).

In *Cypella, C. hauthalii* subsp. *hauthalii, C. hauthalii* subsp. *opalina* Ravenna and *C. hauthalii* subsp. *minuticristata* Chauveau & L.Eggers stand out from the rest of the genus forming a fully supported group with *Onira unguiculata* (Baker) Ravenna and *Kelissa brasiliensis* (Baker) Ravenna (100/100/1.00).The other species of *Cypella*, remain in the same group with moderate support (96.9/93.0/1.00). *Cypella discolor* Ravenna and *C. ravenniana* Deble & F.S. Alves are in the base of the clade, followed by *C. pusilla* (Link & Otto) Benth. & Hook. f. ex B.D. Jacks. , *C. charruana* Deble & F.S. Alves, *C. aquatilis* Ravenna, *C. laxa* Ravenna and *C. laeta* Ravenna. A large group fully supported with *Cypella herbertii* (Lind.) Herb. was formed, including the subspecies analysed, and *C. pabstiana* Ravenna, *C. guttata* Deble & F.S. Alves and *C. osteniana* Beauverd at the base of the clade (100/100/1.00). The

remaining taxa (*C. fucata* Ravenna, *C.* aff. *fucata*, *C. altouruguaya* Chauveau & L.Eggers, *C. amplimaculata* Chauveau & L.Eggers and *C. rivularis* Chauveau & L.Eggers) were recovered with strong support, with *C. magnicristata* Deble at the base of the clade (99.7/100/1.00).

In clade B, *Alophia* and *Gelasine* do not correspond to monophyletic genera. For this clade, the species are divided into two groups. The first one included *Alophia* and *Lethia* fully supported (100/100/1.00). *Alophia* was recovered as not monophyletic because *Alophia* aff. *drummondii* (Graham) R.C. Foster belong to the same clade with *Lethia umbellata* (Klatt) Ravenna, strongly supported (89.1/99.0/1.00). The second clade includes *Gelasine*, *Eleutherine* and *Phalocallis* with weak or without support (--/70.6/--). *Gelasine* was recovered as not monophyletic once *Gelasine elongata* belongs to the clade formed by *Phalocallis coelestis* (Lehm.) Ravenna with strong support for ML and PP (--/100.0/1.00). The group formed by other species of *Gelasine*, as *G. coerulea* (Vell.) Ravenna, *G. gigantea* Ravenna and *Gelasine* sp. was recovered as monophyletic with full support (100/100/1.00). Finally, *Eleutherine* was recovered as monophyletic with full support (100/100/1.00).

Structural observations of leaf anatomy— Leaf anatomy micrographs are shown in Figure 3 to Figure 7 and their order are based on structural differences. The codified results are available in Appendix S9.

Epidermis— In the species analysed, the unistratified epidermis consist mainly of isodiametric cells in transverse section, characterized by a thickened wall, predominantly the outer and inner periclinal wall. Cell thickness often varied in the same leaf, mainly at marginal, keels and above sclerenchyma regions. Differences found in the cell wall in the epidermis were above sclerenchyma region: cells thickened only in horizontal walls (periclinal) (Fig. 3A) for all species of clade A, *Gelasine coerulea* (Vell.) Ravenna and *Alophia* (clade B); cells thickened in horizontal and vertical walls (anticlinal + periclinal) but with non-circular cell lumen that occur only in *Gelasine gigantea* Ravenna (Fig. 3B), and cells thickened equally in horizontal and vertical walls (anticlinal + periclinal) but with circular cell lumen that occur in *Hesperoxiphion*, *Lethia*, *Eleutherine*, *Phalocallis* and *Gelasine elongata* and *Gelasine* sp. (Fig. 3C-3D). In addition, the epidermis may exhibit groups of bulliform like cells with pectin content, which may be present in "flat" extension or at shallow depressions. Bulliform like cells with pectin content occur in regular small groups at all genera belongs to the Clade A. In the Clade B, these cells were not observed in *Alophia medusa* (Baker) Goldblatt and *A.* cf. *medusa*, *Phalocallis coelestis* and *Eleutherine.*

Stomata distribution can be restricted to shallow depressions, and this type is common in *Calydorea* and *Cipura* (Fig. 4 A-B and D). Stomata have guard cells with thickened inner and outer walls, and the subsidiary cells, or accessory cells may be in the form of papillae, as in *Calydorea basaltica* (Fig. 4E). In the epidermis we also observed papillae and non-branched unicellular hairs in some species. The papillae occur only at shallow depressions in some species of *Calydorea*, as *C. basaltica* (Fig. 4E) and *C. crocoides*, and *Cipura* species, as *C. paradisiaca*, *Cipura* sp. 1, *Cipura* sp. 2 and *C. xanthomelas*. Non-branched unicellular hairs are present only above sclerenchyma in *Calydorea crocoides*, *C. xiphioides*, *Cipura* sp. 2 and *C. xanthomelas.* (Figs. 4F-G).

Mesophyll— The mesophyll is of the homogeneous type in most species, with cells more or less regular in size and shape, and some with phenolic content. In some species, as *Cipura xanthomelas* and *Calydorea alba*, cells of the mesophyll are radially elongated, palisade-like, but not as palisade tissue characteristic layer.

Vascular bundles— The vascular system is composed of collateral vascular bundles in different calibers that are present along the entire length of the leaf surface, where the xylem-phloem axis is perpendicular to the cross-section axis of the leaf blade in relation of the arrangement of the vascular bundles. The marginal vascular bundle may be twisted in some species and the xylem-phloem axis was to the cross-sectional axis of the leaf blade, with a phloem facing the leaf margin (Figs. 5E-F). The first order vascular bundles were considered the ones with the biggest diameter. The number of first order vascular bundles varied between one to 12 bundles per leaf blade in the analysed species. The number of second order bundles between first order bundles are variable, usually from two to seven.

In the first order vascular bundles, both outer and inner bundle sheaths are often continuous. The discontinuity of this character was observed at not marginal vascular bundles, in *Phalocallis coelestis* (discontinuity in xylem pole) (Fig. 5C) and *Gelasine coerulea* (discontinuity in phloem pole). Normally, outer sheath is a layer of thin parenchymatous cells, and often indistinguishable from surrounding mesophyll. The inner bundle sheath or pericycle is usually sclerenchymatous and often discontinuous, and can vary as continuous, discontinuous at xylem or xylem pole, or discontinuous at xylem and phloem pole.

The marginal vascular bundles of first order are exclusive of some genera and present lignified sclerenchyma, which encircle partially the xylem or phloem pole, and never encircle completely the vascular bundles. At first order marginal vascular bundles, phloem can be

completely encircled by lignified sclerenchyma fibers in all species of clade B, except by *Alophia* aff. *drummondii*, *Hesperoxiphion peruvianum* (Baker) Baker (Fig. 5G), *Lethia umbellata* and *Gelasine* sp. 1. At first order marginaled vascular bundles, only *Phalocallis coelestis* and *Gelasine elongata* has lignified sclerenchyma in the xylem pole (Fig. 5E).

At first order not-marginal vascular bundles, phloem can be completely encircled by lignified sclerenchyma fibers, *e.g.* in species of Clade B as *Alophia medusa, Phalocallis coelestis, Eleutherine angusta* Ravenna, *E. bulbosa* (Mill.) Urb., *Gelasine coerulea, G. elongata, G. gigantea* and Clade A *Cipura* sp. 2, partially encircled, *e.g. Cipura* aff. *campanulata* Ravenna, *Alophia* aff. *drummondii*, *Hesperoxiphion peruvianum* and *Gelasine* sp. 1. In addition, phloem can be not encircled by lignified sclerenchyma fibers as the majority of Clade A species, except *Lethia umbellata* (of clade B). In some genera as *Herbertia*, *Kelissa*, *Onira* and the subspecies of *Cypella hauthalii* sclerenchyma fibers in vascular bundles are not present. At first order not-marginal vascular bundles, *Phalocallis coelestis, Eleutherine angusta, Gelasine elongata, G. gigantea*, *Gelasine* sp. 1 and *Cipura* species (except *C. formosa* and *C. paradisiaca*) have many layers of lignified sclerenchyma in the xylem pole. But only in *Phalocallis coelestis* this sclerenchyma extended until the epidermis (Fig. 5C).

Sclerenchyma— Three types of sclerenchyma were identified and characterized in analysed species. The first type is a subepidermal sclerenchyma, not associated with a vascular bundle and pectin rich was frequently identified present at margins, at ends of ridges or foliose extensions, or at all extension of leaf surface in species of Clade A (Fig. 6A-N). This sclerenchyma is frequently present at margins and ends of ridges or foliose extensions, in margins and short intercalated areas and in margins with long intercalated areas.

Second type, a subepidermal lignified sclerenchyma constituted by few cells (more or less three cells), not associated with vascular bundle and lignin rich was identified only in *Gelasine coerulea* (Fig. 7F-G). Finally, the third type is a lignified sclerenchyma, associated with vascular bundles and present and/or not at phloem and xylem pole (Fig. 7K and 7P).

Character states reconstruction— Ten variable leaf anatomical characters were selected to reconstruct their evolutionary history. The character evolution was optimized into the maximum likelihood (ML) best-scoring tree, obtained from cpDNA analysis, and includes one individual for each species. Characters were optimized on the tree using the method of maximum parsimony (MP) and maximum likelihood (ML).

Character 1– *Leaf outline and* Character 2– *Number of foliose extension in foliate leaves*

Parsimony reconstruction of leaf outline was represented in Fig. 8 (Mirror Tree of MP and ML reconstruction is shown in Appendix S10 and S11). The plicate leaf outline seems to be the ancestral state for Tigridieae. The genera belonging to the Clade B, as *Eleutherine*, *Gelasine*, *Phalocallis*, *Lethia*, *Hesperoxiphion* and *Alophia* aff. *drummondii* have plicate leaves (Fig. 8i-j), except *Alophia medusa*, that has terete leaves (Fig. 8k).

In the Clade A, the leaf outline is more variable, though the plicate leaf was recovered as ancestral state for this clade. In *Cipura*, some species have plicate leaves (Fig. 8a-b), one species was foliate in one side leaf outline (Fig. 8c) and five have foliate both sides leaf outline. This type of leaf outline possibly evolved different times in this genus.

In *Cypella*, and the monotypic genera *Catila*, *Kelissa* and *Onira*, all species are foliate in the both sides of the leaf surface (Fig. 8f), corresponding to the ancestral state for this genera (Fig. 8h). However, in *Herbertia* it was observed convergent for foliate leaves in one side of the leaf surface in *H. lahue* (Molina) Goldblatt, *H. pulchella*, and *H. quareimana* Ravenna (Fig. 8g). In *Calydorea*, all species are foliate in both sides; the only exceptions are *C. crocoides* and *C. basaltica* (Fig. 8e).

Character 3– *Subepidermal sclerenchyma and* Character 4– *Subepidermal sclerenchyma distribution*

Parsimony and maximum likelihood reconstruction of subepidermal sclerenchyma presence and distribution were represented in Fig. 9 and Mirror Tree in Appendix S12 (MP) and S13 (ML). The reconstruction of the states showed that the evolution of the subepidermal sclerenchyma occurred at the base of clade A, and is present in all species analysed.

The presence of pectin rich subepidermal sclerenchyma in margins and long interleaved areas is the ancestral state was recovered only for the *Cipura*, however three reversions were observed in three species (*Cipura paludosa*, *C. campanulata* and *Cipura* sp.1) (Fig. 9). In genera *Cypella*, *Herbertia*, *Kelissa* and *Onira* this sclerenchyma is present only at margins and end of ridges or foliose extensions.

Additionally, we estimated the ratio of subepidermal sclerenchyma and total perimeter of leaf outline and we observed that a variation occurred in this ratio, and besides that, this variation follows the phylogeny (represented in bars (Fig. 9) (Appendix S9 – character 28). This ratio can vary from 0.98 in *Cipura formosa* to 0.046 in *Herbertia pulchella*.

In an attempt to relate the presence and ratio of sclerenchyma with the area of occurrence of each analysed species (Appendix S14), we represented this area in a MP reconstruction tree of

sclerenchyma and distribution (Fig. 9). We observed that species with distribution of subepidermal sclerenchyma in margins and long interleaved areas, where the ratio varies between 0.7 until 0.98.

Character 5 – *Bulliform like cells with pectin content and* Character 6 – *Arrangement of bulliform like cells with pectin content*

Reconstruction of the distribution and arrangement of bulliform like cells with pectin content are available in Appendix S15 (MP) and S16 (ML). Bulliform like cells with pectin content are present in all species of Clade A and in Clade B only in *Lethia, Hesperoxiphion, Alophia* aff. *drummondii* and *Gelasine* (except *G. elongata*). The absence of bulliform like cells with pectin content is observed in *Eleutherine*, *Phalocallis*, *Gelasine elongata* and *Alophia medusa*.

Character 7 – *Vascular bundles first order (marginal bundles) at leaf margins* and Character 8 – *Vascular bundles first order (marginal bundles) - Extension of lignified sclerenchyma at phloem pole*

Reconstruction of this character is available in Appendix S17 (MP) and S18 (ML). The presence of marginal vascular bundles of first order was recovered for ancestral state of Clade B, and the absence in *Lethia* and *Alophia* is a reversion. In addition, all species with marginal vascular bundles at first order have large layers of lignified sclerenchyma and extending until margin only in *Hesperoxiphion* this is not observed.

Character 9 – *Vascular bundles first order (not marginal bundles) – Extension of lignified sclerenchyma at phloem pole* and Character 10 – *Vascular bundles first order (not marginal bundles) – Extension of lignified sclerenchyma at xylem pole.*

Two characters do not present clear phylogenetic pattern: extension of lignified sclerenchyma at phloem and xylem pole, in not marginal at vascular bundles of first order (Appendix S19 and 20), and was not possible to estimate the evolution of these characters.

DISCUSSION

In this study, we used leaf anatomy of Tigridieae in a phylogenetic context to investigate the evolution of the characters. Information on evolutionary patterns was used to delimit diagnostic characters and investigate the evolution of characters leaf outline and subepidermal sclerenchyma.

Phylogeny — The phylogenetic reconstruction supports Tigridieae as monophyletic, however the circumscription of the two subtribes Cipurinae and Tigridiinae as monophyletic groups is not supported and the tribe remains divided into two clades as proposed by Chauveau et al. (2012) (Clade A and B). The present phylogeny counted with the inclusion of additional markers in relation to those used by Chauveau et al. (2012). Furthermore, sampling for all genera was expanded, mainly for species of *Cypella*, *Herbertia*, *Cipura*, *Calydorea*, *Catila*, *Kelissa* and *Onira*. Among the analysed genera, four were not monophyletic, *Gelasine*, *Cypella* and *Calydorea*, which had already been proposed by Chauveau et al. (2012), in addition to *Alophia*, as evidenced in the present study.

Indeed, the generic delimitations within Tigridieae have been widely discussed, but there are still doubts regarding the circumscription of some genera. It was evidenced for species richness genera such *Tigridia*, *Cypella* and *Calydorea* (Rodríguez and Sytsma, 2006, Goldblatt and Manning, 2008, Goldblatt et al., 2008). The present phylogeny shows that *Calydorea* is clearly not monophyletic. The genus is divided into three clusters, being *C. alba* sister to the other clades. *Catila amabilis*, considered in this study as a monospecific genus, was synonymized in *Calydorea* (Goldblatt and Manning, 2008), and even if *Catila* were considered here in the broad sense, the species type *C. xyphioides* is not totally related to the other species of the genus. *Cypella* species were divided into two clusters; the first one included all species of the genus except *C. hauthalii*, which remained in the same cluster with the monospecific genera *Kelissa* and *Onira*.

In the clade B of Tigridieae, *Gelasine* is clearly not monophyletic, whereas the species type *G. elongata* is grouped with *Phalocallis*, the other species of the genus remain in the same clade. Previous phylogenies, found a similar pattern, and even sampling only two species of *Gelasine* (*G. elongata* and *G. coerulea*), the authors suggested that the circumscription of the genus was misunderstood and should be reviewed. *Phalocallis coelestis* seems closely related to *Gelasine elongata*, and both species are related to *Eleutherine* with a high support.

The generic boundaries within Tigridieae have been discussed for a long time. The similarities between the morphological characters in the genera and the difficulty to define diagnostic characters hinder the interpretation of the phylogenetic results. The lack of clear diagnostic characters caused, for example, *Kelissa* and *Onira* to be synonymized in *Cypella* (Roitman and Castilhos, 2007, Goldblatt and Manning, 2008), *Catila*, *Itysa*, *Lethia* and *Tamia* synonymized in *Calydorea* by Goldblatt & Manning (2008). Other example is *Phalocallis*, that

was synonymized in *Cypella* based on floral morphology characters (Roitman and Castillo 2007; Goldblatt and Manning, 2008). The absence of clear morphological differences between genera of Tigridieae suggests that additional characters must be used for taxonomic studies.

Leaf anatomy — Leaf anatomy data presented in these study confirmed previous studies (Rudall 1991, 1995) and provide important diagnostic characters for taxonomy of the Tigridieae. Studies in Iridaceae and other families, as Amaryllidaceae and Zygophyllaceae (Rudall 1986; Rudall and Goldblatt, 1991; Mashayekhi and Columbus, 2014; Lauterbach et al., 2016), demonstrated the occurrence of several leaf outline modifications and these proved to be significant to taxonomy and phylogeny. Our study evidenced that leaf outline is an important diagnostic character, for example, to distinguish *Phalocallis* (with plicate leaves), and *Cypella* (with foliate leaves). Research with *Allium* L. (Amaryllidaceae: Asparagales) evidenced similar results, and proposed that the evolution of leaf outline, specially terete leaves, as important to taxonomy and evolution of subgenus *Amerallium* Traub (Mashayekhi and Columbus, 2014)

Our anatomical and histochemical results report for the first time the occurrence of subepidermal sclerenchyma as an exclusive character from clade A of Tigridieae. Rudall (1995) suggested that although the subepidermal sclerenchyma occurs throughout the entire Iridaceae, including representatives of the subfamily Iridoideae (*Orthrosanthus* Sweet), this character is absent for the Mariceae group (currently Trimezieae), suggesting that this character may be an apomorphy for the group Cipurinae with foliated leaves (Tigridieae). The presence of subepidermal sclerenchyma can be a diagnostic character for clade A, thus, could sustain a new circumscription of Cipurinae and Tigridiinae, two subtribes proposed by Goldblatt (1982). Cipurinae would include all genera present in Clade A and with subepidermal sclerenchyma: *Calydorea*, *Catila*, *Cipura*, *Cypella*, *Herbertia*, *Kelissa*, *Onira,* and consequently Tigridiinae would include all genera present in Clade B and without subepidermal sclerenchyma *Eleutherine*, *Gelasine*, *Hesperoxiphion*, *Phalocallis* and *Alophia*.

Evolution of characters—The evolutionary analysis of characters in Tigridieae shows that the subepidermal sclerenchyma evolved in the common ancestral for clade A. The evolution of sclerenchyma observed in clade A can be related to the distribution areas of the species an adaptation to dry regions. We observed through reconstruction of the distribution area of analysed species that most species that have sclerenchyma distributed throughout the leaf extension, represented by 70% to 98% of leaf outline, occur in the Brazilian cerrado. The

province of the cerrado, as defined by Morrone (2014), has savanna physiognomies with soils that are poor in nutrients with high aluminum content (Ratter et al., 1997; Fiaschi and Pirani, 2009). The region is peculiar because there are rainy periods, but also a very strong dry season, mainly between April and September (Ratter et al., 1997). Selection pressures can initially favor the development of "xeromorphic" characteristics in leaves and these could then be incorporated into other adaptive functions (Haworth et al., 2008). Climatic conditions could select larger leaf growth strategies during the rainy season (Keeley et al., 2011). On the other hand, soil oligotrophy selects foliar anatomical characteristics associated with leaf persistence (Rossatto et al., 2009). These conditions of cerrado can have driven the development of large subepidermal sclerenchyma in *Cipura* as an ecological alternative of the species to environmental stress. Leaf anatomical characters are reported to others genera as ecological adaptations, in *Oxalis* L. (Oxalidaceae), for example, are highly variable and subject to environmentally-induced variation (Joostle et al., 2016). In Bromeliaceae, leaf anatomical characters are related as adaptation and expansion into arid areas (Santos-Silva et. al., 2016).

The reconstruction of leaf outline evolution in Tigridieae showed that plicate leaf evolved in the common ancestor for de tribe, and convergent evolution for foliate leaves was observed in clade A. According to Rudall (1995), early developmental stages from plicate and foliate leaves are similar, only foliate type has “more extensive outgrowth” (or “foliose extension” - as defined in the present study) in the keels. Different leaf outline, plicate and foliate, presumably would be conditions that would increase the total area for photosynthesis and, also, foliate type would be related to reduce habit and reduce leaf size, adaptations to adverse conditions (Rudall 1995).

The development of leaf area is an important factor to be taken into account as adaptation to climatic conditions, mainly dry areas (Bolhàr-Nordenkampf and Draxler 1993; Passioura, 1996; Lauterbach et al., 2016). Lauterbach et al., (2016) postulated that the anatomical diversity of the raised leaves in *Tetraena* Maxim. and *Roepera* A. Juss. (Zygophyllaceae) is the result of rapid local adaptation to diverse microhabitat conditions, not regional climatic differences. We observed that there are variations in the leaf outline of *Cipura xanthomelas*, which presents foliate leaves in both sides, and the taxa denominated *Cipura* aff. *xanthomelas* presents plicate leaves.

Conclusions — We used a phylogenetic framework to access the evolution of leaf anatomy in Tigridieae species. Anatomical characters, as leaf outline and subepidermal sclerenchyma, were useful in supporting phylogenetic relationships. In addition, we indicated the presence of subepidermal sclerenchyma as diagnostic characters to Cipurinae and Tigridiinae.

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Table 1. Morphological and anatomical characters evaluated for Tigridieae and Trimezieae species.

1. Leaf outline	(0) flat; (1) plicate; (2) foliate one side; (3) foliate both sides; (4) terete.
Epidermis	
2. Bulliform like cells with pectin content	(0) absent; (1) present in "flat" extension; (2) present at shallow depressions.
3. Arrangement of bulliform like cells with pectin content	(0) not applicable; (1) isolated; (2) regular small groups; (3) irregular small groups.
4. Papillae	(0) absent; (1) present scattered throughout the epidermis; (2) present at shallow depressions (only).
5. Non-branched unicellular hairs;	(0) absent; (1) present
6. Localization of epidermal cells with thickened walls	(0) absent; (1) above sclerenchyma; (2) distributed throughout the entire leaf surface.
7. Cell dimensions periclinal wall x anticlinal wall length:	(0) approximately equal; (1) periclinal wall shorter than anticlinal wall; (2) periclinal wall longer than anticlinal wall.
8. Epidermal cells above subepidermal sclerenchyma	(0) not applicable; (1) thickened only in horizontal walls (periclinal); (2) thickened equally in horizontal and vertical walls (anticlinal + periclinal) but with circular cell lumen; (3) thickened in horizontal and vertical walls; but with non-circular cell lumen;
9. Stomata distribution	(0) restricted to shallow depressions; (1) not restricted to shallow depressions.
Vascular bundles (general)	
10. Second order bundles between first order bundles (not in foliose extensions)	(0) not applicable; (1) usually one; (2) usually two; (3) usually three; (4) usually four; (5) five, or usually more than five;
11. Vascular bundles (1^{st} Ord) at leaf margins	(0) absent; (1) present; perpendicular to the main axis of the transversal section; (2) present; parallel to the main axis of the transversal section.
12. Vascular bundles organization in the foliose extensions	(0) not applicable; (1) present and unifacial;
13. Number of vascular bundles (2^{nd}) in the foliose extensions	(0) zero; (1) usually one; (2) usually two; (3) usually three; (4) usually four; (5) not applicable;
14. Number of foliose extensions at leaf margins	(0) zero; (1) usually one; (2) usually two; (3) usually three; (4) usually four; (5) usually five; (6) usually six; (7) not applicable;
15.Presence of pecti-rich cells at not marginal vascular bundles (1^{st} Ord)	(0) absent; (1) present.
16. Presence of pecti-rich cells at marginal vascular bundles (1^{st} Ord)	(0) absent; (1) present; (2) not applicable;
Vascular bundles first order (not marginal)	

17. Outer bundle sheath (=endoderm) continuity	(0) present and continuous; (1) present and discontinuous.
Vascular bundles first order (marginal bundles)	
18. Outer bundle sheath (=endoderm) continuity	(0) present and continuous; (1) present and discontinuous.
vascular bundles first order (not marginal) - inner vascular bundle (=pericycle)	
19. Pericycle continuity	(0) continuous; (1) discontinuous xylem pole; (2) discontinuous phloem pole; (3) discontinuous xylem and phloem pole.
20. Phloem pole: relative position of sclerenchyma with phloem	(0) not applicable; (1) phloem not encircle; (2) phloem partially encircle; (3) phloem completely encircle.
21. Extension of sclerenchyma at phloem pole	(0) not applicable; (1) thin layer ((2) layers); (2) large layer (+(2) layers); (3) extending as girders.
22. Extension of sclerenchyma at xylem pole	(0) not applicable; (1) thin layer ((2) layers); (2) large layer (+(2) layers); (3) extending as girders.
Vascular bundles first order (marginal bundles) - inner vascular bundle (=pericycle)	
23. Pericycle continuity	(0) continuous; (1) discontinuous xylem pole; (2) discontinuous phloem pole. (3) discontinuous xylem and phloem pole.
24. Phloem pole: relative position of sclerenchyma with phloem	(0) not applicable; (1) phloem not encircle; (2) phloem partially encircle; (3) phloem completely encircle.
25. Extension of sclerenchyma at phloem pole	(0) absent; (1) thin layer ((2) layers); (2) large layer(+(2) layers); (3) extending as girders.
26. Extension of sclerenchyma at xylem pole	(0) absent; (1) thin layer ((2) layers); (2) large layer(+(2) layers); (3) extending as girders.
Sclerenchyma	
27. Subepidermal sclerenchyma	(0) absent; (1) present.
Subepidermal sclerenchyma	
28. Ratio of subepidermal sclerenchyma perimeter x total leaf perimeter	(0-1)
29. Subepidermal sclerenchyma distribution	(0) not applicable; (1) only at margins and end of ridges or foliose extensions; (2) margins and short interleaved areas; (3) margins and long interleaved areas.
Subepidermal lignified sclerenchyma	
30. Subepidermal lignified sclerenchyma distribution	(0) absent; (1) present only at margins and end of ridges or foliose extensions.
Mesophyll	
31. Layers of mesophyll (endoderm) cells between epidermis and lignified sclerenchyma of the vascular bundle (^{1st} Ord) at margins	(0) not applicable; (1) usually zero; (2) usually one.

32. Layers of mesophyll (endoderm) cells between epidermis and lignified sclerenchyma at the xylem pole of the vascular bundle (^{1st} Ord) at leaf surface (not margins)	(0) not applicable; (1) usually zero; (2) usually one.
33. Layers of mesophyll cells between epidermis and lignified sclerenchyma at the phloem pole of the vascular bundle (^{1st} Ord) at leaf surface (not margins)	(0) not applicable; (1) usually zero; (2) usually one.

Figure 1 Flowers in apical view of species of Clade A. A- *Calydorea alba* (Pastori et al. 125), B- *Cipura paludosa* (Chauveau & Aguiar 906C), C- *Cipura xanthomelas* (Eggers et al. 924), D- *Cypella amplimaculata* (Eggers & Souza-Chies 664), E- *Cypella luteogibbosa* (Eggers *et al.* 791), F- *Cypella charruana* (Pastori et al. 203), G- *Kelissa brasiliensis* (Pastori et al. 145), H- *Onira unguiculata* (Pastori et al. 162), I- *Herbertia lahue* (Pastori et al. 132), Clade B: J- *Gelasine elongata* (Pastori et al. 202), K- *Phalocallis coelestis* (photo by Marcio Verdi) and the outgroup L- *Trimezia spathata* (Pastori & Chauveau 177). Information inside brackets corresponds to collector name and number, as listed in Table 1. Scale bar = 1 cm.

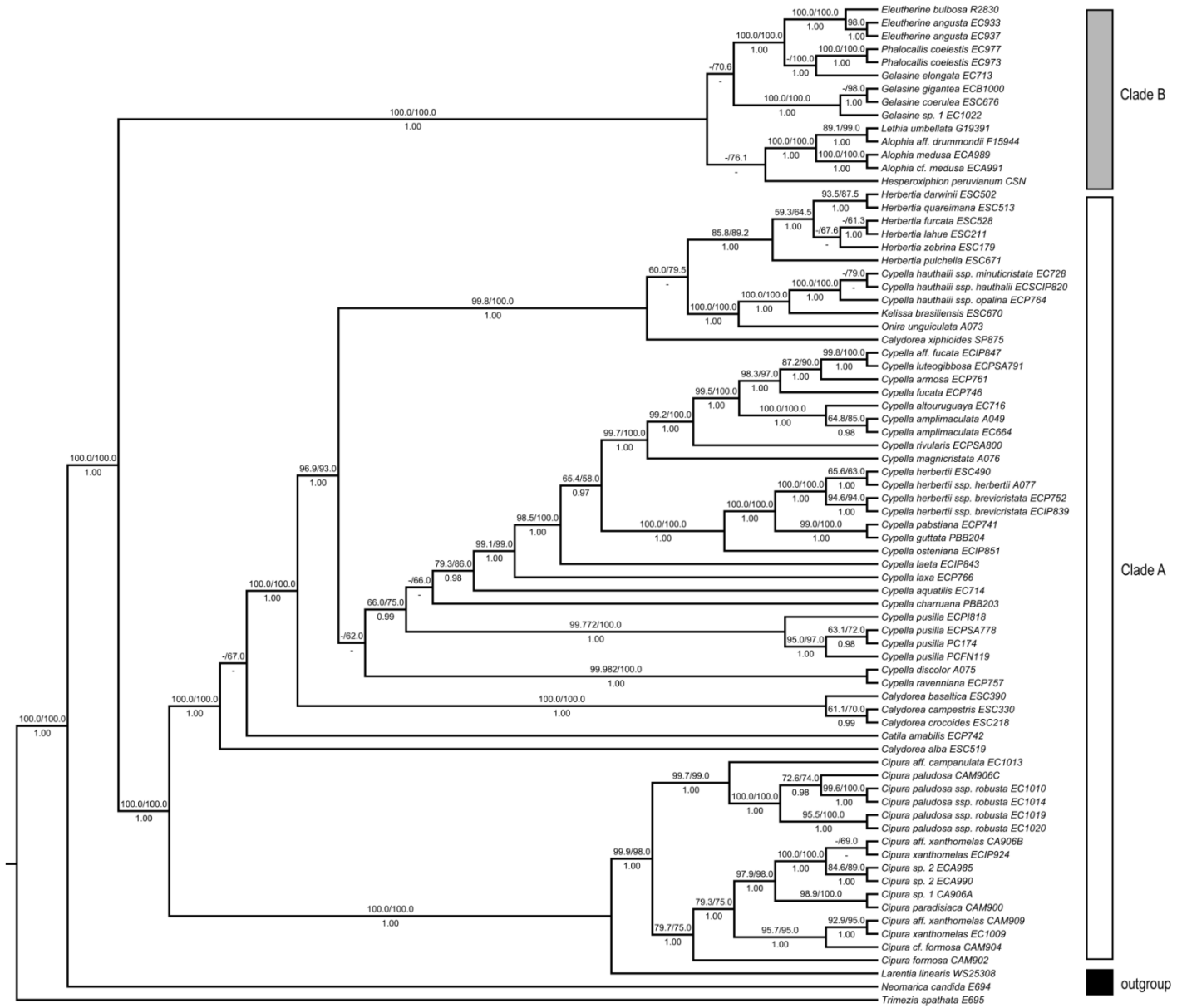
Eleutherine bulbosa R2830
Eleutherine angusta EC933
Eleutherine angusta EC937
Phalocallis coelestis EC977
Phalocallis coelestis EC973
Gelasine elongata EC713
Gelasine gigantea ECB1000
Gelasine coerulea ESC676
Gelasine sp. 1 EC1022
Lethia umbellata G19391
Alophia aff. drummondii F15944
Alophia medusa ECA989
Alophia cf. medusa ECA991
Hesperoxiphion peruvianum CSN
Herbertia darwinii ESC502
Herbertia quareimana ESC513
Herbertia furcata ESC528
Herbertia lahue ESC211
Herbertia zebrina ESC179
Herbertia pulchella ESC671
Cypella hauthalii ssp. minuticristata EC728
Cypella hauthalii ssp. hauthalii ECSCIP820
Cypella hauthalii ssp. opalina ECP764
Kelissa brasiliensis ESC670
Onira unguiculata A073
Calydorea xiphioides SP875
Cypella aff. fucata ECIP847
Cypella luteogibbosa ECPSA791
Cypella armosa ECP761
Cypella fucata ECP746
Cypella altouruguaya EC716
Cypella amplimaculata A049
Cypella amplimaculata EC664
Cypella rivularis ECPSA800
Cypella magnicristata A076
Cypella herbertii ESC490
Cypella herbertii ssp. herbertii A077
Cypella herbertii ssp. brevicristata ECP752
Cypella herbertii ssp. brevicristata ECIP839
Cypella pabstiana ECP741
Cypella guttata PBB204
Cypella osteniana ECIP851
Cypella laeta ECIP843
Cypella laxa ECP766
Cypella aquatilis EC714
Cypella charruana PBB203
Cypella pusilla ECPI818
Cypella pusilla ECPSA778
Cypella pusilla PC174
Cypella pusilla PCFN119
Cypella discolor A075
Cypella ravenniana ECP757
Calydorea basaltica ESC390
Calydorea campestris ESC330
Calydorea crocoides ESC218
Catila amabilis ECP742
Calydorea alba ESC519
Cipura aff. campanulata EC1013
Cipura paludosa CAM906C
Cipura paludosa ssp. robusta EC1010
Cipura paludosa ssp. robusta EC1014
Cipura paludosa ssp. robusta EC1019
Cipura paludosa ssp. robusta EC1020
Cipura aff. xanthomelas CA906B
Cipura xanthomelas ECIP924
Cipura sp. 2 ECA985
Cipura sp. 2 ECA990
Cipura sp. 1 CA906A
Cipura paradisiaca CAM900
Cipura aff. xanthomelas CAM909
Cipura xanthomelas EC1009
Cipura cf. formosa CAM904
Cipura formosa CAM902
Larentia linearis WS25308
Neomarica candida E694
Trimezia spathata E695
Clade B
Clade A
outgroup

Figure 2 ML best-scoring cladogram obtained from the analyses of the combined chloroplast data sets. Support values indicated above branches are parsimony bootstrap (PBS)/likelihood bootstrap (LBS), whereas Bayesian posterior probability (PP) is reported below branches. Bootstrap supports and posterior probabilities for a given node are provided only if one of the values reached the following thresholds: PBS ≥60% or LPB ≥60% or PP ≥0.95. A dash (-) indicates support value of less than 50% for PBS and LPB or less than 0.95 for PP.

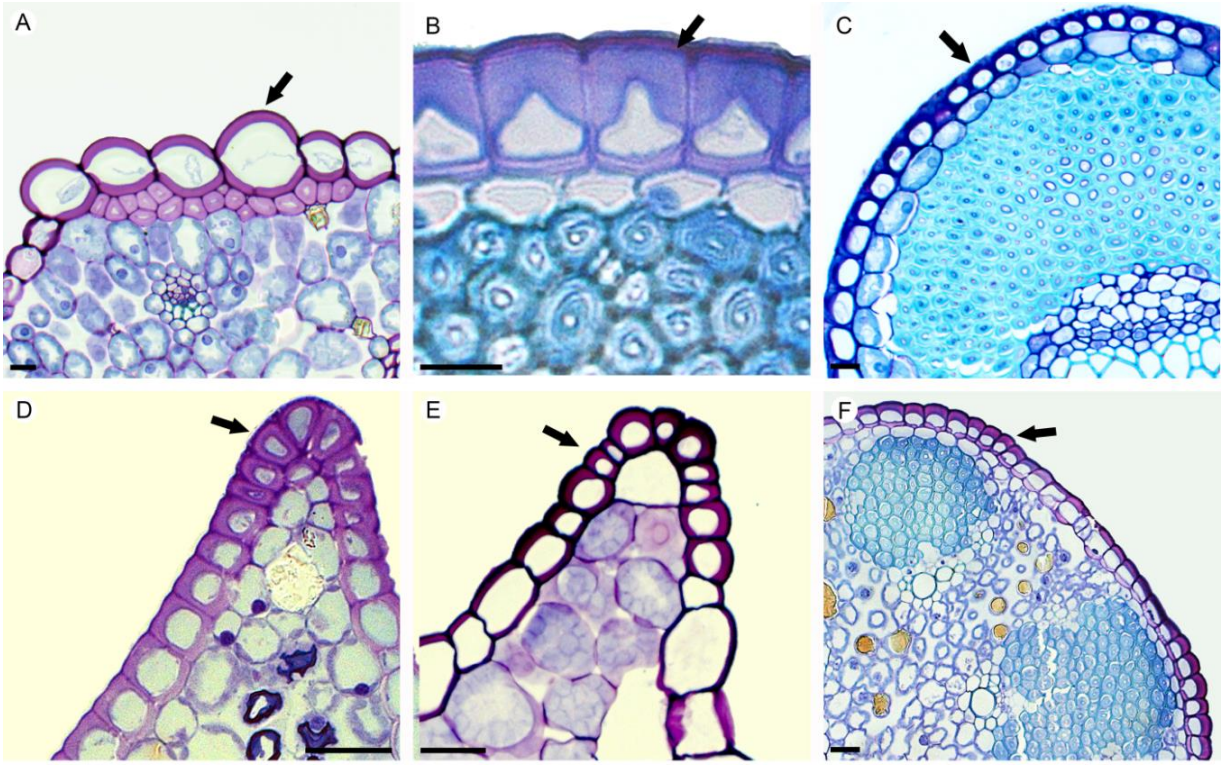

Figure 3 Transverse section with black arrow in epidermis showing the different types cell wall thickening; A- *Calydorea alba*, B- *Gelasine gigantea*, C- *Gelasine elongata*, D- *Gelasine* sp.(1026), E- *Lethia umbellata*, F- *Alophia* cf. *medusa*. Scale bar = 10 µm.

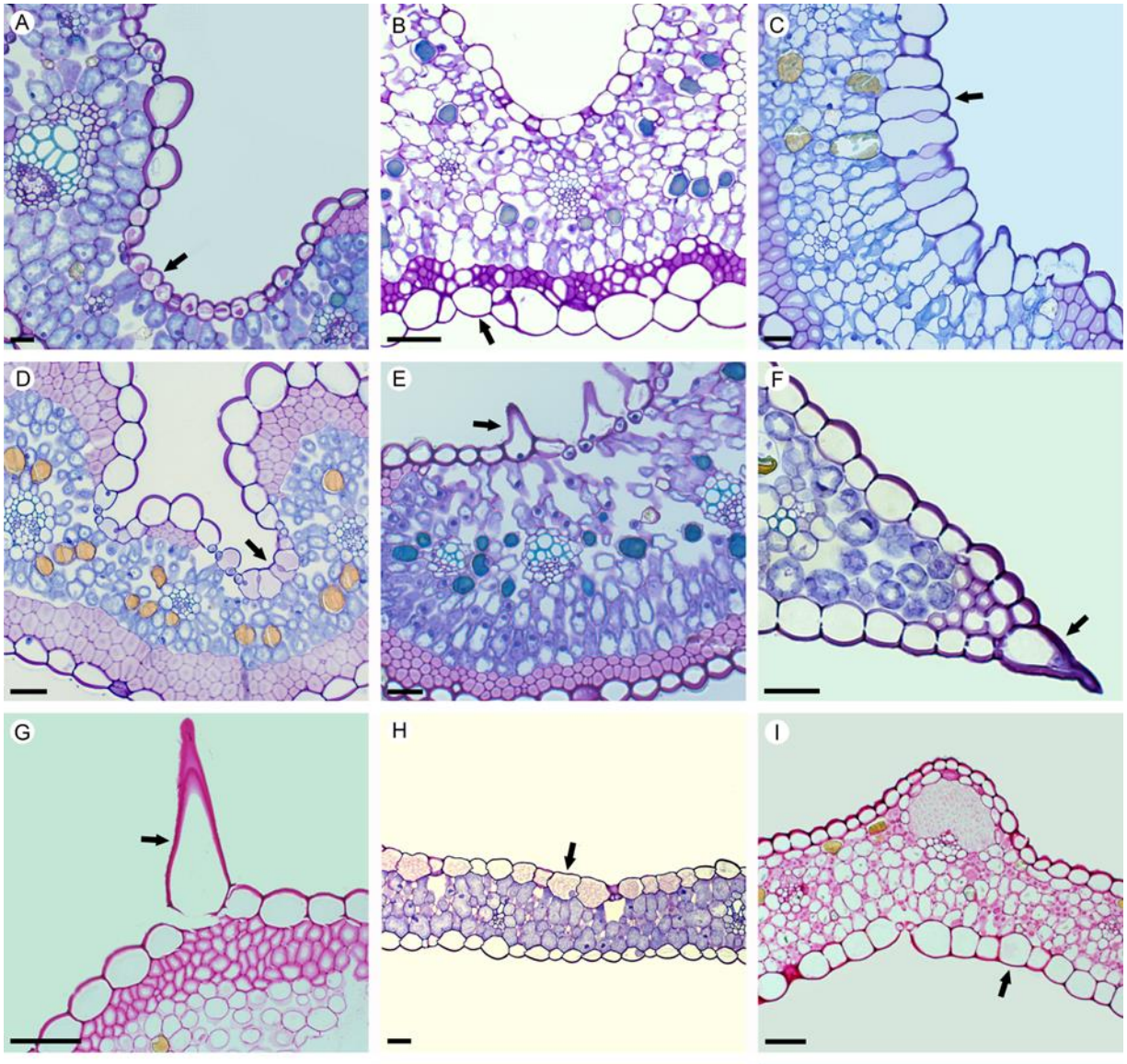

Figure 4 Transverse section of leaves with black arrow showing details of epidermis cells. A- *Calydorea alba* with bulliform like cells with pectin content in shallow depression, B- *Calydorea campestris* with large irregular cells, C- *Cipura xanthomelas* 987 with bulliform like cells with pectin content, D- *Cipura formosa* with bulliform like cells with pectin content, E- *Calydorea basaltica* with papillae, F- *Calydorea xiphioides* with trichome, above sclerenchyma at marginal region., G- *Cipura* sp. 2 with trichome, above sclerenchyma not at marginal region., H- *Lethia umbellata* with bulliform like cells with pectin content in flat extension, I- *Eleutherine angusta* with bulliform like cells with pectin content, above sclerenchyma not at marginal region. Scale bar = 10 µm.

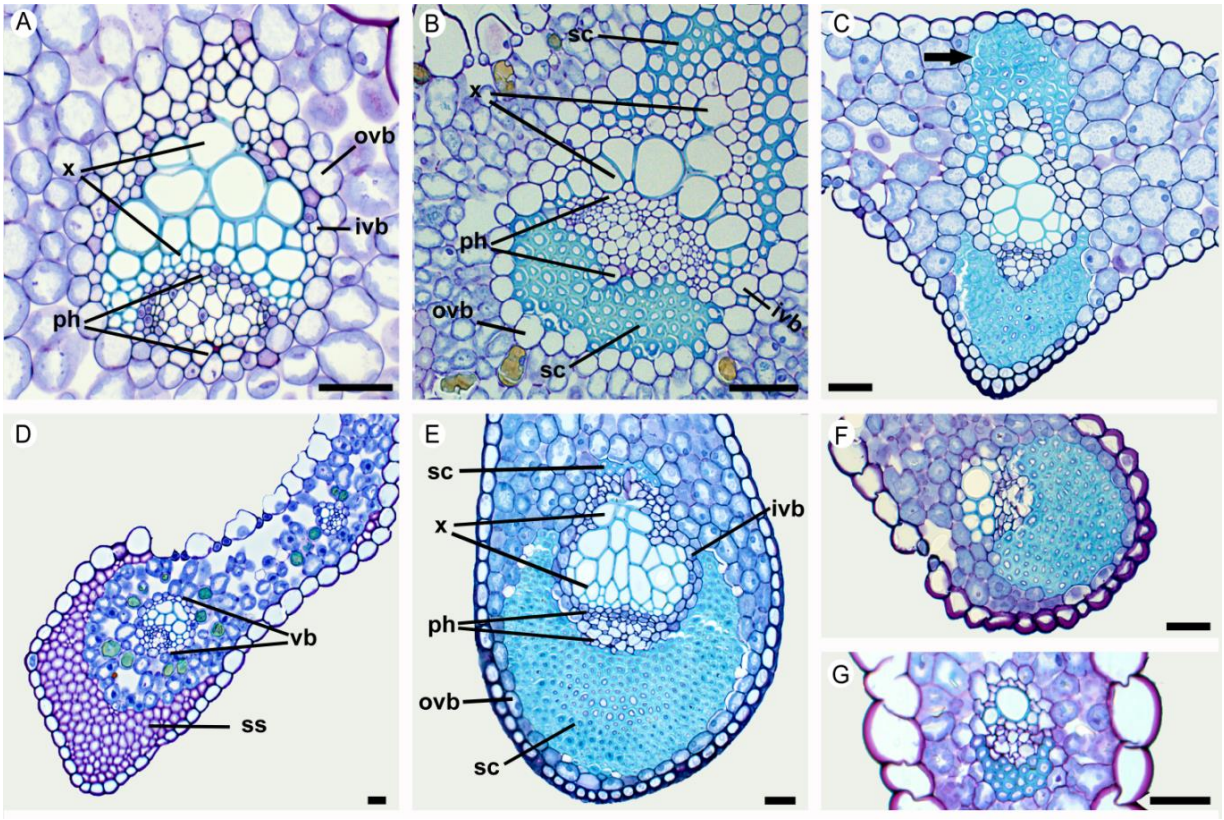

Figure 5 Vascular bundles of first order in not marginal region (A-C) and marginal region (D-G). A- *Cypella herbertii*, B- *Cipura* sp. 2, C- *Phalocallis coelestis*, D- *Cypella discolor*, E- *Gelasine coerulea*, F- *Eleutherine angusta*, G- *Hesperoxiphion peruvianum.* x= xylem; ph=phloem; ovb=outer vascular bundle; ivb= inner vascular bundle; sc= sclerenchyma lignified; ss= subepidermal sclerenchyma pectin rich; black arrow indicates the continuity of sclerenchyma to the epidermis in *Phalocallis coelestis*. Scale bar = 10 µm.

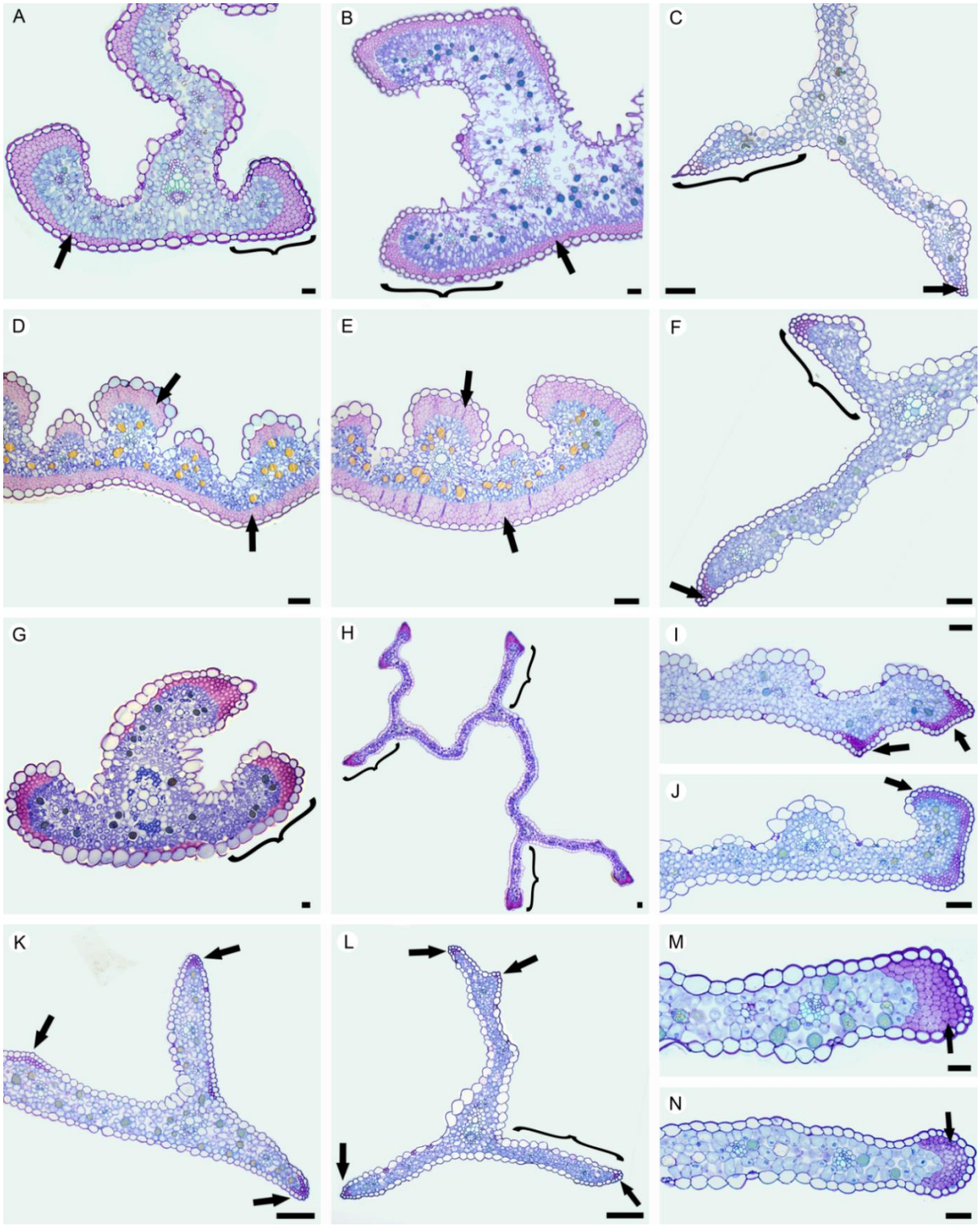

Figure 6 Transverse section of leaves of species of Clade A with black arrow showing details of subepidermical sclerenchyma and curly brackets showing the foliose extension; A- *Calydorea alba*, B- *Calydorea basaltica*, C- *Calydorea xiphioides*, D-E- *Cipura formosa*, F- *Cipura paludosa*, G- *Cipura* sp. 1, H- *Cypella discolor*, I- *Cypella rivularis*, J- *Cypella altouruguaya*, K- *Cypella aquatilis*, L- *Herbertia zebrina*, M- *Herbertia quareimana*, N- *Herbertia lahue*. Scale bar = 50 µm.

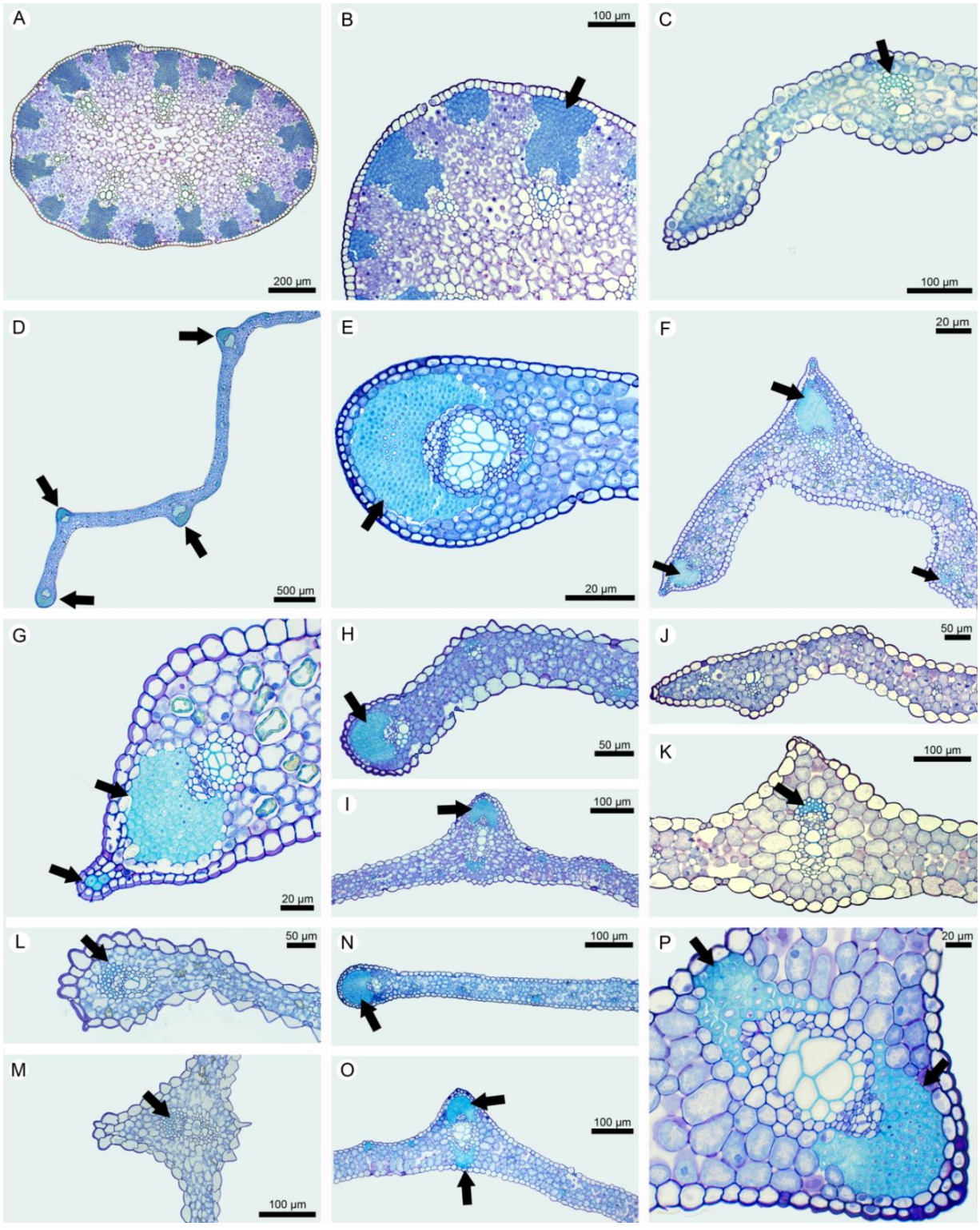

Figure 7 Transverse section of leaves of species of Clade B with black arrow showing details of lignified sclerenchyma, A-B- *Alophia medusa;* C- *Alophia* aff. *drummondii,* D- E- *Gelasine elongata,* F-G- *Gelasine coerulea,* H-I- *Eleutherine bulbosa,* J-K- *Lethia umbellata,* L-M- *Hesperoxiphion peruvianum,* N-O-P- *Phalocallis coelestis*.

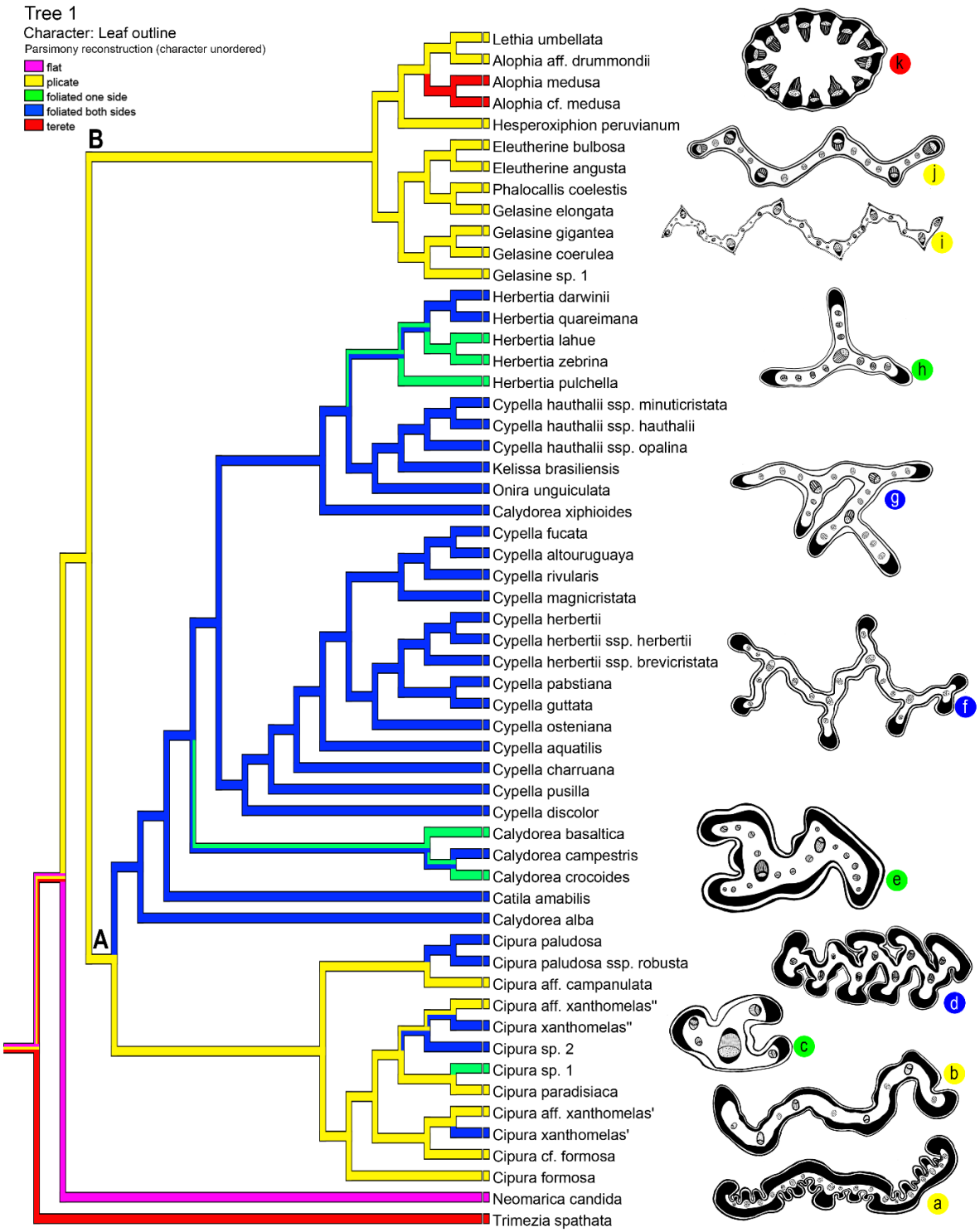

Figure 8 Leaf outline character evolution optimization into the maximum likelihood (ML) best-scoring tree, obtained from cpDNA analysis. Characters were optimized on the tree using maximum parsimony (MP) method. Illustrations on the right side show representative leaf transversal sections of the different leaf outline. The color match with the leaf outline type, and the letters indicate: a) *Cipura formosa* and b) *Cipura paradisiaca*, both plicate; c) *Cipura* sp. 1, foliate one side; d) *Cipura* sp. 2, foliate both sides; e) *Calydorea basaltica* foliate one side; f) *Cypella osteniana* and g) *Kelissa brasiliensis*, both foliate both sides; h) *Herbertia zebrina*, foliate one side; i) *Gelasine coerulea*, and j) *Eleutherine angusta*, plicate and k) *Alophia* cf. *medusa*, terete. Representation of sclerenchyma in wide black; phloem in dots, xylem in stripes.

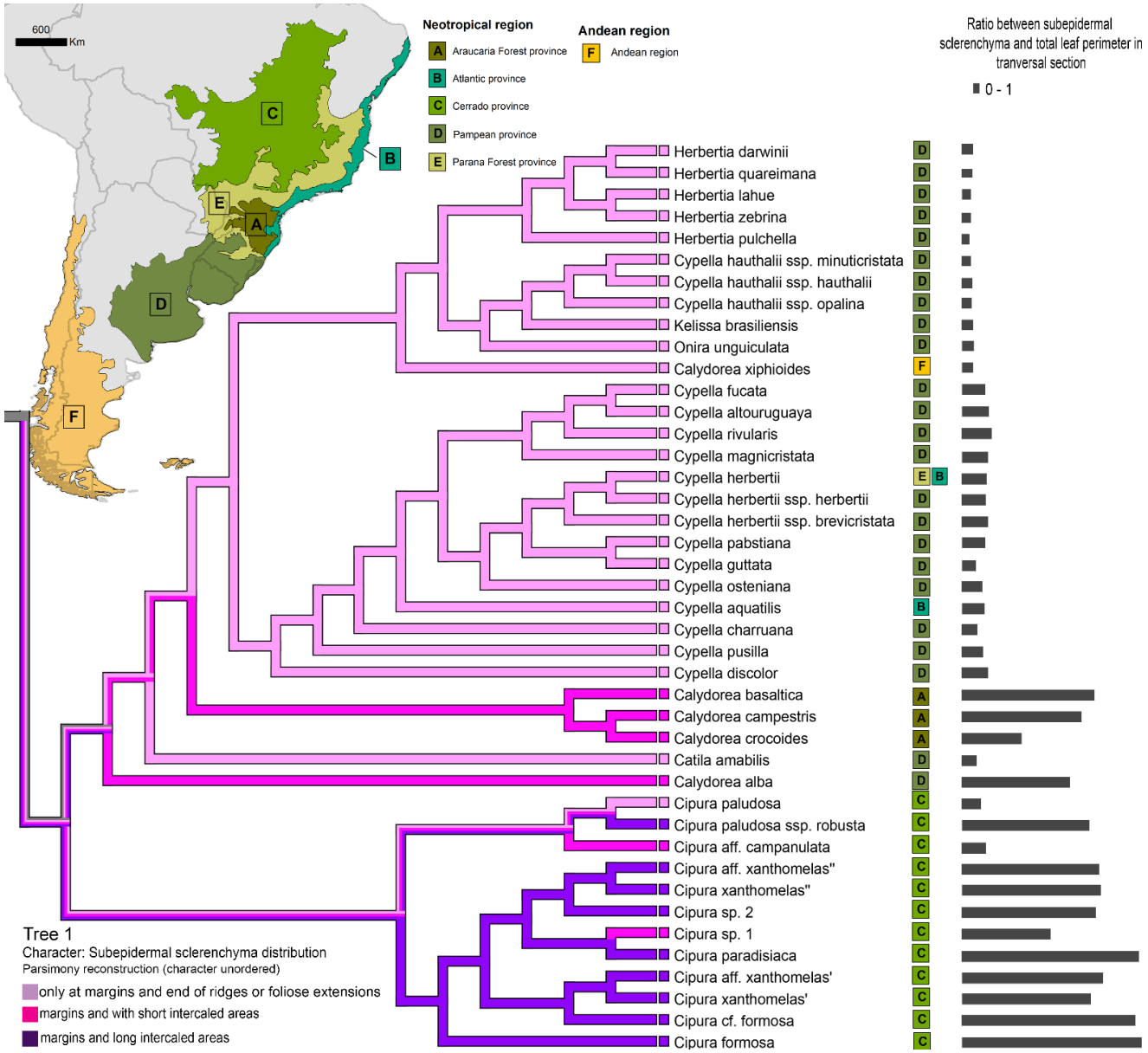
600
Km
Neotropical region
A Araucaria Forest province
B Atlantic province
C Cerrado province
D Pampean province
E Parana Forest province
Andean region
F Andean region
Ratio between subepidermal sclerenchyma and total leaf perimeter in tranversal section
0 - 1
Herbertia darwinii
Herbertia quareimana
Herbertia lahue
Herbertia zebrina
Herbertia pulchella
Cypella hauthalii ssp. minuticristata
Cypella hauthalii ssp. hauthalii
Cypella hauthalii ssp. opalina
Kelissa brasiliensis
Onira unguiculata
Calydorea xiphioides
Cypella fucata
Cypella altouruguaya
Cypella rivularis
Cypella magnicristata
Cypella herbertii
Cypella herbertii ssp. herbertii
Cypella herbertii ssp. brevicristata
Cypella pabstiana
Cypella guttata
Cypella osteniana
Cypella aquatilis
Cypella charruana
Cypella pusilla
Cypella discolor
Calydorea basaltica
Calydorea campestris
Calydorea crocoides
Catila amabilis
Calydorea alba
Cipura paludosa
Cipura paludosa ssp. robusta
Cipura aff. campanulata
Cipura aff. xanthomelas"
Cipura xanthomelas"
Cipura sp. 2
Cipura sp. 1
Cipura paradisiaca
Cipura aff. xanthomelas'
Cipura xanthomelas'
Cipura cf. formosa
Cipura formosa
Tree 1
Character: Subepidermal sclerenchyma distribution
Parsimony reconstruction (character unordered)
only at margins and end of ridges or foliose extensions
margins and with short intercaled areas
margins and long intercaled areas

Figure 9 Subepidermal sclerenchyma distribution character evolution optimization into the maximum likelihood (ML) best-scoring tree, obtained from cpDNA analysis. Characters were optimized on the tree using maximum parsimony (MP) method. On the left side of the tree is the map of South America with the biogeographic regions and the area of occurrence of each species is indicated on the right side of the tree (A; B; C; D; E; F), according to Morrone (2014). On the right side, the grey bars indicate the ratio between subepidermal sclerenchyma and the total leaf perimeter in cross section. Variation is between 0 and 1, the shortest bar being close to zero and the longest bar being close to one (see Appendix 9).

SUPPLEMENTARY DATA

Appendix S1. Voucher information and geographical origin of species sampled to leaf anatomy of Tigridieae (ingroup) and Trimezieae (outgroup).

Species	Voucher	Geographical origin	Phylo	Anato
Ingroup				
Calydorea alba Roitman & A. Castillo	Pastori et al. 125 (ICN)	BR: RS, Alegrete		x
	Eggers & Souza-Chies 519 (ICN)	BR: RS, Uruguaiana	x	
Calydorea basaltica Ravenna	Eggers et al. 916 (ICN)	BR: SC, Água Doce		x
	Eggers & Souza-Chies 390 (ICN)	BR: PR, Palmas	x	
Calydorea campestris (Klatt) Baker	Eggers et al. 921 (ICN)	BR: PR, Palmas		x
	Eggers & Souza-Chies 330 (ICN)	BR:PR, Balsa Nova	x	
Calydorea crocoides Ravenna	Eggers & Souza-Chies 677 (ICN)	BR: RS, São Francisco de Paula		x
	Eggers & Souza-Chies 218 (ICN)	BR: RS, São José dos Ausentes	x	
Calydorea xiphioides (Poepp.) Espinosa	Chauveau H11001 (cultivated at UPSBG)	CH: Libueno (Region VII)		x
Catila amabilis Ravenna	Eggers et al. 742 (ICN)	BR: RS, Barra do Quaraí	x	x
Cipura aff. xanthomelas Maxim. ex Klatt	Chauveau & Aguiar 906B (ICN)	BR: GO, Cavalcante	x	x
	Chauveau & Aguiar 909 (ICN)	BR: DF, Brasília	x	x
Cipura aff. campanulata Ravenna	Eggers & Chauveau 1013 (ICN)	BR: MT, Rosário do Oeste	x	x
Cipura cf. *formosa* Ravenna	Eggers et al. 996 (ICN)	BR: GO, Alto Paraíso de Goiás	x	x
	Chauveau et al. 904 (ICN)	BR: GO, Alto Paraíso de Goiás	x	x
Cipura formosa Ravenna	Eggers et al. 994 (ICN)	BR: GO, Alto Paraíso de Goiás	x	x
	Eggers et al. 902 (ICN)	BR: GO, Cavalcante	x	x
Cipura paludosa subsp. *robusta* Ravenna	Eggers & Chauveau 1010 (ICN)	BR: MT, Rosário do Oeste	x	x
	Eggers & Chauveau 1014 (ICN)	BR: MT, Rosário do Oeste	x	x
	Eggers & Chauveau 1019 (ICN)	BR: MT, Cuiabá	x	x
	Eggers & Chauveau 1020 (ICN)	BR: MT, Jangada	x	x
Cipura paludosa Aubl.	Chauveau & Aguiar 906C (ICN)	BR: DF, Brasília	x	x
Cipura paradisiaca Ravenna	Chauveau et al. 900 (ICN)	BR: GO, Teresina de Goiás	x	x
	Eggers et al. 993 (ICN)	BR: GO, Cavalcante	x	x
Cipura sp. 1	Chauveau & Aguiar 906A (ICN)	BR: GO, Cavalcante	x	x
Cipura sp. 2	Eggers et al. 984 (ICN)	BR: TO, Taguatinga	x	x
	Eggers et al. 985 (ICN)	BR: TO, Ponte Alta do Bom Jesus	x	x
	Eggers et al. 990 (ICN)	BR: TO, Ponte Alta do Bom Jesus	x	x

Species	Voucher	Geographical origin	Phylo	Anato
Ingroup				
Cipura xanthomelas Maxim. ex Klatt	Eggers et al. 924 (ICN)	BR: MG, Prata	x	x
	Eggers et al. 987 (ICN)	BR: TO, Dianópolis	x	x
	Eggers & Chauveau 1009 (ICN)	BR: MT, Rosário do Oeste	x	x
Cypella altouruguaya Chauveau & L.Eggers	Eggers & Chauveau 716 (ICN)	BR: RS, Trindade do Sul	x	x
Cypella amplimaculata Chauveau & L.Eggers	Aita 49 (ICN)	BR: RS, Pelotas	x	
	Eggers & Souza-Chies 664 (MBM)	BR:RS, Porto Alegre	x	
Cypella aquatilis Ravenna	Eggers & Chauveau 714 (ICN)	BR: RS, Muitos Capões	x	x
Cypella armosa Ravenna	Eggers *et al.* 761 (ICN)	BR: RS, São Borja	x	
Cypella charruana Deble & F.S.Alves	Pastori et al. 203 (ICN)	BR: RS, Quaraí	x	x
Cypella discolor Ravenna	Aita 75 (ICN)	BR: RS, Quaraí	x	x
Cypella fucata Ravenna	Eggers *et al.* 746 (ICN)	BR: RS, Uruguaiana	x	
	Pastori et al. 122 (ICN)	BR: RS, Alegrete		x
Cypella aff. *fucata* Ravenna	Eggers *et al.* 847 (ICN)	UR: Paysandú, Chapicuy	x	
Cypella guttata Deble & F.S. Alves	Pastori et al. 204 (ICN)	BR: RS, Quaraí	x	x
Cypella hauthalii (Kuntze) R.C. Foster	Eggers *et al.* 820 (ICN)	AR: Misiones, Posadas	x	x
Cypella hauthalii subsp. *opalina* Ravenna	Pastori et al. 128 (ICN)	BR: RS, Manoel Viana	x	x
Cypella hauthalii subsp. *minuticristata* Chauveau & L.Eggers	Eggers & Chauveau 728 (ICN)	BR: RS, Salto do Jacuí	x	x
Cypella herbertii (Lindl.) Herb.	Eggers et al. 725 (ICN)	BR: RS, Salto do Jacuí	x	x
	Eggers et al. 918 (living collection UFRGS)	BR: SC, São Joaquim		x
	Alves 260 (ICN)	BR: SC, Caçador		x
	Eggers & Souza-Chies 490 (ICN)	BR: RS, São Gabriel	x	
Cypella herbertii subsp. *herbertii*	Aita 77 (ICN)	BR: RS, Quaraí	x	
Cypella herbertii subsp. *brevicristata* Ravenna	Eggers *et al.* 839 (ICN)	UR: Artigas, Sequeira	x	x
	Eggers *et al.* 752 (ICN)	BR: RS, Itaqui	x	x
Cypella laeta Ravenna	Eggers *et al.* 843 (ICN)	UR: Paysandú, Chapicuy	x	
Cypella laxa Ravenna	Eggers *et al.* 766 (living collection UFRGS)	BR: RS, Santo Antônio das Missões	x	
Cypella luteogibbosa Deble	Eggers *et al.* 791 (ICN)	BR: RS, Quaraí	x	
Cypella magnicristata Deble	Aita 76 (ICN)	BR: RS, Quaraí	x	x
Cypella osteniana Beauverd	Eggers et al. 851 (ICN)	UR: Lavalleja, Minas	x	x
Cypella pabstiana Ravenna	Eggers et al. 741 (ICN)	BR: RS, Barra do Quaraí	x	x
Cypella pusilla (Link & Otto) Benth. & Hook. f. ex B.D. Jacks.	Pastori et al. 119 (ICN)	BR: RS, São Vicente do Sul	x	x
	Pastori & Chauveau 174 (ICN)	BR: RS, Amaral Ferrador	x	x
	Eggers *et al.* 778 (ICN)	BR: RS, Lavras	x	x
	Eggers et al. 818 (ICN)	BR: RS, Viamão	x	x

Species	Voucher	Geographical origin	Phylo	Anato
Ingroup				
Cypella ravenniana Deble & F.S.Alves	Eggers *et al.* 757 (ICN)	BR, RS, São Borja	x	X
Cypella rivularis Chauveau & L.Eggers	Eggers et al. 800 (ICN)	BR: RS, Uruguaiana	x	
Herbertia darwinii Roitman & J.A. Castillo	Eggers & Souza-Chies 502 (ICN)	BR: RS, Santana de Livramento	x	x
	Pastori et al. 123 (ICN)	BR: RS, Alegrete		x
	Pastori et al. 140 (ICN)	BR: RS, Santo Antônio das Missões		x
Herbertia furcata (Klatt) Ravenna	Eggers & Souza-Chies 528 (ICN)	BR: RS, Uruguaiana	x	
Herbertia lahue (Molina) Goldblatt	Eggers & Souza-Chies 211 (ICN)	BR: RS, Taquara	x	
	Eggers & Souza-Chies 495 (ICN)	BR: RS, Santana de Livramento		x
	Pastori et al. 132 (ICN)	BR: RS, São Borja		x
Herbertia lahue subsp. *lahue* (Molina) Goldblatt	Eggers & Souza-Chies 488 (ICN)	BR: RS, São Gabriel		x
	Eggers & Souza-Chies 504 (ICN)	BR: RS, Santana de Livramento		x
Herbertia pulchella Sweet	Eggers & Souza-Chies 671 (ICN)	BR: RS, Cristal	x	
	Pastori & Chauveau 182 (ICN)	BR: RS, Pinheiro Machado		x
Herbertia quareimana Ravenna	Eggers & Souza-Chies 513 (ICN)	BR: RS, Quaraí	x	x
Herbertia zebrina Deble	Eggers & Souza-Chies 179 (ICN)	BR: RS, Encruzilhada do Sul	x	
	Pastori & Chauveau 175 (ICN)	BR: RS, Amaral Ferrador		x
	Pastori et al. 110 (ICN)	BR: RS, Amaral Ferrador		x
Kelissa brasiliensis (Baker) Ravenna	Pastori et al. 109 (ICN)	BR: RS, Amaral Ferrador		x
	Eggers & Souza-Chies 670 (ICN)	BR: RS, Cristal	x	
	Pastori et al. 145 (ICN)	BR: RS, São Pedro do Sul		x
Onira unguiculata (Baker) Ravenna	Aita 73 (ICN)	BR:RS, Quaraí	x	
	Pastori et al. 162 (ICN)	BR: RS, Candiota		x
Alophia aff. *drummondii* (Graham) R.C. Foster	Felix 15944 (cultivated at UFRGS)	BR: PB, Esperança	x	x
Alophia medusa (Baker) Goldblatt	Chauveau & Lizarazo 1007 (ICN)	BR: TO, Novo Jardim	x	x
	Eggers et al. 989 (ICN)	BR: TO, Novo Jardim	x	x
Alophia cf. *medusa* (Baker) Goldblatt	Eggers et al. 991 (ICN)	BR: TO, Ponte Alta do Bom Jesus	x	x
Hesperoxiphion peruvianum Baker	Chauveau *s.n.* (ICN 190682)	PE: cultivated material from B&T World Seeds	x	x
Lethia umbellata (Klatt) Ravenna	Guerra 1939/1(Eggers 1059 (ICN))	BR: PB, São João do Tigre	x	x

Species	Voucher	Geographical origin	Phylo	Anato
Ingroup				
Phalocallis coelestis (Lehm.) Ravenna	Eggers & Chauveau 973 (ICN)	BR: RS, Cambará do Sul	x	X
	Eggers & Chauveau 977 (ICN)	BR: RS, Cambará do Sul	x	x
Eleutherine angusta Ravenna	Eggers & Chauveau 933 (ICN)	BO: Santa Cruz, Nũflo de Chavez	x	x
	Eggers & Chauveau 937 (ICN)	BO: Santa Cruz, Chiquitos	x	x
Eleutherine bulbosa (Mill.) Urb.	Souza *s.n.* (UFP 82763)	BR: BA, Ipiaú	x	x
Gelasine coerulea (Vell.) Ravenna	Eggers & Souza-Chies 676 (ICN)	BR: RS, São Francisco de Paula	x	x
Gelasine elongata (Graham) Ravenna	Eggers & Chauveau 713 (ICN)	BR: RS, Quaraí	x	x
	Pastori et al. 202 (ICN)	BR: RS, Quaraí	x	x
Gelasine gigantea Ravenna	Chauveau & Lizarazo 1000 (ICN)	BR: GO, Pirenópolis	x	x
	Chauveau & Lizarazo 1000-B (ICN)	BR: GO, Pirenópolis	x	x
Gelasine sp. 1	Eggers & Chauveau 1022 (ICN)	BR: RS, Cambará do Sul	x	x
	Eggers & Chauveau 1026 (ICN)	BR: RS, Cambará do Sul	x	x
Outgroup				
Trimezia juncifolia (Klatt) Benth. & Hook. f.	Eggers et al. 926 (ICN)	BR: MG, Araxá		x
Trimezia juncifolia subsp. *speciosa* Ravenna	Eggers et al. 928 (ICN)	BR: MG, São Roque de Minas		x
Trimezia spathata (Klatt) Baker	Eggers et al. 929 (ICN)	BR: MG, Camanducaia		x
	Eggers 695 (ICN)	BR: RS, Derrubadas	x	
	Pastori & Chauveau 177 (ICN)	BR: RS, Encruzilhada do Sul		x
Neomarica candida (Hassl.) Sprague	Eggers 694 (PABG- JB004)	BR:RS, Porto Alegre	x	
Neomarica aff. *caerulea* (Ker Gawl.) Sprague	Eggers et al. 931 (ICN)	BR: MG, Camanducaia		x

Notes: ICN = Herbarium of the Federal University of Rio Grande do Sul (Brazil); UFP = Herbarium of the Universidade Federal de Pernambuco; UFRGS = Universidade Federal do Rio Grande do Sul (Brazil); UPSBG = Botanical Garden of the University Paris-Sud (France). Countries: AR= Argentina; BO= Bolivia; BR = Brazil; CH = Chile; UR = Uruguay; States of Brazil: BA = Bahia; DF = Distrito Federal; GO = Goiás; MG = Minas Gerais; MT = Mato Grosso; PB = Pernambuco; PR = Paraná; RS = Rio Grande do Sul; SC = Santa Catarina; TO = Tocantins;

Appendix S2 Primers used for PCR amplification and DNA sequencing.

Primer name	Direction	PCR, sequencing	Primer sequence 5'–3'	Source
rps4 + spacer *rps4-trnS*				
rps4-f	Forward	PCR, Seq.	ATGTCCCGTTATCGAGGACCT	Souza-Chies et al. 1997
t trnS-r	Reverse	PCR, Seq.	TACCGAGGGTTCGAATC	Souza-Chies et al. 1997
rbcL				
rbcL-1f	Forward	PCR, Seq.	ATGAGTTGTAGGGAGGGACT	Reeves et al. 2001
rbcL-1360r	Reverse	PCR, Seq.	CTTCACAAGCAGCAGCTAGTTC	Reeves et al. 2001
rbcL-656f	Forward	Seq.	TGCGTTGGAGAGACCGTTTC	Chauveau et al. 2012
rbcL-675r	Reverse	Seq.	GAAACGGTCTCTCCAACGC	Chauveau et al. 2012
matK + *matK*-5'*trnK* intron				
matK-f1	Forward	PCR, Seq.	ATGGAAGAATTACAAGGATAT	Chauveau et al. 2012
rnK-2r	Reverse	PCR, Seq.	AACTAGTCGGATGGAGTAG	Johnston and Soltis 1995
matK-f4	Forward	Seq.	GGTATCAATCGTACAGGAT	Pastori et al. 2018
matK-r2	Reverse	Seq.	AGTTTGATAATTGGTTTATATG	Chauveau et al. 2012
trnQ-rps16				
trnQ-S1f	Forward	PCR, Seq.	GCGTGGCCAAGTGGTAAGGC	Shaw et al. 2007
rps16-S1r	Reverse	PCR, Seq.	GTTGCTTTCTACCACATCGTTT	Shaw et al. 2007
trnQ-f6	Forward	Seq.	AACTCTTGATACTCGAGAAGAAGTG	Pastori et al. 2018
trnQ-r5	Reverse	Seq.	TACGCCCGTTATTTGGACTTTC	Pastori et al. 2018
rps16 intron				
rps16-f	Forward	PCR, Seq.	GTGGTAGAAAGCAACGTGCGACT	Oxelman et al., 1997
rps16-r2	Reverse	PCR, Seq.	TCGGGATCGAACATCAATTGCAA	Oxelman et al., 1997
psbA-trnH				
psbA	Forward	PCR, Seq.	GTTATGCATGAACGTAATGCTC	Shaw et al. 2005
$trnH^{GUG}$	Reverse	PCR, Seq.	CGCGCATGGTGGATTCACAATCC	Shaw et al. 2005

Notes: Seq. = sequencing

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Appendix S3. PCR profiles for DNA amplification. (1) Initial denaturation; (2) Number of cycles; (3) Denaturation, annealing, and elongation steps for each cycle; (4) final elongation step. Temperature and duration are indicated for each step.

Locus	PCR profile
rps4 + spacer *rps4-trnS*	(1) 95°C–2 mn; (2) 40; (3) 94°C–1 mn, 50°C–40 s, 72°C–2 mn; (4) 72°C, 5 mn
rbcL	(1) 95°C–2 mn; (2) 40; (3) 94°C–1 mn, 56°C–40 s, 72°C–2 mn; (4) 72°C, 5 mn
matK + *matK*-5'*trnK* intron	(1) 95°C–2 mn; (2) 40; (3) 94°C–1 mn, 53°C–40 s, 72°C–2 mn; (4) 72°C–5 mn
trnQ-rps16	(1) 95°C–2 mn; (2) 40; (3) 94°C–45 s, 62°C–40 s, 72°C–1:30 mn; (4) 72°C, 5 mn
rps16 intron	(1) 95°C–2 mn; (2) 40; (3) 94°C–1 mn, 50°C–40 s, 72°C–1:30 mn; (4) 72°C, 5 mn
psbA-trnH	(1) 95°C–2 mn; (2) 40; (3) 94°C–1 mn, 53°C–40 s, 72°C–1:30 mn; (4) 72°C, 5 mn

Appendix S4. Dataset partitions for Maximum Likelihood (ML) and Bayesian Inference (BI) analyses and evolutionary models used in BI.

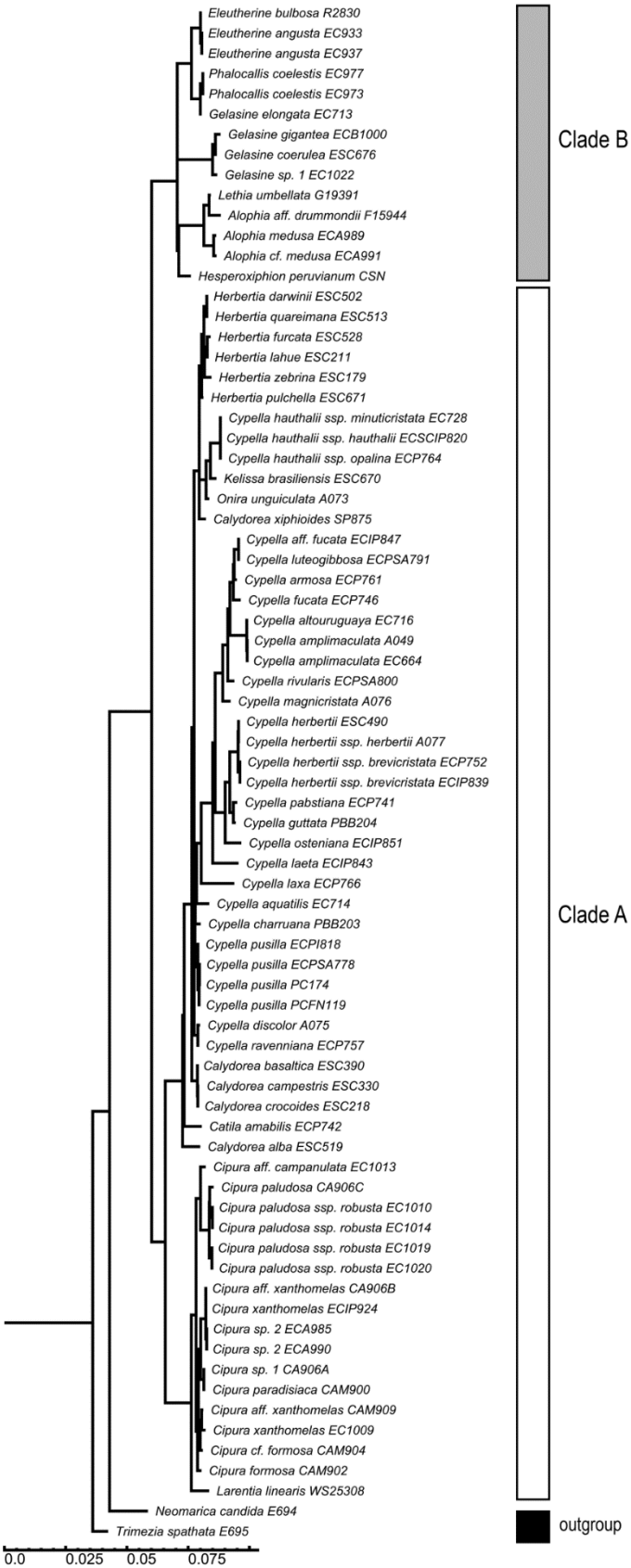
Data partition	Data partition	Partition by codon position	Model
trnQ-rps16 spacer (complete sequence)	1209		GTR
rps16 intron (complete sequence)	872		HKY
matK (partial sequence)	1548	X	GTR
matK-5'*trnK* intron (partial sequence)	228		F81
psbA (partial sequence)	53	X	K80
psbA-rps19 spacer (complete sequence)	109		JC
rps19 (complete sequence - negative strand)	278	X	GTR
rps19-trnH spacer (complete sequence)	174		HKY
rps4 (partial sequence)	585	X	GTR
rps4-trnS spacer (complete sequence)	299		HKY
rbcL (partial sequence)	1359	X	GTR

Notes: partition by codon position = partition coding for protein.

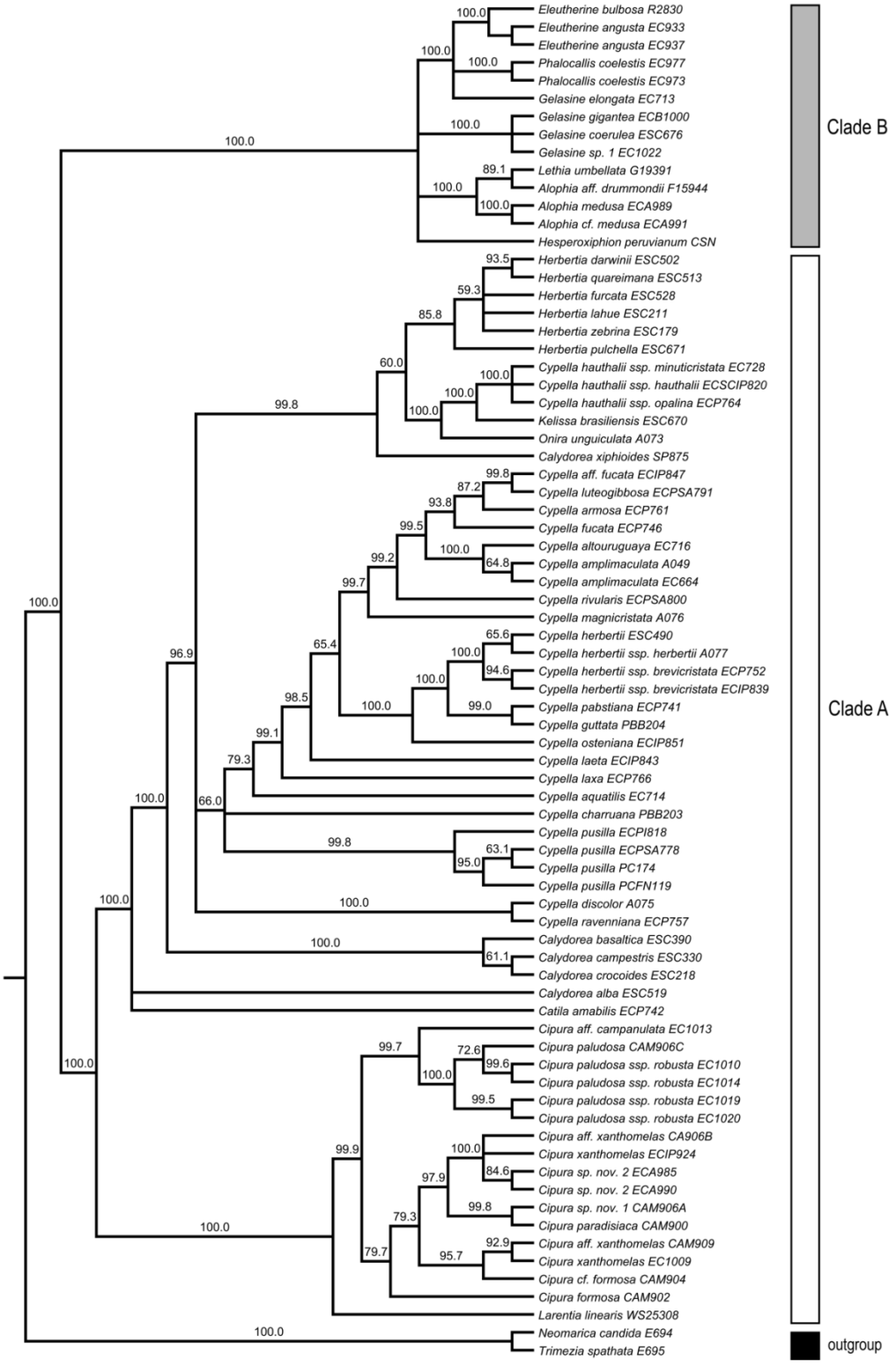
Appendix S5. Lengths and indices for the resulting most parsimonious trees from separated and combined data sets. CI and RI are respectively the consistency and retention indices of most parsimonious topologies.

	Number of characters						
	Total characters	Variable characters / %*	Parsimony informative characters	MP* trees	Tree length	CI	RI
matK-5'trnK + [coded indels]	1834 +[22]	364+[22]/20.8	222+[15]/12.8	42750	569	0.756	0.934
trnH-psbA + [coded indels]	687 +[10]	133 +[10]/20.5	83+[8]/13.1	690	237	0.671	0.856
rbcL	1359	133/9.8	93/6.8	700	204	0.716	0.917
rps4-trnS + [coded indels]	885+[13]	138+[13]/16.8	85+[7]/10.2	38721	207	0.809	0.950
rps16 + [coded indels]	896+[20]	133+[20]/16.7	78+[13]/9.9	14	188	0.888	0.968
trnQ-rps16 + [coded indels]	1468+[68]	342+[68]/26.7	201+[36]/15.4	40984	64	0.938	0.975
Total evidence plastid + [ci]	6871+[133]	1243 +[133]/19.6	762 +[133]/12.8	567	2003	0.773	0.932

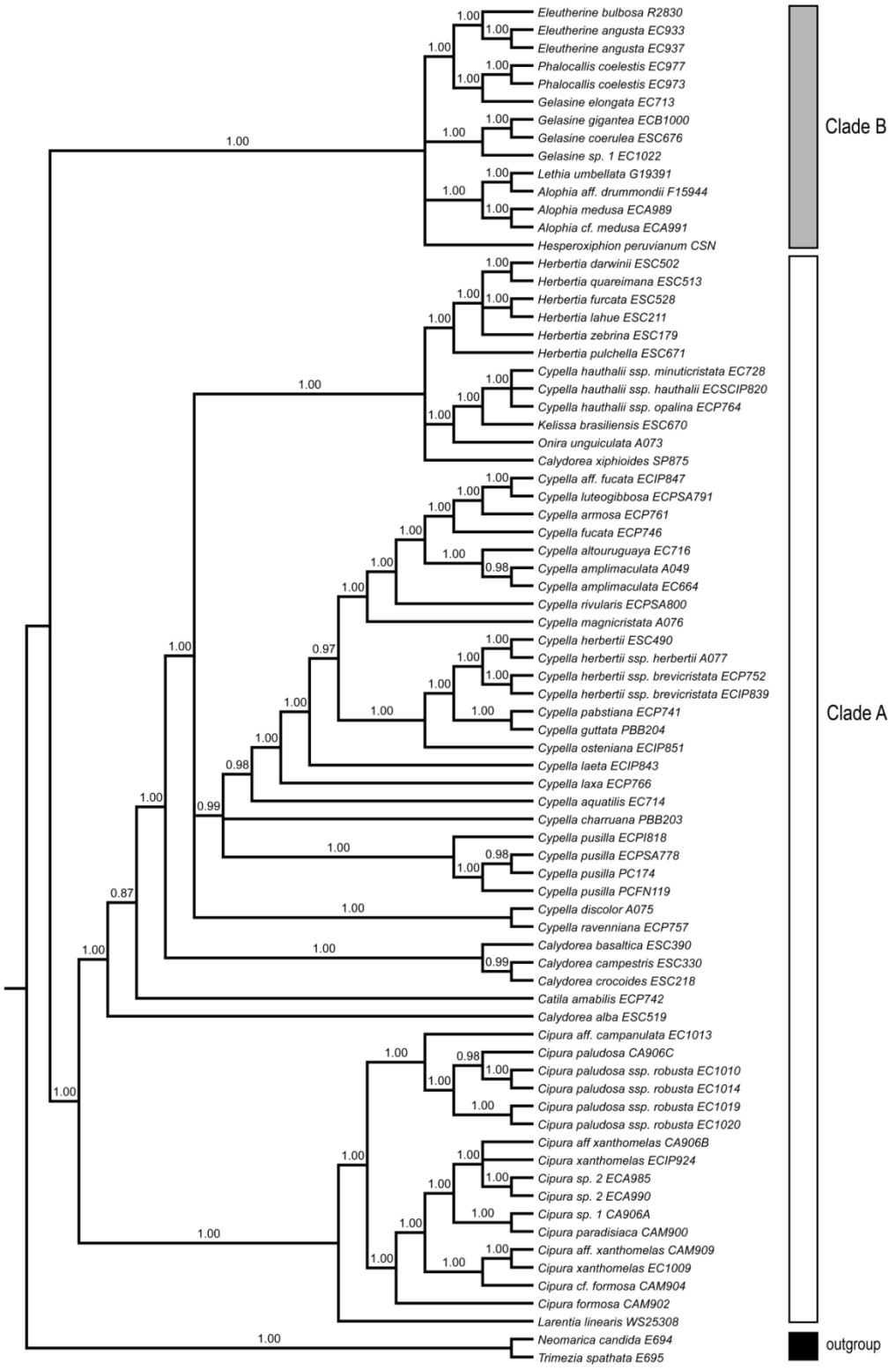
Notes: * = most parsimonious trees; numbers into brackets are numbers of characters resulting from indel-coding.



Appendix S6. Maximum likelihood best-scoring topology obtained from the combined molecular dataset showing branch lengths (-ln likelihood = -23 082).



Appendix S7. Strict consensus tree from maximum parsimony analysis, with support values indicated above branches representing the statistical supports (PBS). The nodes with support value of less than 50% were collapsed. The tree is rooted using *Neomarica candida* and *Trimezia spathata* as outgroup.



Appendix S8. Majority-rule consensus tree for the Bayesian inference, and Bayesian posterior probabilities (PP) values indicated above branches. The nodes with support value of less than 0.95 were collapsed. The tree is rooted using *Neomarica candida* and *Trimezia spathata* as outgroup.

Appendix S9 Morphological and anatomical characters evaluated for Tigridieae and Trimezieae species, states coded according Table 1.

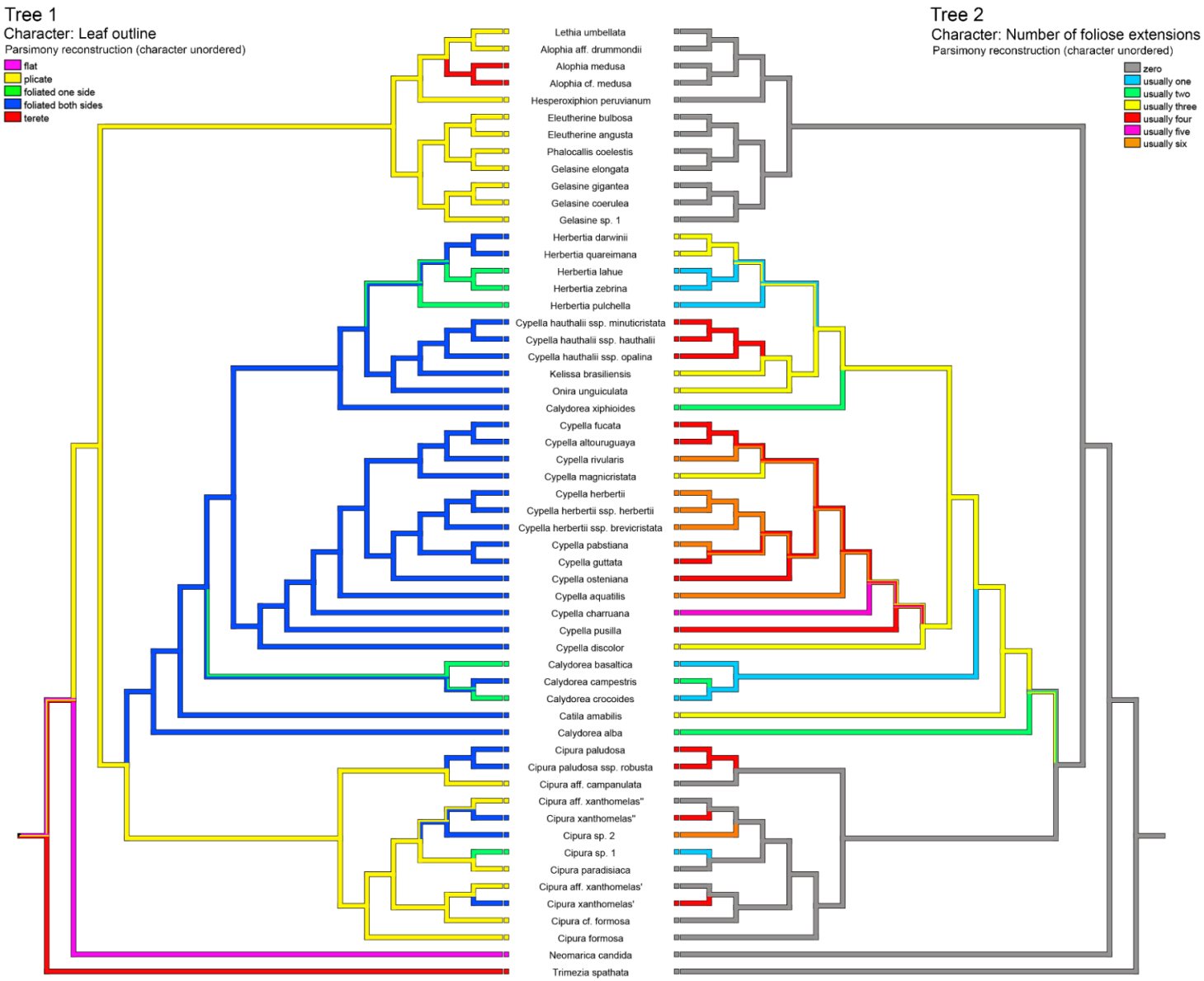
Species	Voucher[a]	Morphological and anatomical characters																																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Calydorea alba	125	3	2	1	0	0	2	2	1	0	3	0	1	4	2	1	2	0	3	4	0	0	0	0	0	0	0	1	0.60	2	0	0	0	0
Calydorea basaltica	916	2	2	1	2	0	2	2	1	0	3	0	1	3	1	0	2	0	3	1	0	0	0	0	0	0	0	1	0.73	2	0	0	0	0
Calydorea campestris	921	3	2	1	0	0	2	2	1	0	2	0	1	3	2	0	2	0	3	1	0	0	0	0	0	0	0	1	0.66	2	0	0	0	0
Calydorea crocoides	677	2	2	1	2	1	2	2	1	1	4	0	1	3	1	0	2	0	3	4	0	0	0	0	0	0	0	1	0.56	2	0	0	0	0
Calydorea xiphioides	SP875	3	2	1	0	1	2	2	1	1	4	0	1	2	2	0	2	0	3	1	0	0	0	0	0	0	0	1	0.06	1	0	0	0	0
Catila amabilis	742	3	2	1	0	0	2	2	1	1	4	0	1	2	3	0	2	0	3	4	0	0	0	0	0	0	0	1	0.08	1	0	0	0	0
Cipura aff. xanthomelas	906B	1	3	1	0	0	2	2	1	1	5	0	0	0	0	0	2	0	3	1	1	1	0	0	0	0	0	1	0.78	3	0	0	0	3
Cipura aff. xanthomelas	909	1	3	1	0	0	2	2	1	1	5	0	0	0	0	0	2	0	3	1	1	1	0	0	0	0	0	1	0.76	3	0	0	0	3
Cipura aff. campanulata	1013	1	3	1	0	0	2	2	1	1	5	0	0	0	0	0	2	0	3	2	2	1	1	0	0	0	0	1	0.13	2	0	0	3	3
Cipura cf. *formosa*	996	1	2	1	0	0	2	2	1	0	5	0	0	0	0	0	2	0	3	1	0	0	0	0	0	0	0	1	0.96	3	0	0	0	0
Cipura formosa	994	1	2	1	0	0	2	2	1	0	5	0	0	0	0	0	2	0	3	1	0	0	0	0	0	0	0	1	0.98	3	0	0	0	0
Cipura formosa	902	1	2	1	0	0	2	2	1	0	5	0	0	0	0	0	2	0	3	1	0	0	0	0	0	0	0	1	0.99	3	0	0	0	0
Cipura paludosa subsp. *robusta*	1010	3	3	1	0	0	2	2	1	1	5	0	0	0	0	0	2	0	3	2	1	1	1	0	0	0	0	1	0.71	3	0	0	3	3
Cipura paludosa subsp. *robusta*	1014	3	3	1	0	0	2	2	1	1	5	0	0	0	0	0	2	0	3	2	1	1	1	0	0	0	0	1	0.69	3	0	0	3	3
Cipura paludosa subsp. *robusta*	1019	3	3	1	0	0	2	2	1	1	5	0	0	0	0	0	2	0	3	2	1	1	1	0	0	0	0	1	0.72	3	0	0	3	3
Cipura paludosa subsp. *robusta*	1020	3	3	1	0	0	2	2	1	1	5	0	0	0	0	0	2	0	3	2	1	1	1	0	0	0	0	1	0.68	3	0	0	3	3
Cipura paludosa	906C	3	3	1	0	0	2	2	1	1	5	0	1	2	4	0	2	0	3	2	1	1	1	0	0	0	0	1	0.10	1	0	0	0	3
Cipura paradisiaca	900	1	3	1	2	0	2	2	1	1	5	0	0	0	0	0	2	0	3	1	1	1	0	0	0	0	0	1	0.97	3	0	0	3	3
Cipura paradisiaca	993	1	3	1	2	0	2	2	1	1	5	0	0	0	0	0	2	0	3	1	1	1	0	0	0	0	0	1	0.99	3	0	0	3	3
Cipura sp. 1	906A	2	2	1	2	0	2	2	1	0	5	0	0	0	0	0	2	0	3	3	2	1	1	0	0	0	0	1	0.49	2	0	0	3	3
Cipura sp. 2	984	3	3	1	2	1	2	2	1	0	5	0	0	0	0	0	2	0	3	4	3	2	2	0	0	0	0	1	0.80	3	0	0	3	3
Cipura sp. 2	985	3	3	1	2	1	2	2	1	0	5	0	0	0	0	0	2	0	3	4	3	2	2	0	0	0	0	1	0.79	3	0	0	3	3
Cipura sp. 2	990	3	3	1	2	1	2	2	1	0	5	0	0	0	0	0	2	0	3	4	3	2	2	0	0	0	0	1	0.79	3	0	0	3	3
Cipura xanthomelas	924	3	3	1	2	1	2	2	1	0	5	0	0	0	0	0	2	0	3	1	1	1	1	0	0	0	0	1	0.14	3	0	0	3	3

Species	Voucher[a]	Morphological and anatomical characters																																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Cipura xanthomelas	987	3	3	1	2	1	2	2	1	0	5	0	0	0	0	0	2	0	3	1	1	1	1	0	0	0	0	1	0.71	3	0	0	3	3
Cipura xanthomelas	1009	3	3	1	2	1	2	2	1	0	5	0	0	0	0	0	2	0	3	1	1	1	1	0	0	0	0	1	0.72	3	0	0	3	3
Cypella altouruguaya	716	3	3	1	0	0	1	2	1	1	5	0	1	2	4	0	2	0	3	4	0	0	0	0	0	0	0	1	0.15	1	0	0	0	0
Cypella aquatilis	714	2	2	1	0	0	1	2	1	1	5	0	1	2	6	0	2	0	3	1	1	1	0	0	0	0	0	1	0.12	1	0	0	3	3
Cypella charruana	203	3	3	1	0	0	1	2	1	1	5	0	1	2	5	0	2	0	3	4	1	1	0	0	0	0	0	1	0.09	1	0	0	0	3
Cypella discolor	75	3	3	1	0	0	1	2	1	1	5	0	1	3	3	0	2	0	3	4	0	0	0	0	0	0	0	1	0.11	1	0	0	0	0
Cypella fucata	122	3	2	1	0	0	1	2	1	1	5	0	1	3	3	0	2	0	3	3	1	1	0	0	0	0	0	1	0.13	1	0	0	0	3
Cypella guttata	204	3	2	1	0	0	1	2	1	1	5	0	1	4	4	0	2	0	3	3	1	1	0	0	0	0	0	1	0.08	1	0	0	3	3
Cypella hauthalii	820	3	2	1	0	0	1	2	1	1	5	0	1	3	4	0	2	0	3	3	0	0	0	0	0	0	0	1	0.06	1	0	0	0	0
Cypella hauthalii subsp. *opalina*	128	3	2	1	0	0	1	2	1	1	5	0	1	3	4	0	2	0	3	3	0	0	0	0	0	0	0	1	0.05	1	0	0	0	0
Cypella hauthalii subsp. *minuticristata*	728	3	2	1	0	0	1	2	1	1	5	0	1	3	4	0	2	0	3	3	0	0	0	0	0	0	0	1	0.05	1	0	0	0	0
Cypella herbertii	725	3	3	1	0	0	1	2	1	1	5	0	1	3	6	0	2	0	3	4	0	0	0	0	0	0	0	1	0.15	1	0	0	0	0
Cypella herbertii	918	3	3	1	0	0	1	2	1	1	5	0	1	3	6	0	2	0	3	4	0	0	0	0	0	0	0	1	0.11	1	0	0	0	0
Cypella herbertii	260	3	3	1	0	0	1	2	1	1	5	0	1	3	6	0	2	0	3	4	0	0	0	0	0	0	0	1	0.16	1	0	0	0	0
Cypella herbertii subsp. herbertii	77	3	3	1	0	0	1	2	1	1	5	0	1	3	6	0	2	0	3	4	0	0	0	0	0	0	0	1	0.15	1	0	0	0	0
Cypella herbertii subsp. *brevicristata*	752	3	3	1	0	0	1	2	1	1	5	0	1	3	6	0	2	0	3	4	0	0	0	0	0	0	0	1	0.15	1	0	0	0	0
Cypella magnicristata	76	3	2	1	0	0	1	2	1	1	5	0	1	3	3	0	2	0	3	4	0	0	0	0	0	0	0	1	0.20	1	0	0	0	0
Cypella osteniana	851	3	3	1	0	0	1	2	1	1	5	0	1	4	5	0	2	0	3	1	1	1	0	0	0	0	0	1	0.11	1	0	0	3	3
Cypella pabstiana	741	3	2	1	0	0	1	2	1	1	5	0	1	3	6	0	2	0	3	4	1	1	0	0	0	0	0	1	0.13	1	0	0	3	3
Cypella pusilla	119	3	2	1	0	0	1	2	1	1	5	0	1	3	4	0	2	0	3	4	0	0	0	0	0	0	0	1	0.12	1	0	0	0	0
Cypella pusilla	174	3	2	1	0	0	1	2	1	1	5	0	1	3	4	0	2	0	3	4	0	0	0	0	0	0	0	1	0.11	1	0	0	0	0
Cypella pusilla	818	3	2	1	0	0	1	2	1	1	5	0	1	3	4	0	2	0	3	4	0	0	0	0	0	0	0	1	0.11	1	0	0	0	0
Cypella rivularis	800	3	3	1	0	0	1	2	1	1	5	0	1	5	6	0	2	0	3	3	1	1	1	0	0	0	0	1	0.08	1	0	0	0	3
Herbertia darwinii	502	3	2	1	0	0	1	2	1	1	2	0	1	2	3	0	2	0	3	4	0	0	0	0	0	0	0	1	0.07	1	0	0	0	0
Herbertia darwinii	123	3	2	1	0	0	1	2	1	1	2	0	1	2	3	0	2	0	3	4	0	0	0	0	0	0	0	1	0.07	1	0	0	0	0
Herbertia darwinii	140	3	2	1	0	0	1	2	1	1	2	0	1	2	3	0	2	0	3	4	0	0	0	0	0	0	0	1	0.07	1	0	0	0	0

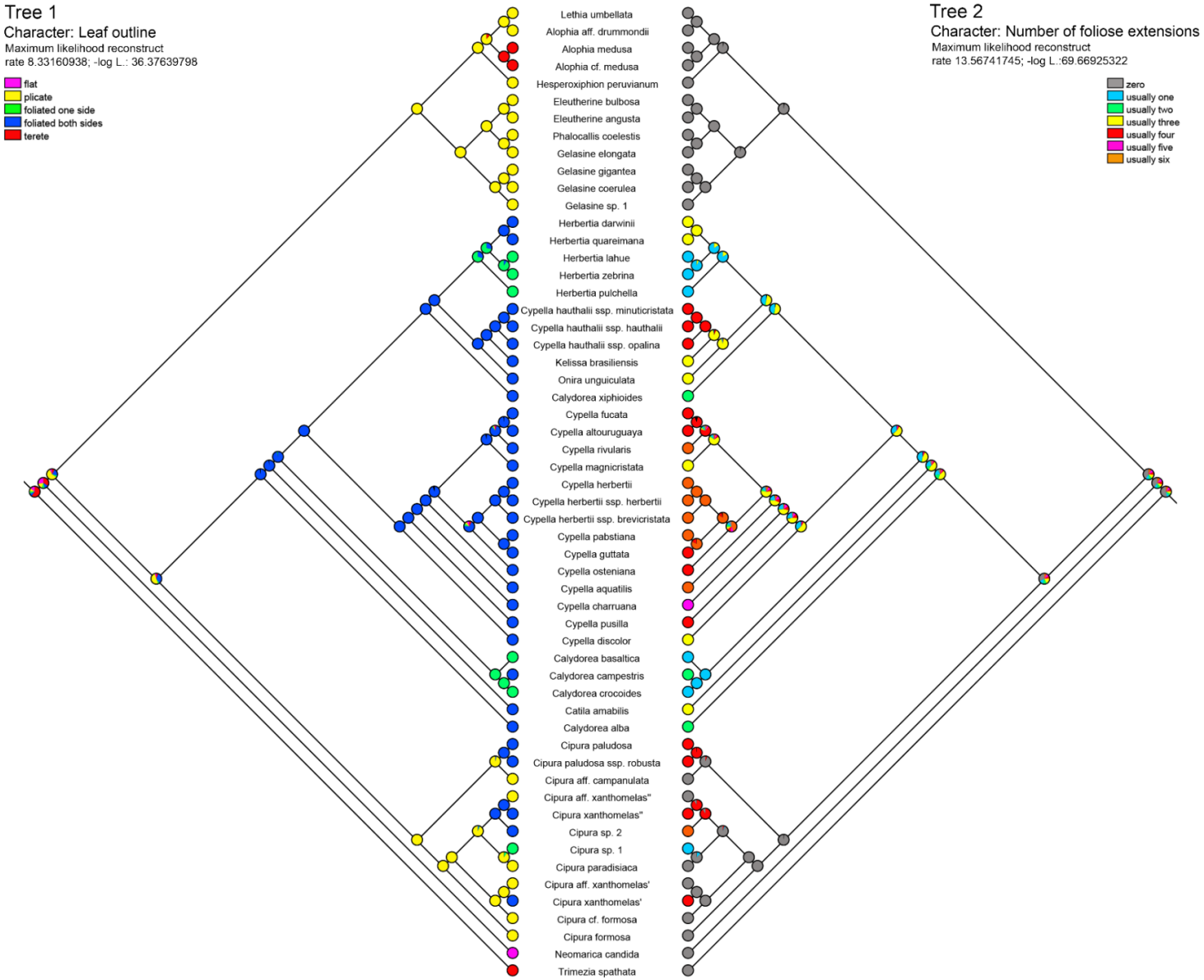
Species	Voucher[a]	Morphological and anatomical characters																																
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**
Herbertia lahue	495	2	2	1	0	0	1	2	1	1	2	0	1	4	1	0	2	0	3	4	0	0	0	0	0	0	0	1	0.05	1	0	0	0	0
Herbertia lahue	132	2	2	1	0	0	1	2	1	1	2	0	1	4	1	0	2	0	3	4	0	0	0	0	0	0	0	1	0.06	1	0	0	0	0
Herbertia lahue subsp. *lahue*	488	2	2	1	0	0	1	2	1	1	2	0	1	4	1	0	2	0	3	4	0	0	0	0	0	0	0	1	0.07	1	0	0	0	0
Herbertia lahue subsp. *lahue*	504	2	2	1	0	0	1	2	1	1	2	0	1	4	1	0	2	0	3	4	0	0	0	0	0	0	0	1	0.06	1	0	0	0	0
Herbertia pulchella	182	2	3	1	0	0	1	2	1	1	2	0	1	5	1	0	2	0	3	4	0	0	0	0	0	0	0	1	0.05	1	0	0	0	0
Herbertia quareimana	513	2	2	1	0	0	1	2	1	1	2	0	1	3	3	0	2	0	3	4	0	0	0	0	0	0	0	1	0.06	1	0	0	0	0
Herbertia zebrina	175	2	3	1	0	0	1	2	1	1	2	0	1	2	1	0	2	0	3	4	0	0	0	0	0	0	0	1	0.05	1	0	0	0	0
Herbertia zebrina	110	2	3	1	0	0	1	2	1	1	2	0	1	2	1	0	2	0	3	4	0	0	0	0	0	0	0	1	0.06	1	0	0	0	0
Kelissa brasiliensis	109	3	2	1	0	0	1	2	1	1	2	0	1	3	4	0	2	0	3	3	0	0	0	0	0	0	0	1	0.07	1	0	0	0	0
Kelissa brasiliensis	145	3	2	1	0	0	1	2	1	1	2	0	1	3	4	0	2	0	3	3	0	0	0	0	0	0	0	1	0.06	1	0	0	0	0
Onira unguiculata	162	3	2	1	0	0	1	2	1	1	2	0	1	3	4	0	2	0	3	3	0	0	0	0	0	0	0	1	0.07	1	0	0	0	0
Alophia aff. *drummondii*	15944	1	3	1	0	0	2	2	1	1	?	2	0	5	7	0	0	0	0	1	2	2	0	0	0	0	0	0	-	0	0	0	0	2
Alophia medusa	1007	4	0	0	0	0	2	2	1	1	0	1	0	5	7	0	2	1	3	2	3	2	0	0	0	0	0	0	-	0	0	1	0	0
Alophia medusa	989	4	0	0	0	0	2	2	1	1	0	1	0	5	7	0	2	1	3	2	3	2	0	0	0	0	0	0	-	0	0	1	0	0
Alophia cf. *medusa*	991	4	0	0	0	0	2	2	1	1	0	1	0	5	7	0	0	0	3	4	3	2	0	0	0	0	0	0	-	0	0	2	0	0
Hesperoxiphion peruvianum	190682	1	3	1	0	0	1	0	3	1	5	2	0	5	7	0	0	0	0	1	2	2	0	2	2	2	0	0	-	0	0	0	0	3
Lethia umbellata	1939/1	1	3	2	0	0	1	0	3	1	5	0	0	5	7	0	0	0	0	4	1	1	0	0	0	0	0	0	-	0	0	0	0	3
Phalocallis coelestis	973	1	0	0	0	0	2	0	3	1	5	2	0	5	7	0	0	1	1	3	3	3	3	4	3	3	1	0	-	0	0	2	1	2
Phalocallis coelestis	977	1	0	0	0	0	2	0	3	1	5	2	0	5	7	0	0	1	1	3	3	3	3	4	3	3	1	0	-	0	0	2	1	2
Eleutherine angusta	933	1	0	0	0	0	2	0	3	1	5	2	0	5	7	0	0	0	0	1	3	3	2	2	2	3	0	0	-	0	0	2	2	2
Eleutherine angusta	937	1	0	0	0	0	2	0	3	1	5	2	0	5	7	0	0	0	0	1	3	3	2	2	2	3	0	0	-	0	0	2	2	2
Eleutherine bulbosa	82763	1	0	0	0	0	2	0	3	1	5	2	0	5	7	0	0	0	0	1	3	3	2	2	2	3	0	0	-	0	0	2	3	2
Gelasine coerulea	676	1	1	1	0	0	2	2	1	1	5	2	0	5	7	1	1	1	0	1	3	3	1	1	2	3	0	0	-	0	1	2	0	2
Gelasine elongata	713	1	1	1	0	0	2	0	3	1	5	2	0	5	7	0	0	0	0	1	3	3	0	1	3	3	1	0	-	0	0	2	0	2
Gelasine elongata	202	1	1	1	0	0	2	0	3	1	5	2	0	5	7	0	0	0	0	1	3	3	0	1	3	3	1	0	-	0	0	2	0	2
Gelasine gigantea	1000	1	3	1	0	0	1	0	4	1	5	2	0	5	7	0	0	0	0	1	3	3	1	1	2	3	0	0	-	0	0	2	3	2
Gelasine gigantea	1000-B	1	3	1	0	0	1	0	4	1	5	2	0	5	7	0	0	0	0	1	3	3	1	1	2	3	0	0	-	0	0	2	3	2

Species	**Voucher**[a]																																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Gelasine sp. 1	1022	1	3	1	0	0	1	0	3	1	5	2	0	5	7	0	0	0	0	1	2	2	1	2	2	3	0	0	-	0	0	2	3	2
Gelasine sp. 1	1026	1	3	1	0	0	1	0	3	1	5	2	0	5	7	0	0	0	0	1	2	2	1	2	2	3	0	0	-	0	0	2	3	2
Outgroup																																		
Trimezia juncifolia	926	4	0	0	0	0	0	0	0	1	0	1	0	5	7	0	2	0	3	1	0	0	0	0	0	0	0	0	-	0	0	0	0	0
Trimezia juncifolia subsp. *speciosa*	928	4	0	0	0	0	0	0	0	1	0	1	0	5	7	0	2	0	3	1	0	0	0	0	0	0	0	0	-	0	0	0	0	0
Trimezia spathata	929	0	0	0	1	0	0	0	0	1	0	1	0	5	7	0	2	0	0	0	0	0	0	0	0	0	0	0	-	0	0	0	0	0
Trimezia spathata	177	0	0	0	1	0	0	0	0	1	0	1	0	5	7	0	2	0	0	0	0	0	0	0	0	0	0	0	-	0	0	0	0	0
Neomarica aff. *caerulea*	931	0	0	0	0	0	0	0	0	1	0	1	0	5	7	0	2	0	0	0	0	0	0	0	0	0	0	0	-	0	0	0	0	0

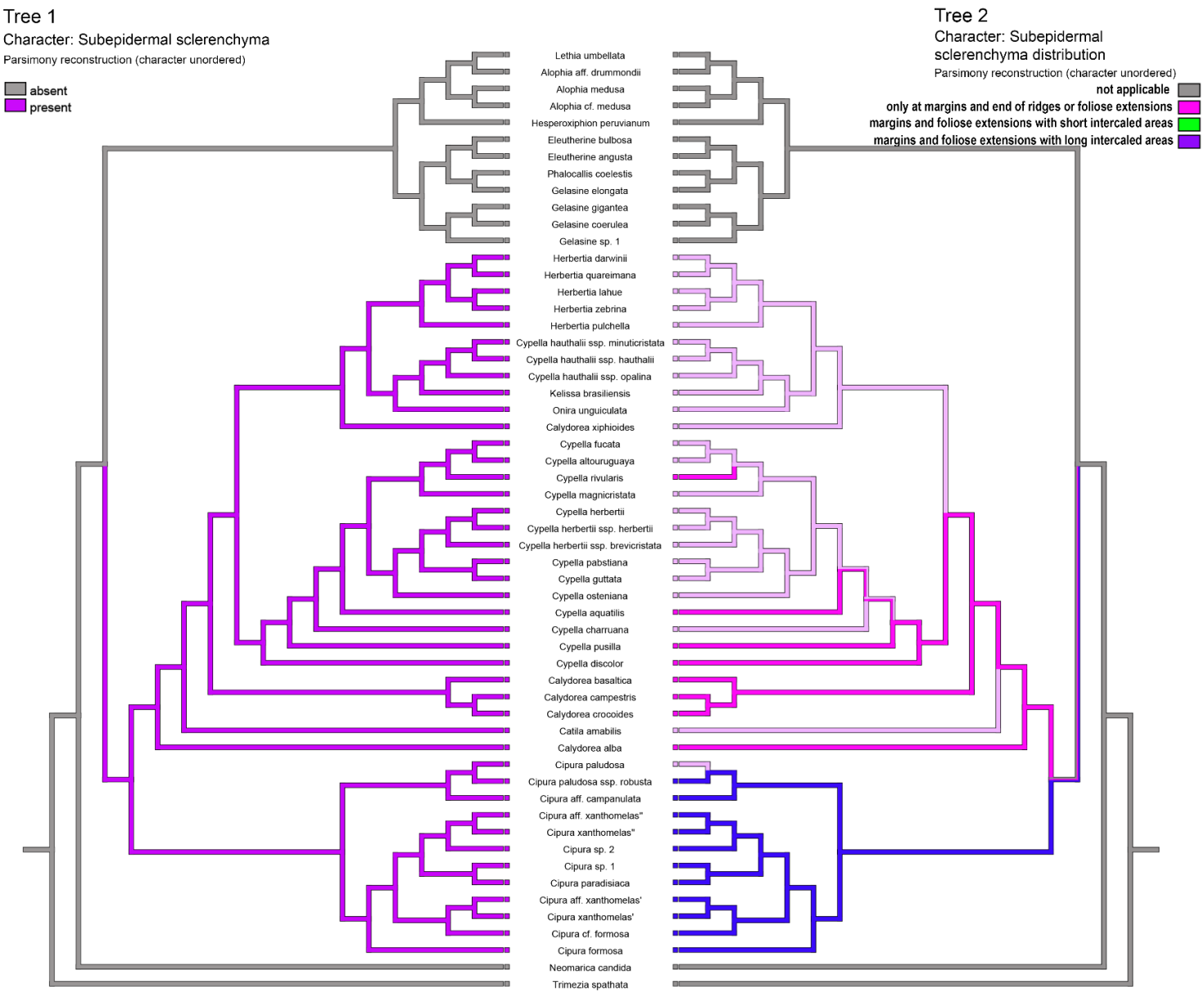
Notes: a= Voucher information according Appendix S1.


Tree 1
Character: Leaf outline
Parsimony reconstruction (character unordered)
flat
plicate
foliated one side
foliated both sides
terete
Tree 2
Character: Number of foliose extensions
Parsimony reconstruction (character unordered)
zero
usually one
usually two
usually three
usually four
usually five
usually six
Lethia umbellata
Alophia aff. drummondii
Alophia medusa
Alophia cf. medusa
Hesperoxiphion peruvianum
Eleutherine bulbosa
Eleutherine angusta
Phalocallis coelestis
Gelasine elongata
Gelasine gigantea
Gelasine coerulea
Gelasine sp. 1
Herbertia darwinii
Herbertia quareimana
Herbertia lahue
Herbertia zebrina
Herbertia pulchella
Cypella hauthalii ssp. minuticristata
Cypella hauthalii ssp. hauthalii
Cypella hauthalii ssp. opalina
Kelissa brasiliensis
Onira unguiculata
Calydorea xiphioides
Cypella fucata
Cypella altouruguaya
Cypella rivularis
Cypella magnicristata
Cypella herbertii
Cypella herbertii ssp. herbertii
Cypella herbertii ssp. brevicristata
Cypella pabstiana
Cypella guttata
Cypella osteniana
Cypella aquatilis
Cypella charruana
Cypella pusilla
Cypella discolor
Calydorea basaltica
Calydorea campestris
Calydorea crocoides
Catila amabilis
Calydorea alba
Cipura paludosa
Cipura paludosa ssp. robusta
Cipura aff. campanulata
Cipura aff. xanthomelas"
Cipura xanthomelas"
Cipura sp. 2
Cipura sp. 1
Cipura paradisiaca
Cipura aff. xanthomelas'
Cipura xanthomelas'
Cipura cf. formosa
Cipura formosa
Neomarica candida
Trimezia spathata

Appendix S10. Characters evolution optimized into the maximum likelihood (ML) best-scoring tree, obtained from cpDNA analysis, and includes one individual for each species. Characters were optimized on the tree using maximum parsimony (MP) method. Tree 1: Leaf outline and Tree 2: Number of foliose extensions.


Tree 1
Character: Leaf outline
Maximum likelihood reconstruct
rate 8.3316093 8; -log L.: 36.37639798
flat
plicate
foliated one side
foliated both sides
terete
Lethia umbellata
Alophia aff. drummondii
Alophia medusa
Alophia cf. medusa
Hesperoxiphion peruvianum
Eleutherine bulbosa
Eleutherine angusta
Phalocallis coelestis
Gelasine elongata
Gelasine gigantea
Gelasine coerulea
Gelasine sp. 1
Herbertia darwinii
Herbertia quareimana
Herbertia lahue
Herbertia zebrina
Herbertia pulchella
Cypella hauthalii ssp. minuticristata
Cypella hauthalii ssp. hauthalii
Cypella hauthalii ssp. opalina
Kelissa brasiliensis
Onira unguiculata
Calydorea xiphioides
Cypella fucata
Cypella altouruguaya
Cypella rivularis
Cypella magnicristata
Cypella herbertii
Cypella herbertii ssp. herbertii
Cypella herbertii ssp. brevicristata
Cypella pabstiana
Cypella guttata
Cypella osteniana
Cypella aquatilis
Cypella charruana
Cypella pusilla
Cypella discolor
Calydorea basaltica
Calydorea campestris
Calydorea crocoides
Catila amabilis
Calydorea alba
Cipura paludosa
Cipura paludosa ssp. robusta
Cipura aff. campanulata
Cipura aff. xanthomelas''
Cipura xanthomelas''
Cipura sp. 2
Cipura sp. 1
Cipura paradisiaca
Cipura aff. xanthomelas'
Cipura xanthomelas'
Cipura cf. formosa
Cipura formosa
Neomarica candida
Trimezia spathata
Tree 2
Character: Number of foliose extensions
Maximum likelihood reconstruct
rate 13.56741745; -log L.:69.66925322
zero
usually one
usually two
usually three
usually four
usually five
usually six

Appendix S11. Characters evolution optimized into the maximum likelihood (ML) best-scoring tree, obtained from cpDNA analysis, and includes one individual for each species. Characters were optimized on the tree using maximum likelihood (ML) method. Tree 1: Leaf outline and Tree 2: Number of foliose extensions.


Tree 1
Character: Subepidermal sclerenchyma
Parsimony reconstruction (character unordered)
absent
present
Tree 2
Character: Subepidermal sclerenchyma distribution
Parsimony reconstruction (character unordered)
not applicable
only at margins and end of ridges or foliose extensions
margins and foliose extensions with short intercaled areas
margins and foliose extensions with long intercaled areas
Lethia umbellata
Alophia aff. drummondii
Alophia medusa
Alophia cf. medusa
Hesperoxiphion peruvianum
Eleutherine bulbosa
Eleutherine angusta
Phalocallis coelestis
Gelasine elongata
Gelasine gigantea
Gelasine coerulea
Gelasine sp. 1
Herbertia darwinii
Herbertia quareimana
Herbertia lahue
Herbertia zebrina
Herbertia pulchella
Cypella hauthalii ssp. minuticristata
Cypella hauthalii ssp. hauthalii
Cypella hauthalii ssp. opalina
Kelissa brasiliensis
Onira unguiculata
Calydorea xiphioides
Cypella fucata
Cypella altouruguaya
Cypella rivularis
Cypella magnicristata
Cypella herbertii
Cypella herbertii ssp. herbertii
Cypella herbertii ssp. brevicristata
Cypella pabstiana
Cypella guttata
Cypella osteniana
Cypella aquatilis
Cypella charruana
Cypella pusilla
Cypella discolor
Calydorea basaltica
Calydorea campestris
Calydorea crocoides
Catila amabilis
Calydorea alba
Cipura paludosa
Cipura paludosa ssp. robusta
Cipura aff. campanulata
Cipura aff. xanthomelas"
Cipura xanthomelas"
Cipura sp. 2
Cipura sp. 1
Cipura paradisiaca
Cipura aff. xanthomelas'
Cipura xanthomelas'
Cipura cf. formosa
Cipura formosa
Neomarica candida
Trimezia spathata

Appendix S12. Characters evolution optimized into the maximum likelihood (ML) best-scoring tree, obtained from cpDNA analysis, and includes one individual for each species. Characters were optimized on the tree using maximum parsimony (MP) method. Tree 1: Subepidermal sclerenchyma and Tree 2: Subepidermal sclerenchyma distribution.


Tree 1
Character: Subepidermal sclerenchyma
Maximum likelihood reconstruct
rate 4.07739534; -log. : 5.4877745
absent
present
Lethia umbellata
Alophia aff. drummondii
Alophia medusa
Alophia cf. medusa
Hesperoxiphion peruvianum
Eleutherine bulbosa
Eleutherine angusta
Phalocallis coelestis
Gelasine elongata
Gelasine gigantea
Gelasine coerulea
Gelasine sp. 1
Herbertia darwinii
Herbertia quareimana
Herbertia lahue
Herbertia zebrina
Herbertia pulchella
Cypella hauthalii ssp. minuticristata
Cypella hauthalii ssp. hauthalii
Cypella hauthalii ssp. opalina
Kelissa brasiliensis
Onira unguiculata
Calydorea xiphioides
Cypella fucata
Cypella altouruguaya
Cypella rivularis
Cypella magnicristata
Cypella herbertii
Cypella herbertii ssp. herbertii
Cypella herbertii ssp. brevicristata
Cypella pabstiana
Cypella guttata
Cypella osteniana
Cypella aquatilis
Cypella charruana
Cypella pusilla
Cypella discolor
Calydorea basaltica
Calydorea campestris
Calydorea crocoides
Catila amabilis
Calydorea alba
Cipura paludosa
Cipura paludosa ssp. robusta
Cipura aff. campanulata
Cipura aff. xanthomelas"
Cipura xanthomelas"
Cipura sp. 2
Cipura sp. 1
Cipura paradisiaca
Cipura aff. xanthomelas'
Cipura xanthomelas'
Cipura cf. formosa
Cipura formosa
Neomarica candida
Trimezia spathata
Tree 2
Character: Subepidermal sclerenchyma distribution
Maximum likelihood reconstruct
rate 14.17435672 -log L.:39.9771837
not applicable
only at margins and end of ridges or foliose extensions
margins and foliose extensions with short intercaled areas
margins and foliose extensions with long intercaled areas

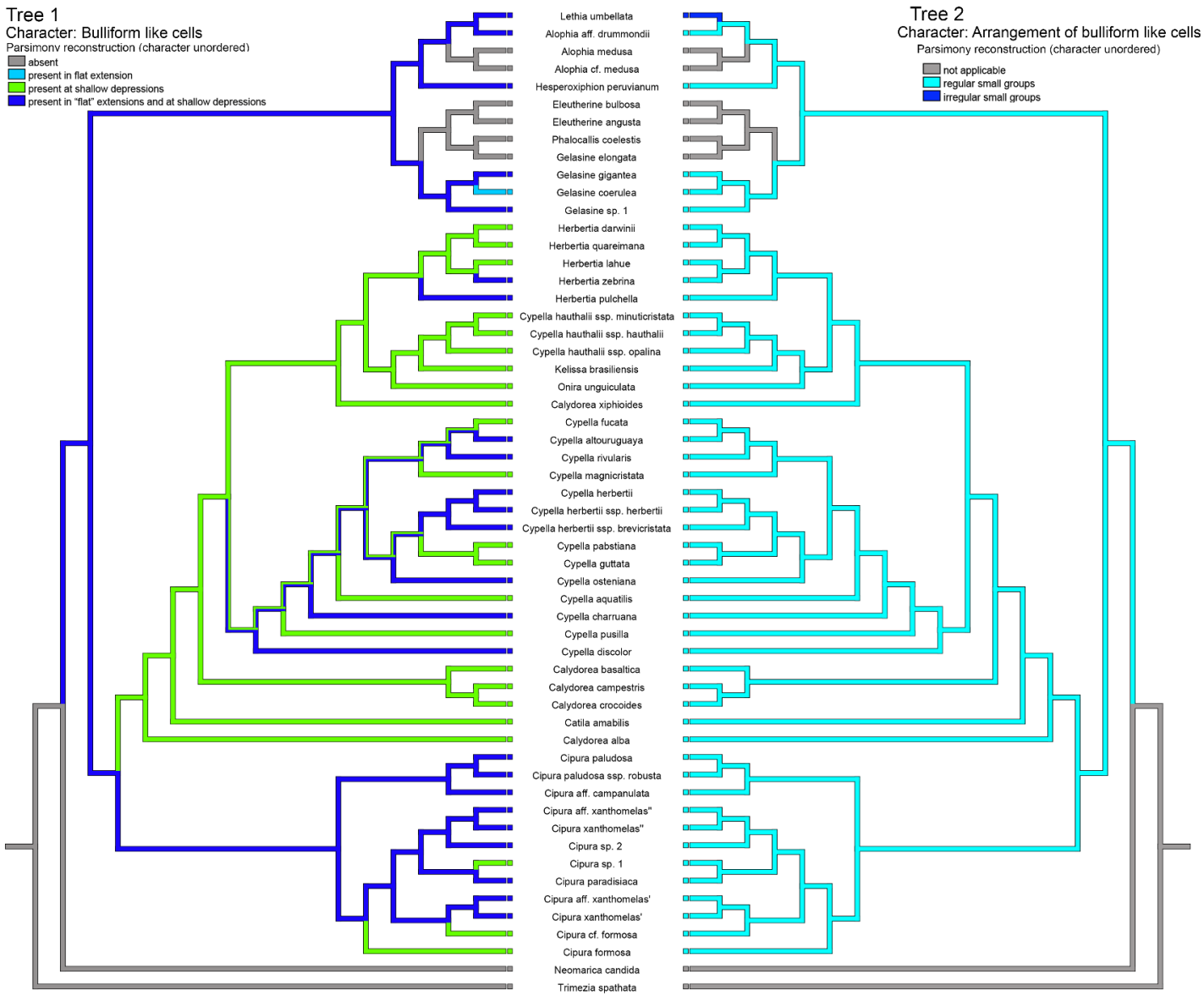
Appendix S13. Characters evolution optimized into the maximum likelihood (ML) best-scoring tree, obtained from cpDNA analysis, and includes one individual for each species. Characters were optimized on the tree using maximum likelihood (ML) method. Tree 1: Subepidermal sclerenchyma and Tree 2: Subepidermal sclerenchyma distribution.

Appendix S14. Species of Tigridieae (Clade A) analysed according to the biogeographical dominions, regions and province for South America (Morrone 2014).

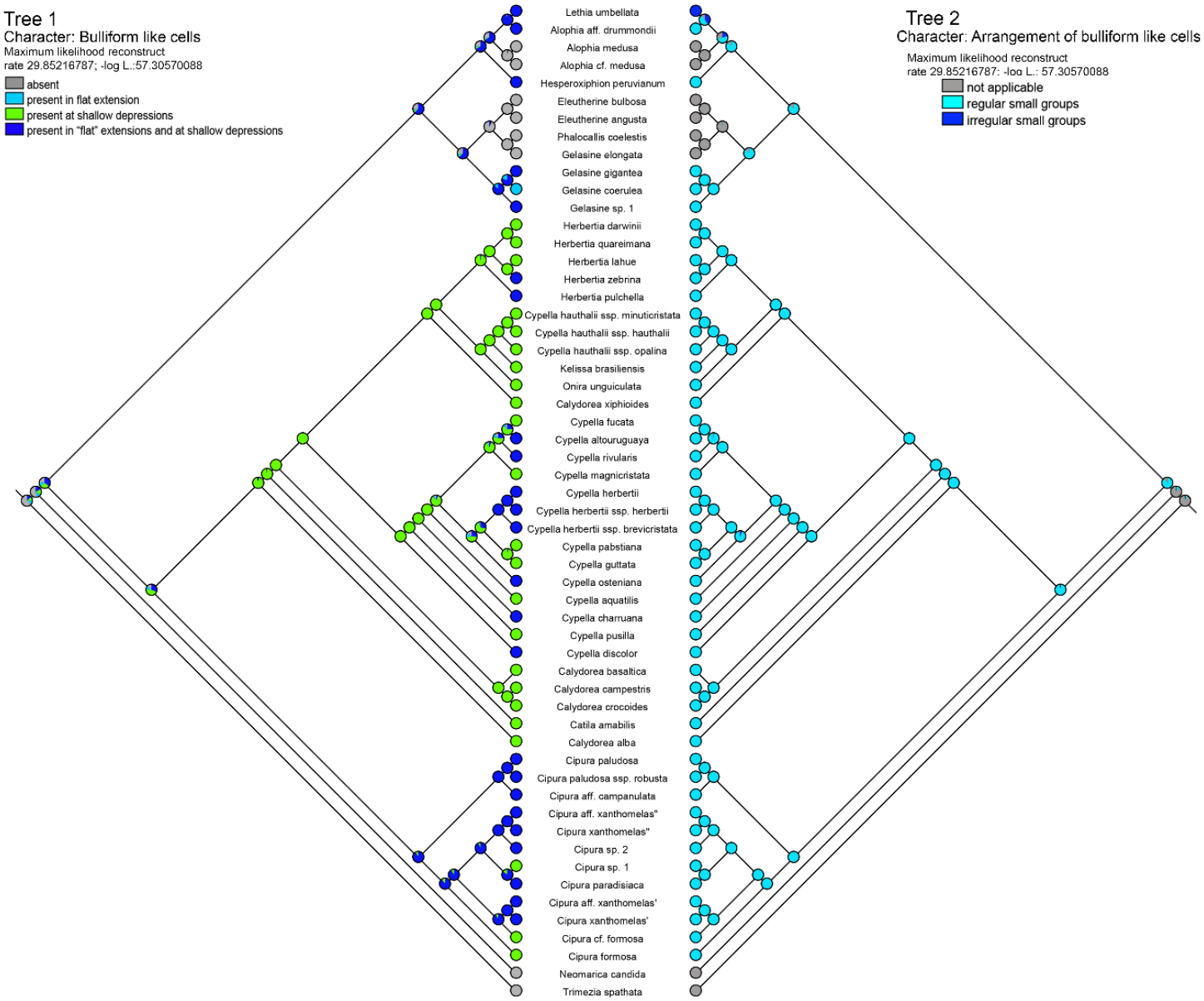
Species	*Biogeographic regions*			
	Voucher	Province	Region	Dominions
Calydorea alba	125	D	G	H
Calydorea basaltica	916	D	G	H
Calydorea campestris	921	A	G	I
Calydorea crocoides	677	A	G	I
Calydorea xiphioides	SP875	-	F	
Catila amabilis	742	D	G	H
Cipura aff. xanthomelas	906B	C	G	H
Cipura aff. xanthomelas	909	C	G	H
Cipura aff. campanulata	1013	C	G	H
Cipura cf. *formosa*	996	C	G	H
Cipura formosa	994	C	G	H
Cipura formosa	902	C	G	H
Cipura paludosa subsp. *robusta*	1010	C	G	H
Cipura paludosa subsp. *robusta*	1014	C	G	H
Cipura paludosa subsp. *robusta*	1019	C	G	H
Cipura paludosa subsp. *robusta*	1020	C	G	H
Cipura paludosa	906C	C	G	H
Cipura paradisiaca	900	C	G	H
Cipura paradisiaca	993	C	G	H
Cipura sp. 1	906A	C	G	H
Cipura sp. 2	984	C	G	H
Cipura sp. 2	985	C	G	H
Cipura sp. 2	990	C	G	H
Cipura xanthomelas	924	C	G	H
Cipura xanthomelas	987	C	G	H
Cipura xanthomelas	1009	C	G	H
Cypella altouruguaya	716	C	G	H
Cypella aquatilis	714	A	G	I
Cypella charruana	203	D	G	H
Cypella discolor	75	D	G	H
Cypella fucata	122	D	G	H
Cypella guttata	204	D	G	H
Cypella hauthalii	820	D	G	H
Cypella hauthalii subsp. *opalina*	128	D	G	H
Cypella hauthalii subsp. *minuticristata*	728	D	G	H
Cypella herbertii	725	E	G	I
Cypella herbertii	918	B	G	I
Cypella herbertii	260	D	G	H
Cypella herbertii subsp. herbertii	77	D	G	H

Biogeographic regions				
Species	Voucher	Province	Region	Dominions
Cypella herbertii subsp. *brevicristata*	752	D	G	H
Cypella magnicristata	76	D	G	H
Cypella osteniana	851	D	G	H
Cypella pabstiana	741	D	G	H
Cypella pusilla	119	D	G	H
Cypella pusilla	174	D	G	H
Cypella pusilla	818	D	G	H
Cypella rivularis	800	D	G	H
Herbertia darwinii	502	D	G	H
Herbertia darwinii	123	D	G	H
Herbertia darwinii	140	D	G	H
Herbertia lahue	495	D	G	H
Herbertia lahue	132	D	G	H
Herbertia lahue subsp. *lahue*	488	D	G	H
Herbertia lahue subsp. *lahue*	504	D	G	H
Herbertia pulchella	182	D	G	H
Herbertia quareimana	513	D	G	H
Herbertia zebrina	175	D	G	H
Herbertia zebrina	110	D	G	H
Kelissa brasiliensis	109	D	G	H
Kelissa brasiliensis	145	D	G	H
Onira unguiculata	162	D	G	H

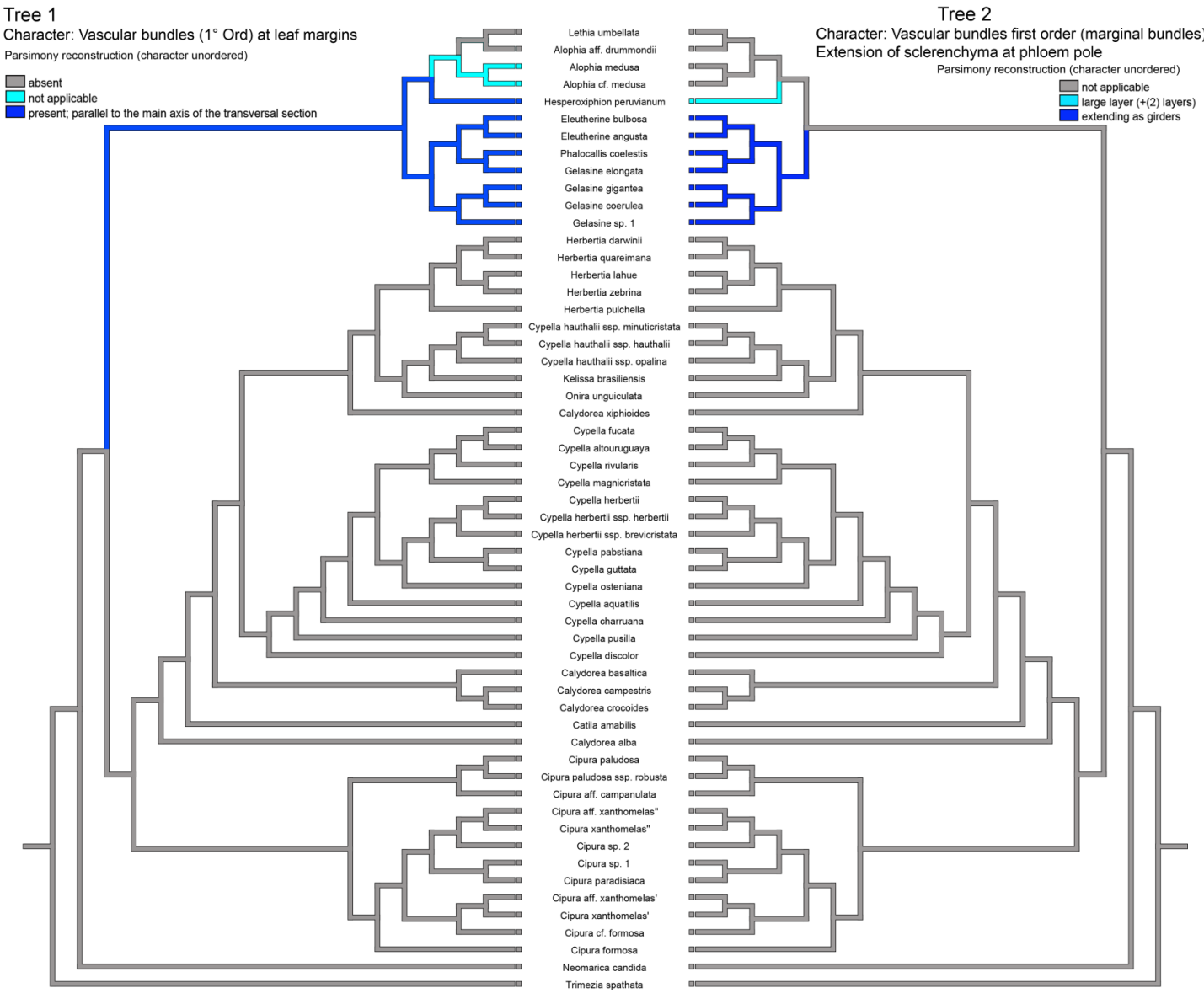
Notes: A = Araucaria Forest province; B= Atlantic province; C=Cerrado province; D =Pampean province; E= Parana Forest, F= Andean region; G= Neotropical region; H= Chacoan dominion; I= Parana dominion .


Tree 1
Character: Bulliform like cells
Parsimony reconstruction (character unordered)
absent
present in flat extension
present at shallow depressions
present in "flat" extensions and at shallow depressions
Lethia umbellata
Alophia aff. drummondii
Alophia medusa
Alophia cf. medusa
Hesperoxiphion peruvianum
Eleutherine bulbosa
Eleutherine angusta
Phalocallis coelestis
Gelasine elongata
Gelasine gigantea
Gelasine coerulea
Gelasine sp. 1
Herbertia darwinii
Herbertia quareimana
Herbertia lahue
Herbertia zebrina
Herbertia pulchella
Cypella hauthalii ssp. minuticristata
Cypella hauthalii ssp. hauthalii
Cypella hauthalii ssp. opalina
Kelissa brasiliensis
Onira unguiculata
Calydorea xiphioides
Cypella fucata
Cypella altouruguaya
Cypella rivularis
Cypella magnicristata
Cypella herbertii
Cypella herbertii ssp. herbertii
Cypella herbertii ssp. brevicristata
Cypella pabstiana
Cypella guttata
Cypella osteniana
Cypella aquatilis
Cypella charruana
Cypella pusilla
Cypella discolor
Calydorea basaltica
Calydorea campestris
Calydorea crocoides
Catila amabilis
Calydorea alba
Cipura paludosa
Cipura paludosa ssp. robusta
Cipura aff. campanulata
Cipura aff. xanthomelas"
Cipura xanthomelas"
Cipura sp. 2
Cipura sp. 1
Cipura paradisiaca
Cipura aff. xanthomelas'
Cipura xanthomelas'
Cipura cf. formosa
Cipura formosa
Neomarica candida
Trimezia spathata
Tree 2
Character: Arrangement of bulliform like cells
Parsimony reconstruction (character unordered)
not applicable
regular small groups
irregular small groups

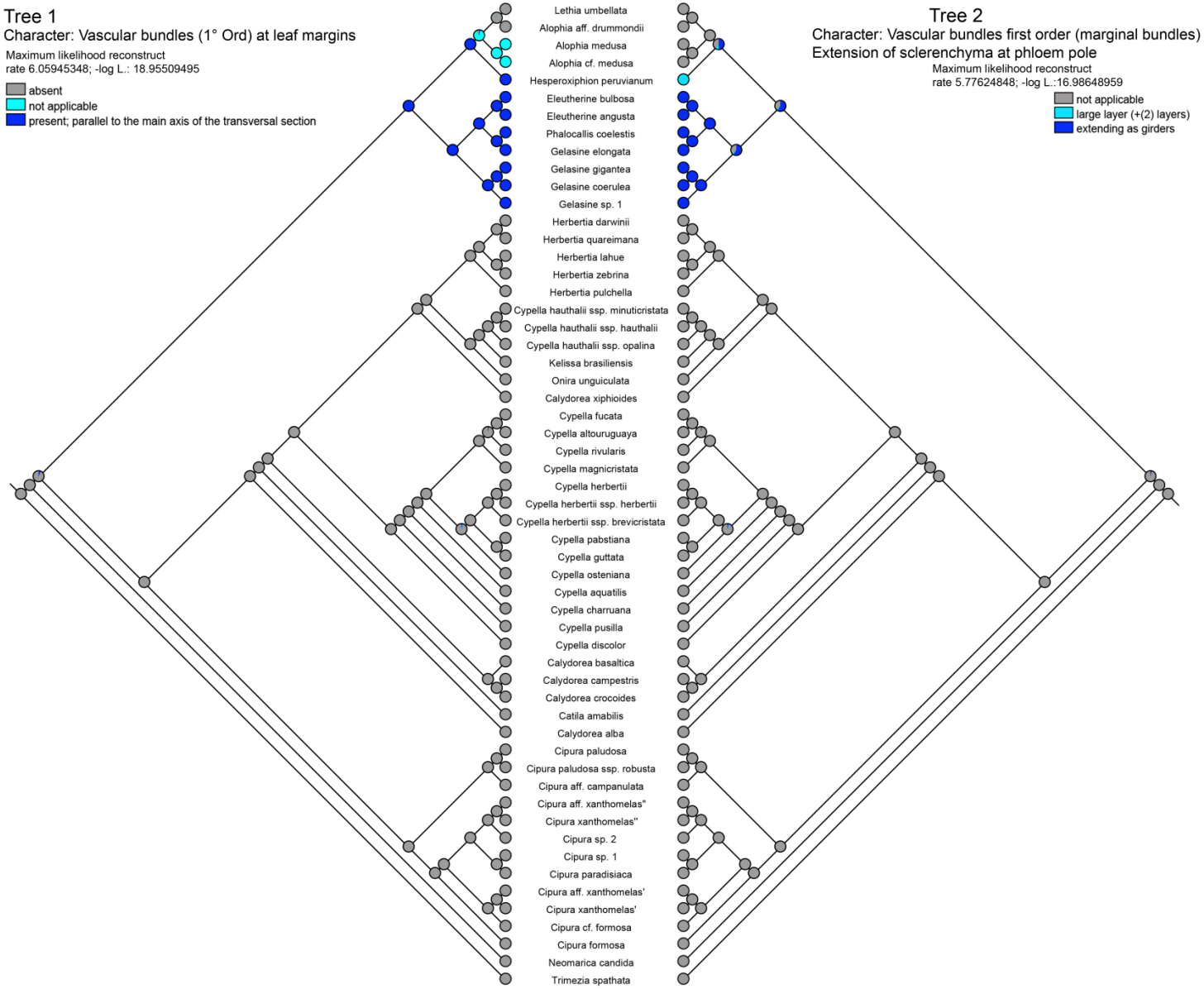
Appendix S15. Characters evolution optimized into the maximum likelihood (ML) best-scoring tree, obtained from cpDNA analysis, and includes one individual for each species. Characters were optimized on the tree using maximum parsimony (MP) method. Tree 1: Bulliform like cells with pectin content and Tree 2: Arrangement of bulliform like cells with pectin content**.**


Tree 1
Character: Bulliform like cells
Maximum likelihood reconstruct
rate 29.85216787; -log L.:57.30570088
absent
present in flat extension
present at shallow depressions
present in "flat" extensions and at shallow depressions
Tree 2
Character: Arrangement of bulliform like cells
Maximum likelihood reconstruct
rate 29.85216787; -log L.: 57.30570088
not applicable
regular small groups
irregular small groups
Lethia umbellata
Alophia aff. drummondii
Alophia medusa
Alophia cf. medusa
Hesperoxiphion peruvianum
Eleutherine bulbosa
Eleutherine angusta
Phalocallis coelestis
Gelasine elongata
Gelasine gigantea
Gelasine coerulea
Gelasine sp. 1
Herbertia darwinii
Herbertia quareimana
Herbertia lahue
Herbertia zebrina
Herbertia pulchella
Cypella hauthalii ssp. minuticristata
Cypella hauthalii ssp. hauthalii
Cypella hauthalii ssp. opalina
Kelissa brasiliensis
Onira unguiculata
Calydorea xiphioides
Cypella fucata
Cypella altouruguaya
Cypella rivularis
Cypella magnicristata
Cypella herbertii
Cypella herbertii ssp. herbertii
Cypella herbertii ssp. brevicristata
Cypella pabstiana
Cypella guttata
Cypella osteniana
Cypella aquatilis
Cypella charruana
Cypella pusilla
Cypella discolor
Calydorea basaltica
Calydorea campestris
Calydorea crocoides
Catila amabilis
Calydorea alba
Cipura paludosa
Cipura paludosa ssp. robusta
Cipura aff. campanulata
Cipura aff. xanthomelas"
Cipura xanthomelas"
Cipura sp. 2
Cipura sp. 1
Cipura paradisiaca
Cipura aff. xanthomelas'
Cipura xanthomelas'
Cipura cf. formosa
Cipura formosa
Neomarica candida
Trimezia spathata

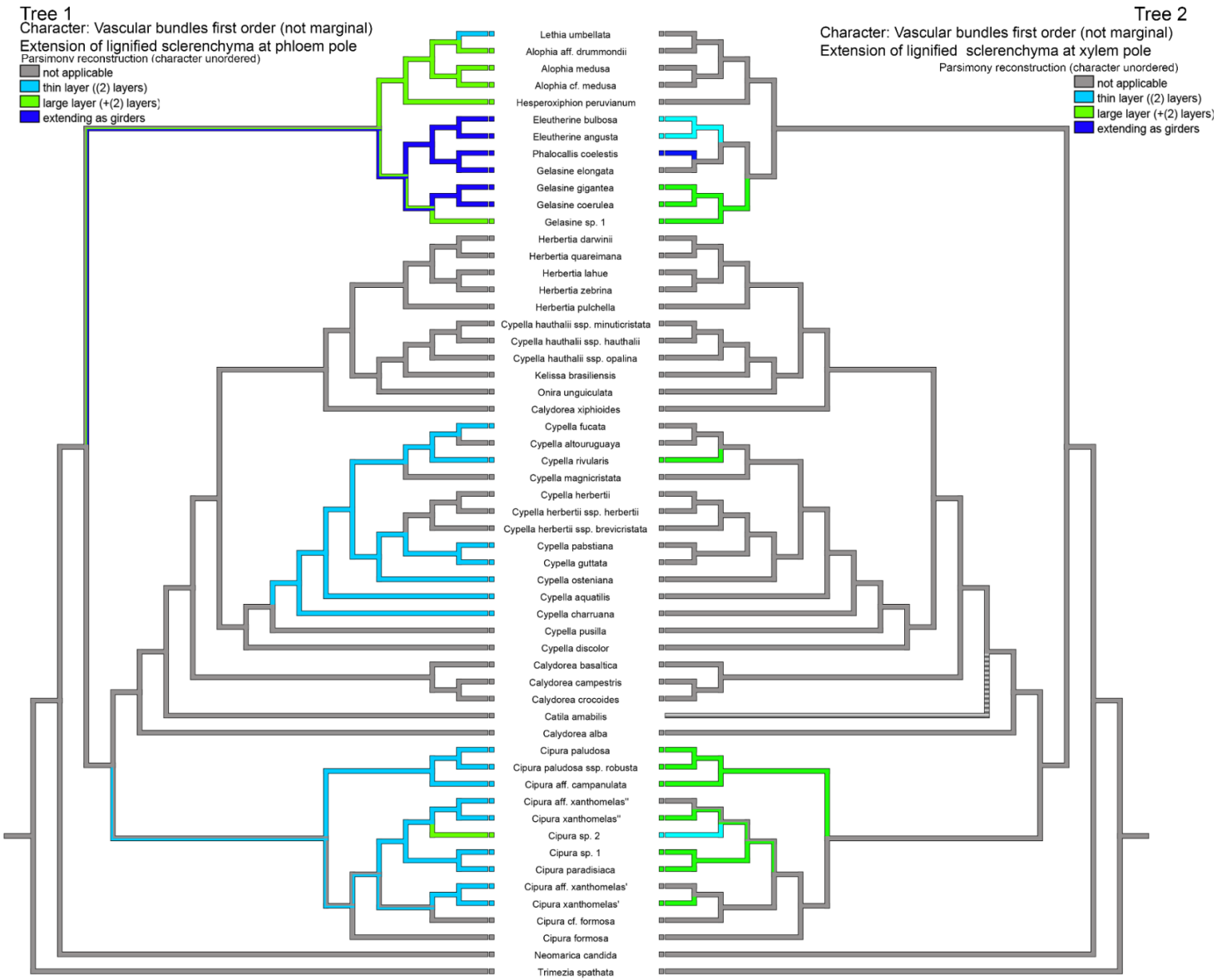
Appendix S16. Characters evolution optimized into the maximum likelihood (ML) best-scoring tree, obtained from cpDNA analysis, and includes one individual for each species. Characters were optimized on the tree using maximum likelihood (ML) method. Tree 1: Bulliform like cells with pectin content and Tree 2: Arrangement of bulliform like cells with pectin content**.**


Tree 1
Character: Vascular bundles (1° Ord) at leaf margins
Parsimony reconstruction (character unordered)
absent
not applicable
present; parallel to the main axis of the transversal section
Tree 2
Character: Vascular bundles first order (marginal bundles)
Extension of sclerenchyma at phloem pole
Parsimony reconstruction (character unordered)
not applicable
large layer (+(2) layers)
extending as girders
Lethia umbellata
Alophia aff. drummondii
Alophia medusa
Alophia cf. medusa
Hesperoxiphion peruvianum
Eleutherine bulbosa
Eleutherine angusta
Phalocallis coelestis
Gelasine elongata
Gelasine gigantea
Gelasine coerulea
Gelasine sp. 1
Herbertia darwinii
Herbertia quareimana
Herbertia lahue
Herbertia zebrina
Herbertia pulchella
Cypella hauthalii ssp. minuticristata
Cypella hauthalii ssp. hauthalii
Cypella hauthalii ssp. opalina
Kelissa brasiliensis
Onira unguiculata
Calydorea xiphioides
Cypella fucata
Cypella altouruguaya
Cypella rivularis
Cypella magnicristata
Cypella herbertii
Cypella herbertii ssp. herbertii
Cypella herbertii ssp. brevicristata
Cypella pabstiana
Cypella guttata
Cypella osteniana
Cypella aquatilis
Cypella charruana
Cypella pusilla
Cypella discolor
Calydorea basaltica
Calydorea campestris
Calydorea crocoides
Catila amabilis
Calydorea alba
Cipura paludosa
Cipura paludosa ssp. robusta
Cipura aff. campanulata
Cipura aff. xanthomelas"
Cipura xanthomelas"
Cipura sp. 2
Cipura sp. 1
Cipura paradisiaca
Cipura aff. xanthomelas'
Cipura xanthomelas'
Cipura cf. formosa
Cipura formosa
Neomarica candida
Trimezia spathata

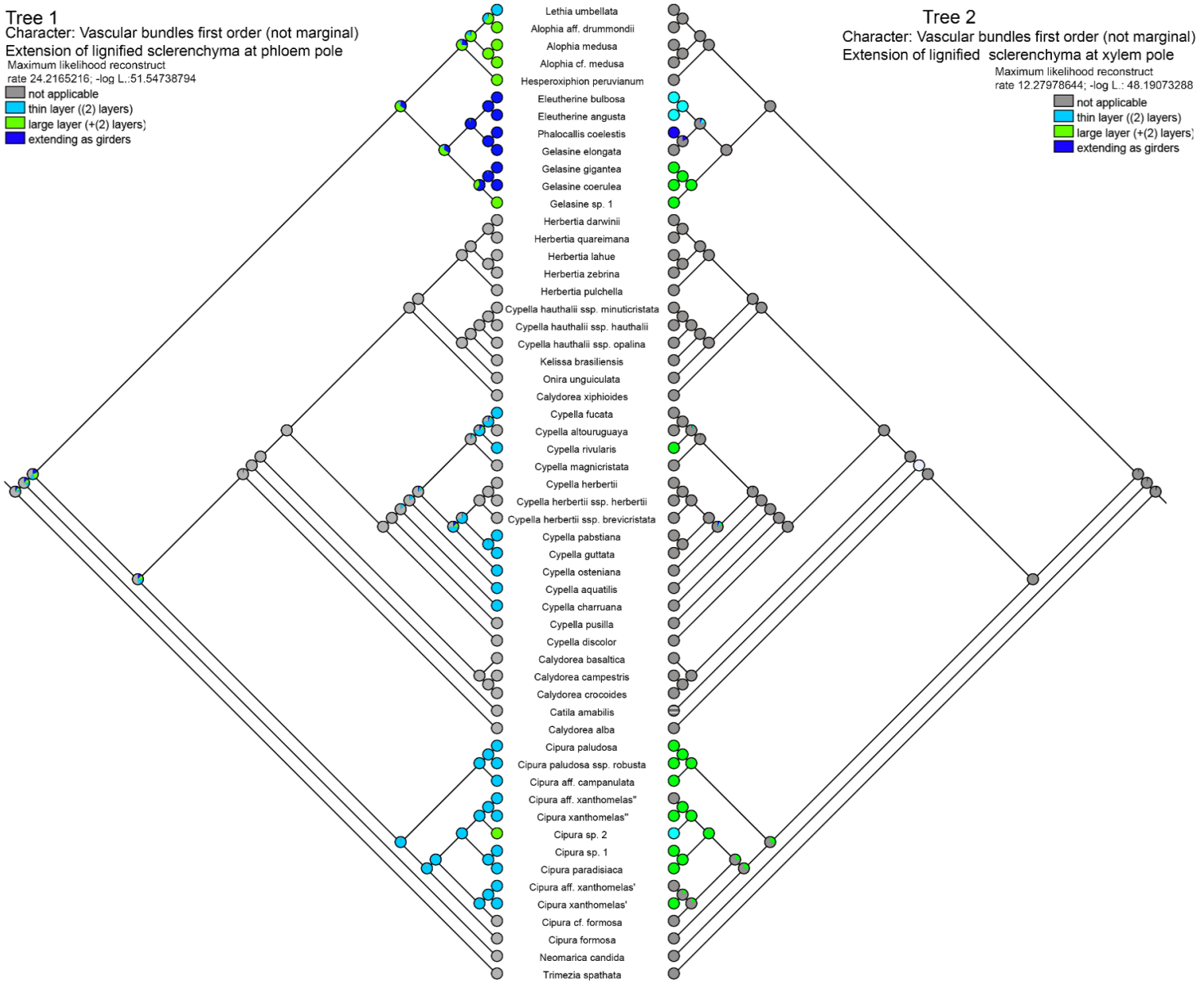
Appendix S17. Characters evolution optimized into the maximum likelihood (ML) best-scoring tree, obtained from cpDNA analysis, and includes one individual for each species. Characters were optimized on the tree using maximum parsimony (MP) method. Tree 1: Vascular bundles (1st ord.) at leaf margins and Tree 2: Extension of sclerenchyma at phloem pole.


Tree 1
Character: Vascular bundles (1° Ord) at leaf margins
Maximum likelihood reconstruct
rate 6.05945348; -log L.: 18.95509495
absent
not applicable
present; parallel to the main axis of the transversal section
Tree 2
Character: Vascular bundles first order (marginal bundles)
Extension of sclerenchyma at phloem pole
Maximum likelihood reconstruct
rate 5.77624848; -log L.:16.98648959
not applicable
large layer (+(2) layers)
extending as girders
Lethia umbellata
Alophia aff. drummondii
Alophia medusa
Alophia cf. medusa
Hesperoxiphion peruvianum
Eleutherine bulbosa
Eleutherine angusta
Phalocallis coelestis
Gelasine elongata
Gelasine gigantea
Gelasine coerulea
Gelasine sp. 1
Herbertia darwinii
Herbertia quareimana
Herbertia lahue
Herbertia zebrina
Herbertia pulchella
Cypella hauthalii ssp. minuticristata
Cypella hauthalii ssp. hauthalii
Cypella hauthalii ssp. opalina
Kelissa brasiliensis
Onira unguiculata
Calydorea xiphioides
Cypella fucata
Cypella altouruguaya
Cypella rivularis
Cypella magnicristata
Cypella herbertii
Cypella herbertii ssp. herbertii
Cypella herbertii ssp. brevicristata
Cypella pabstiana
Cypella guttata
Cypella osteniana
Cypella aquatilis
Cypella charruana
Cypella pusilla
Cypella discolor
Calydorea basaltica
Calydorea campestris
Calydorea crocoides
Catila amabilis
Calydorea alba
Cipura paludosa
Cipura paludosa ssp. robusta
Cipura aff. campanulata
Cipura aff. xanthomelas"
Cipura xanthomelas"
Cipura sp. 2
Cipura sp. 1
Cipura paradisiaca
Cipura aff. xanthomelas'
Cipura xanthomelas'
Cipura cf. formosa
Cipura formosa
Neomarica candida
Trimezia spathata

Appendix S18. Characters evolution optimized into the maximum likelihood (ML) best-scoring tree, obtained from cpDNA analysis, and includes one individual for each species. Characters were optimized on the tree using maximum likelihood (ML) method. Tree 1: Vascular bundles (1st ord.) at leaf margins and Tree 2: Extension of sclerenchyma at phloem pole.


Tree 1
Character: Vascular bundles first order (not marginal)
Extension of lignified sclerenchyma at phloem pole
Parsimony reconstruction (character unordered)
not applicable
thin layer ((2) layers)
large layer (+(2) layers)
extending as girders
Tree 2
Character: Vascular bundles first order (not marginal)
Extension of lignified sclerenchyma at xylem pole
Parsimony reconstruction (character unordered)
not applicable
thin layer ((2) layers)
large layer (+(2) layers)
extending as girders
Lethia umbellata
Alophia aff. drummondii
Alophia medusa
Alophia cf. medusa
Hesperoxiphion peruvianum
Eleutherine bulbosa
Eleutherine angusta
Phalocallis coelestis
Gelasine elongata
Gelasine gigantea
Gelasine coerulea
Gelasine sp. 1
Herbertia darwinii
Herbertia quareimana
Herbertia lahue
Herbertia zebrina
Herbertia pulchella
Cypella hauthalii ssp. minuticristata
Cypella hauthalii ssp. hauthalii
Cypella hauthalii ssp. opalina
Kelissa brasiliensis
Onira unguiculata
Calydorea xiphioides
Cypella fucata
Cypella altouruguaya
Cypella rivularis
Cypella magnicristata
Cypella herbertii
Cypella herbertii ssp. herbertii
Cypella herbertii ssp. brevicristata
Cypella pabstiana
Cypella guttata
Cypella osteniana
Cypella aquatilis
Cypella charruana
Cypella pusilla
Cypella discolor
Calydorea basaltica
Calydorea campestris
Calydorea crocoides
Catila amabilis
Calydorea alba
Cipura paludosa
Cipura paludosa ssp. robusta
Cipura aff. campanulata
Cipura aff. xanthomelas''
Cipura xanthomelas''
Cipura sp. 2
Cipura sp. 1
Cipura paradisiaca
Cipura aff. xanthomelas'
Cipura xanthomelas'
Cipura cf. formosa
Cipura formosa
Neomarica candida
Trimezia spathata

Appendix S19. Characters evolution optimized into the maximum likelihood (ML) best-scoring tree, obtained from cpDNA analysis, and includes one individual for each species. Characters were optimized on the tree using maximum parsimony (MP) method. Character: Vascular bundles first order (not marginal). Tree 1: Extension of lignified sclerenchyma at phloem pole and Tree 2: Extension of lignified sclerenchyma at xylem pole.


Tree 1
Character: Vascular bundles first order (not marginal)
Extension of lignified sclerenchyma at phloem pole
Maximum likelihood reconstruct
rate 24.2165216; -log L.:51.54738794
not applicable
thin layer ((2) layers)
large layer (+(2) layers)
extending as girders
Lethia umbellata
Alophia aff. drummondii
Alophia medusa
Alophia cf. medusa
Hesperoxiphion peruvianum
Eleutherine bulbosa
Eleutherine angusta
Phalocallis coelestis
Gelasine elongata
Gelasine gigantea
Gelasine coerulea
Gelasine sp. 1
Herbertia darwinii
Herbertia quareimana
Herbertia lahue
Herbertia zebrina
Herbertia pulchella
Cypella hauthalii ssp. minuticristata
Cypella hauthalii ssp. hauthalii
Cypella hauthalii ssp. opalina
Kelissa brasiliensis
Onira unguiculata
Calydorea xiphioides
Cypella fucata
Cypella altouruguaya
Cypella rivularis
Cypella magnicristata
Cypella herbertii
Cypella herbertii ssp. herbertii
Cypella herbertii ssp. brevicristata
Cypella pabstiana
Cypella guttata
Cypella osteniana
Cypella aquatilis
Cypella charruana
Cypella pusilla
Cypella discolor
Calydorea basaltica
Calydorea campestris
Calydorea crocoides
Catila amabilis
Calydorea alba
Cipura paludosa
Cipura paludosa ssp. robusta
Cipura aff. campanulata
Cipura aff. xanthomelas"
Cipura xanthomelas"
Cipura sp. 2
Cipura sp. 1
Cipura paradisiaca
Cipura aff. xanthomelas'
Cipura xanthomelas'
Cipura cf. formosa
Cipura formosa
Neomarica candida
Trimezia spathata
Tree 2
Character: Vascular bundles first order (not marginal)
Extension of lignified sclerenchyma at xylem pole
Maximum likelihood reconstruct
rate 12.27978644; -log L.: 48.19073288
not applicable
thin layer ((2) layers)
large layer (+(2) layers)
extending as girders

Appendix S20. Characters evolution optimized into the maximum likelihood (ML) best-scoring tree, obtained from cpDNA analysis, and includes one individual for each species. Characters were optimized on the tree using maximum likelihood (ML) method. Character: Vascular bundles first order (not marginal). Tree 1: Extension of lignified sclerenchyma at phloem pole and Tree 2: Extension of lignified sclerenchyma at xylem pole.

CAPÍTULO IV

Chemical of floral oils: the role of non-volatile lipids on evolution of the Tigridieae (Iridoideae: Iridaceae)

Abstract This study aim to identify, characterize and compare the principal chemical components in oil-offering species of Tigridieae and test the hypothesis about the relation of these components with the evolution and diversification of the tribe in South America. Were sampled species from *Cypella*, *Cipura*, *Herbertia*, *Kelissa* and *Onira*. Floral oil was extracted with hexane and derivatized, the extract was analysed with Gas Chromatography. Results were obtained from *Cypella*, *Cipura*, *Herbertia* and *Kelissa* and the floral oil of *Onira unguiculata* was not derivatized in any of the samples, so this species was not analysed. The results made possible identify free fatty acids (FFA's esters of fatty acid (FAMES Fatty acids methyl ou ethyl ester), alcohols, aldehydes, ketones, hydrocarbon and monoglycerides. The free fatty acids present in higher percentage of area were: Tetracosenoic acid, Butanoic acid, Hexacosanoic acid, Pentacosanoic acid, Octadecadienoic acid, Dodecanoic acid, Docosenoic acid, Octadecenoic acid and Nonanoic acid. Chemical analyses were used to investigate the composition of the floral non-volatile oil in closely related genera of South American Tigridieae. This study confirmed the occurence of fatty acids in elaiophores of *Cypella*, *Cipura*, *Herbertia* and *Kelissa*. This work provided an important characterization for future studies in pollination biology and hypothesis for the utilization of this oil for the collecting bees.

Keywords: BSTFA/TMCS, *Cipura*, *Cypella*, elaiophores, floral lipids, Gas Chromatography, *Herbertia*, *Kelissa*, oil offering flowers, oil secretion,

Este capítulo será preparado para publicação e contou com a participação dos seguintes pesquisadores: Tiago Schena, Lilian Eggers, Elina Bastos Caramão, Olivier Chauveau, Rosangela Assis Jacques

INTRODUCTION

Floral rewards are an important strategy used by angiosperms to attract pollinators and promote cross-pollination. The nature of the floral rewards is variable among the species, generally pollen and nectar are present (Bernardello, 2007). Specialized rewards as resins, mucilages and floral oils are offered by a restrict group of angiosperms. Oil-offering flowers are present in 11 families from seven unrelated orders (Renner & Schaefer, 2010). In most of the families, occurred only one transition to the production of floral lipids, except in Orchidaceae and Iridaceae, where the lipid-secreting structures in flowers occurred several times independently (Vogel, 1981; Manning & Goldblatt, 2005).

The floral oils are produced in structures called elaiophores. In Iridaceae, elaiophores are observed in taxa of the American tribes of Iridoideae, especially in species of *Herbertia* Sweet and *Cypella* Herb. (Tigridieae) and *Trimezia* Salisb. ex Herb. (Trimezieae) where they are mainly located in tepals (Chauveau et al., 2012), but also in the staminal tube of species of the genus *Sisyrinchium* L. (Sisyrinchieae) (Vogel, 1974, Chauveau et al., 2011, Silvério et al., 2012). Only one African species of Iridaceae, *Tritoniopsis parviflora* (Jacq.) G.J. Lewis, belonging to Crocoideae, is recognized as an oil-offering flower (Manning & Goldblatt, 2005; Goldblatt & Manning, 2008). Elaiophores occur in other genera as: *Alophia* Herb., *Ennealophus* N.E.Br. *Sphenostigma* Baker, *Tigridia* Juss., *Trimezia*, *Cardenanthus* R.C. Foster, *Catila* Ravenna, *Cipura* Aubl., *Ennealophus*, *Hesperoxiphion* Baker, *Mastigostyla* I.M. Johnst, *Kelissa* Ravenna and *Onira* Ravenna (Buchmann, 1987; Chauveau et al., 2012; Pastori et al., 2013, Pastori, 2014). Chauveau et al. (2012), in the latest comprehensive phylogeny of Tigridieae showed that in Iridoideae (Iridaceae), oil-producing flowers probably evolved independently in three tribes, Sisyrinchieae, Trimezieae and Tigridieae. The optimization of the characters for the Tigridieae suggested that the ancestral state is the presence of trichomatic elaiophores only in the internal tepals (Chauveau et al., 2012). *Cypella* stands out from other American genera of Iridoideae for presenting three types of resources to pollinators (Vogel, 1974; Devoto & Medan, 2008; Pastori, 2014). This pattern of floral rewards has only been identified in Iridaceae in the South African species *Tritoniopsis parviflora*, Crocoideae, which secretes floral oils in epithelial elahophores added to nectar secretion in septal nectars (Manning & Goldblatt, 2002).

This flower oil is offered to pollinators as a specialized reward and are colourless or yellow, constitute mainly by non-volatile lipids and without odour (Vogel, 1974; Buchmann, 1987). Floral oils are composed of lipids such as fatty acids and mono- and di- glycerides (Buchmann 1981, Dumri 2008). Floral oils are complex mixtures, and in addition to lipids, small amounts of carbohydrates, aldehydes, phenolic compounds, hydrocarbons, ketones, and amino acids can be found (Vogel 1974, Buchmann, 1987, Dumri, 2008). This composition varies among plant families, genera and species. In Iridaceae, few chemical characterization studies were performed, and the information available show that free fatty acids and acylglycerols are the main components of oil (Vogel, 1974; Simpson and Neff, 1981; Dumri, 2008). In Tigridieae, just *Cypella herbertii* (Lindl.) Herb. was investigated until now. The results obtained by Dumri (2008) showed that (3R)-Acetoxypalmitic acid represents the principal component of floral oil (70%), and fatty acids (16%) and acylglycerols (12.4%).

Characterization of chemical components of floral rewards, mainly lipids, is essential for understanding of evolution of Tigridieae in South America, considered the center of diversification of *Cypella* (Chauveau et al., 2014). *Cypella* is characterized by offering nectar and floral oils, however, the relationship between these resources still needs to be clarified. In addition, for *Cypella herbertii*, Dumri (2008) showed that the elaiophores present in the inner tepals secrete floral oil rich in mono and diacylglycerols, which are used by specialized bees (Buchmann, 1987). Questions regarding the functionality, composition and importance of these structures remain misunderstood.

The goal of this study is to identify, characterize and compare the principal chemical components in oil-offering species of Tigridieae, mainly lipids, and discuss the relation of these components with the evolution and diversification of the tribe in South America.

MATERIAL AND METHODS

Plant material—Taxa sampled, sources of plant material and voucher information are listed in Table 1. A total of 23 Tigridieae accessions, representing 20 species, were sampled from Southern and Southeast of Brazil: *Cypella* (12 spp.), *Cipura* (2 spp.), *Herbertia* (4 spp.), *Kelissa* (1 sp.) and *Onira* (1 sp.). Fresh flowers were collected at the beginning of pollen presentation (anthesis stage) from field specimens and from plants grown in the living collection of the Universidade Federal do Rio Grande do Sul (Brazil).

Oil collection in the flowers— To characterize the floral oils, a total of five flowers per species were collected. For each flower analysed, only the oil-producing region was collected, excluding the rest of the tepal. For each species, oil was extracted with three washes of 1 mL of hexane for 50 min.

Sample preparation—After obtaining the extracts, they were solubilized with 1 mL of Hexane and treated with 30 μL of BSTFA/TMCS (99:1) under nitrogen atmosphere (Freitas et al. 2009). The derivatized samples were injected in GC/qMS (gas chromatography with quadrupole mass spectrometric detection). GC/qMS analyses were performed in a Shimadzu Gas Chromatograph 17A coupled to a Mass Spectrometry Detector QP 5050A (GC/qMS), equipped with an OV-5 capillary column (30 m × 0.25 mm I.D., 0.25 μm). Injections were done in split mode (1:10) at 300 °C, using helium (Linde Gases, Canoas, Brazil, 99.999% purity) as carrier gas at a flow rate of 1 mL min^{-1}. Quadrupole mass spectrometry was operated in electronic impact mode (70 eV), and its interface and ion source was kept at 300 °C and 260 °C. It was used scan mode with a mass range of 45-450 Daltons. Column temperature program was as follows: 60 °C for 1 min, heating at 5 °C min^{-1} to 185 °C keeping tthis temperature fo 1 min and then at 2 °C min^{-1} to 300 °C, and this final temperature was kept for 5 min (Von Mühlen & Marriott, 2011).

Identification of chemical compounds— The tentative of identification of chemical compounds of derivatized floral oil was made by mass spectrum comparison between the analyte and library, considering the molecular structure and molecular weight of the compound tentatively identified. Also was used Van den Dool Kratz equation to calculate the linear retention indices with temperature programming for all compounds (LTPRI, Temperature Programmed Linear Retention Indices) (Van den Doll & Kratz, 1963), calculated by:

$$LTPRI = 100n + 100\left(\frac{R_{t(i)} - R_{t(n)}}{R_{t(n+1)} - R_{t(n)}}\right)$$

Where: n = carbon number of the linear alkane which elutes just before the compound of interest; $R_{t(i)}$ = retention time of the peak being analysed in the sample; $R_{t(n)}$ = linear alkane retention time which elutes just before the interest compound, and $R_{t(n+1)}$ = linear alkane retention time that elutes immediately after the interest compound. It was also calculated the area percent of each compound present in the sample in relation to the total area of the considered compounds.

The LPTRI indices were experimentally obtained and compared with those reported in the literature (NIST, National Institute of Standards and Technology- NIST-MS search 2.0) to DB-5 column (5% phenyl - 95% methylpolysiloxane). Retention times of n-alkanes series (from 14

to 37 carbon atoms), under the same experimental conditions used for derivatized floral oil were used for the experimental LTPRI calculus. The maximum difference considered between the experimental LTPRI and literature LTPRI values was 25 units, for tentative identification of a determined compound.

Analysis—The matrixes obtained from de chromatography were analysed with a Principal Components analysis (PCA) using the software SPSS (PASW Statistics 18, SPSS Inc., Chicago, Illinois, USA; 2009). A Monte Carlo simulation approach was applied to determine the significantly statistics eigenvalue in Monte Carlo PCA for Parallel Analysis (Watkins, 2000, 2006), and the number of factors to retain in Principal Component Analysis were selected.

RESULTS

Oil characterization– Areas with oil secretion were generally covered in anthesis with an oil film. The oil producing region is located in the inner tepals of species of *Cypella*, *Cipura* and *Kelissa*, and in outer and inner tepals in *Herbertia*. The floral oil in all analysed species was extravasated by the elaiophore region, and there is no blistering structure in the trichomes. Therefore, the elaiophores were easily washed and all the oil was removed. Unfortunately it was not possible to quantify the total oil produced by elaiophore/flower in microlitres, but we estimated that the quantity is less than one microlitre per flower.

Chemical composition– Oil rewards in species of *Cypella, Cipura, Herbertia* and *Kelissa* were investigated in this study. The floral oil of *Onira unguiculata* (Baker) Ravenna, *Herbertia quareimana* Ravenna, *Cypella pabstiana* Ravenna and *Cypella herbertii* (sample 260) were not analysed since they were not derivatized.

The total ion chromatogram of the Tigridieae species indicated that chemical composition of the analysed species is complex and many compounds were not possible to identify. *Trimethylsilyl* (TMS) derivatives components obtained from GC/-MS chromatography for each species are available in online resource (1-18). The total percentages of area in chromatography are shown in Table 2 and included: total free fatty acids (FFA's), total esters of fatty acid (FAMES Fatty acids methyl ou ethyl ester), alcohols, aldehydes, ketones, hydrocarbon, monoglycerides and not identified components. In this study we focused in lipids, so we exclude from the results and discussion the other chemical compounds.

The total percentage of free fatty acids varied among *Cypella* species from 1.19% in *C. charruana* Deble & F.S. Alves to 75.37% in *C. fucata* Ravenna. For *Herbertia*, *H. pulchella* Sweet had 3.87% of free fatty acids and *H. zebrina* Deble had 72.10%. In *Cipura*, *C. paludosa* Aubl. presented 11.59% and *C. xanthomelas* Maxim. ex Klatt, 68.33%. *Kelissa brasiliensis* (Baker) Ravenna presented a total of 64.61% of free fatty acids.

Our results show that floral oil for each species has a peculiar lipidic chemical composition and different numbers of compounds were identified. *Cypella herbertii* (sample 875) and *Herbertia lahue* (Molina) Goldblatt had the highest number of lipids identified, total of 16, while most species had between 10 and 13 lipids identified: 13 lipids are identified in *Cypella herbertii* (sample 918), *Cypella rivularis* Chaveau & L. Eggers and *Cypella fucata*; 12 in *Cypella altouruguaya* Chaveau & L. Eggers; 11 in *Cypella luteogibbosa* Deble and *Herbertia zebrina*, 10 in *Cypella discolor* Ravenna and *Cypella guttata* Deble & F.S. Alves. Four species was 8 lipids identified: *Cypella aquatilis* Ravenna, *Kelissa brasiliensis*, *Cipura xanthomelas*, and *Cipura paludosa*. The species with the lowest number of compounds identified were: *Cypella amplimaculata* Chaveau & L. Eggers and *Cypella pusilla* (Link & Otto) Benth. & Hook. f. ex B.D. Jacks. (7 lipids) and *Cypella charruana* and *Herbertia pulchella* (6 lipids).

The free fatty acids present in higher percentage of area were: Tetracosenoic acid ($C_{24}H_46O_2$), Butanoic acid ($C_{27}H_{52}O_2$), Hexacosanoic acid ($C_{26}H_{52}O_2$), Pentacosanoic acid ($C^{25}H_{50}O_2$), Octadecadienoic acid ($C_{18}H_{32}O_2$), Dodecanoic acid ($C_{12}H_{24}O_2$), Docosenoic acid ($C_{22}H_{42}O_2$), Octadecenoic acid ($C_{18}H_{34}O_2$) and Nonanoic acid ($C_{29}H_{58}O_2$).Four free fatty acids were present in almost species: Tetracosenoic acid, Octadecadienoic acid, Palmitic acid, Myristic acid ($C_{14}H_{28}O_2$), and Docosenoic acid.

The concentration of each acid varied among different species. The Tetracosenoic acid was present in almost all species and varied from 3.93% in *Cipura paludosa* to 64.66% in *Herbertia zebrina*. Some free fatty acids were present in only one species, as Butanoic acid present in *Herbertia pulchella* (82.66%), *Cypella gutatta* (43,88) and *Cypella herbertii* 875 (13,48%). In *Cypella aquatilis*, for example, the total percentage of Nonadecanoic acid ($C_{19}H_{38}O_2$) was 52.94%, while in the other species there was no record of this acid. The Dodecanoic acid occured in *Kelissa brasiliensis* in higth concentration (38.33%), while in the other species there was no record of this acid, as in *Herbertia*, or its concentration was very low, as in *Cypella*, *e.g. C. luteogibbosa* (0.07%) (Table 4).

Composition of monoglycerides also varies between species: 32.89% in *Cipura paludosa* to 9.41 in *Herbertia zebrina*. Princpal monoglycerides identified are Palmitoylglycerol

($C_{19}H_{38}O_4$) and Oleoylglycerol ($C_{21}H_{40}O_4$). In some species such as: *Cypella aquatilis*, *Cypella gutatta*, *Cypella herbertii*, *Cypella charruana*, *Cypella pusilla* and *Kelissa brasiliensis* it was not possible to identify the monoglycerides.

Principal Component Analysis (PCA) – A PCA was performed with total free fatty acids and esters of fatty acid and the results are available in Table 3. The first three components represented 41.9% of the total variance observed and were selected to dispersion plot (Fig. 1). The component matrixes for the first six components are available in Table 5. The dispersion plot (Fig. 1) shows a central group of species with similar oil composition. *Cypella herbertii*, *C. aquatilis* and *Kelissa brasiliensis* were separated from the other species by the three axes and samples of *Herbertia lahue*, *H. zebrina*, *Cypella rivularis* and *C. amplimaculata* were apparently distinguished by these axes too.

DISCUSSION

Floral oil produced in flowers of Tigridieae was analysed for the first time in this present study. In addition, the present analyses constituted the greatest framework for chemical composition in Iridaceae. Floral oil in Trigridieae has complex chemical composition. The results in this present study confirmed the presence of lipids as free fatty acids and esters of fatty acid as major constituents of floral oils. However we identified in small amounts of alcohols, aldehydes, ketones, hydrocarbons, monoglycerides (acylglycerols or monoacylglycerols) and also not identified components that probably are diglycerides. The results in this present study confirmed the presence of free fatty acids in all species of *Cypella* tested, as well as in *Cipura*, *Kelissa* and *Herbertia*. Our results showed that there are different chemical compositions among the sampled species, but there are also many similarities, as evidenced in PCA analysis. Some free fatty acids Tetracosenoic acid, Octadecadienoic acid, Palmitic acid, Myristic acid and Docosenoic acid are presente in almost species, but on the other hand Nonadecanoic acid, Butanoic acid and others are present in one or few species.

Chemical composition characterization of floral rewards is in general insufficient. Studies are focused mainly on nectar sugars and secondary metabolites (such as alkaloids) (Irwin and Adler, 2008), where the methodology and techniques are well defined in the literature, and do not require prior processes such as toxic solvents as hexane and derivatization (used for floral oils). Studies on chemical composition of floral oils are infrequent, which can be due to the low supply of these resources in angiosperms and the lack of specialized labor. In Iridaceae, few studies are available regarding the chemical composition of floral oils. According to available

study, free fatty acids and acylglycerols (monoglycerides) appear to be the major components of oil (Vogel, 1974; Simpson and Neff, 1981; Dumri, 2008), and our results were in agreement with Dumri (2008). Studies with other plant families indicate that fatty acids are the major components of the oil, but mono-, di- or glycerides are also common (Simpson 1989; Seipold et al., 2004; Reis et al. 2007, Dumri et al. 2008; Cappellari et al. 2011a).

Studies in Scrophulariaceae, especially in *Diascia* Link & Otto, has identified that floral oils produced in epithelial elaiophores are composed of acetylated acylglycerols of (3R) -acetoxy fatty acids (Dumri et al., 2008). This study in *Diascia* also reported the presence of free fatty acids as Myristic acid, Palmitic acid and Stearic acid, as well a the present study with Tigridieae species. In Orchidaceae, a study isolated a new diacylglycerols in *Ornithophora radicans* (Rchb. f.) Garay & Pabst, denominated Oncidinol (Reis et al. 2003). We suggest, as a new step for this study, a detailed analysis of the unidentified compounds in order to identify the diglycerides, which are important compounds already reported as occurring in *Cypella herbertii*.

The present study has shown that it is difficult to establish relationships between species and chemical compounds. It seems that each species or group of species has a specificity secreted lipid in floral oils. Our results showed that two species, *Cypella herbertii* and *Herbertia lahue*, secrete a greater lipid diversity in floral oils. These two species are widely distributed in the campos eco-region from Rio de la Plata Grasslands (RPG) (Medan et al., 2011), and often occur in sympatry with other species of *Cypella*, *Herbertia*, *Kelissa* and *Onira* (Morales et al., 2009; Boldrini et al. 2015). On the other hand, *Kelissa brasiliensis*, which is also well distributed and often sympatric with other species of Tigridieae (Boldrini et al., 2015), secretes floral oils with lower lipid diversity (only 8), but 62% of its composition is based on two fatty acids: Octadecadienoic acid and Dodecanoic acid.The different chemical compositions of the floral oils secreted by Tigridieae can demonstrate distinct strategies of pollination by specialized oil collecting bees. Floral oils with different chemical compositions, mainly with percentages of different free fatty acids, in species that are sympatric could represent specializations by the flowers. The supply of lipids with broad chemical composition could benefit several categories of pollinators, whereas a more restricted chemical composition would benefit specific pollinators.

Oil-collecting behavior is restricted to approximately 500 species of specialized bees (Vogel 1974; Michener 2007). The floral oil is used in different ways by bees. The female solitary bees use oils for the waterproofing of the surface of the nest and also to the nutrition of larvae

(Buchmann, 1987; Schäffler and Dötterl, 2011), on the other hand, the utilization of oils for male bees are unknown (Cappellari et al., 2011b). The oil offering flowers and oil collection behavior of bees were often obtained and lost in the evolutionary history of bees and plants (Renner and Schaefer, 2010). Studies regarding the pollination of Tigridieae species from South America are scarce, visits by specialized oil-collecting bees were only registered for *Cypella herbertii*, mainly bees belonging to the Centridini and Tapinotaspidini of the Apidae (Vogel, 1974; Devoto and Medan, 2008; Pinheiro et al., 2008). *Cipura paludosa* was visited by non-specialized oil-collecting bees, *Augochlora thalia* and *Plebeia* sp. (Santos et al., 2016). According to Souza-Chies et al. (2012), preliminary experiments in *Herbertia* showed that *H. quareimana* and *H. lahue* are visited by an exotic and non-specialized bee, *Apis mellifera* L., which prefer *H. quareimana* flowers and visit *H. lahue* only secondarily. In addition, they also demonstrate that *H. quareimana* is mainly cross-pollinated, while *H. lahue* also produces fruits by self-fertilization. (Souza-Chies et al., 2012). Investigations on the biology of pollination of the Tigridieae species are necessary to identify pollinators and to detect possible variations in the behavior and preferences of the oil-collecting bees.

Interaction with pollinators is indeed the key factor for the success of most angiosperms (Valente et al., 2012; Van der Niet and Johnson, 2012; Forest et al., 2014). Studies have shown that the morphological phenotypic variation observed in angiosperms flowers can be modulated by interaction with pollinators (Valente et al. 2012; Schiest and Johnson, 2013; Van der Niet e Johnson, 2012; Forest *et al.*, 2014; Dai et al, 2017), mainly related to the supply of floral resources. The different flower morphologies observed in the oil-offering flowers of Tigridieae, such as tepals format and the position of the elaiophores, can be related to differences in chemical compositions and adaptations to different pollinators. As shown in Pastori (2014), elaiophores are in different positions in the tepals of Tigridieae. While *Herbertia* elaiophores are located in the proximal portion of the inner and outer tepals, in *Cypella*, *Kelissa* and *Onira* the elaiophores are in the distal portion of the inner tepals. In addition, the orientation of these also varies, ranging from intrusion to extrusion in *Cypella* species. These variations suggest that the supply of floral oils in Tigridieae can be an important mechanism of speciation, which can lead to the isolation of reproductive populations isolated.

Conclusions— Chemical analyses were used to investigate the composition of the floral non-volatile oil in closely related genera of South American Tigridieae. The resulting observations revealed a wide range of variation in the composition of free fatty acids among species. This study confirmed the occurence of fatty acids in elaiophores of *Cypella*, *Cipura*, *Herbertia* and

Kelissa. This work provided an important characterization for future studies in pollination biology and hypothesis for the utilization of this oil for the collecting bees.

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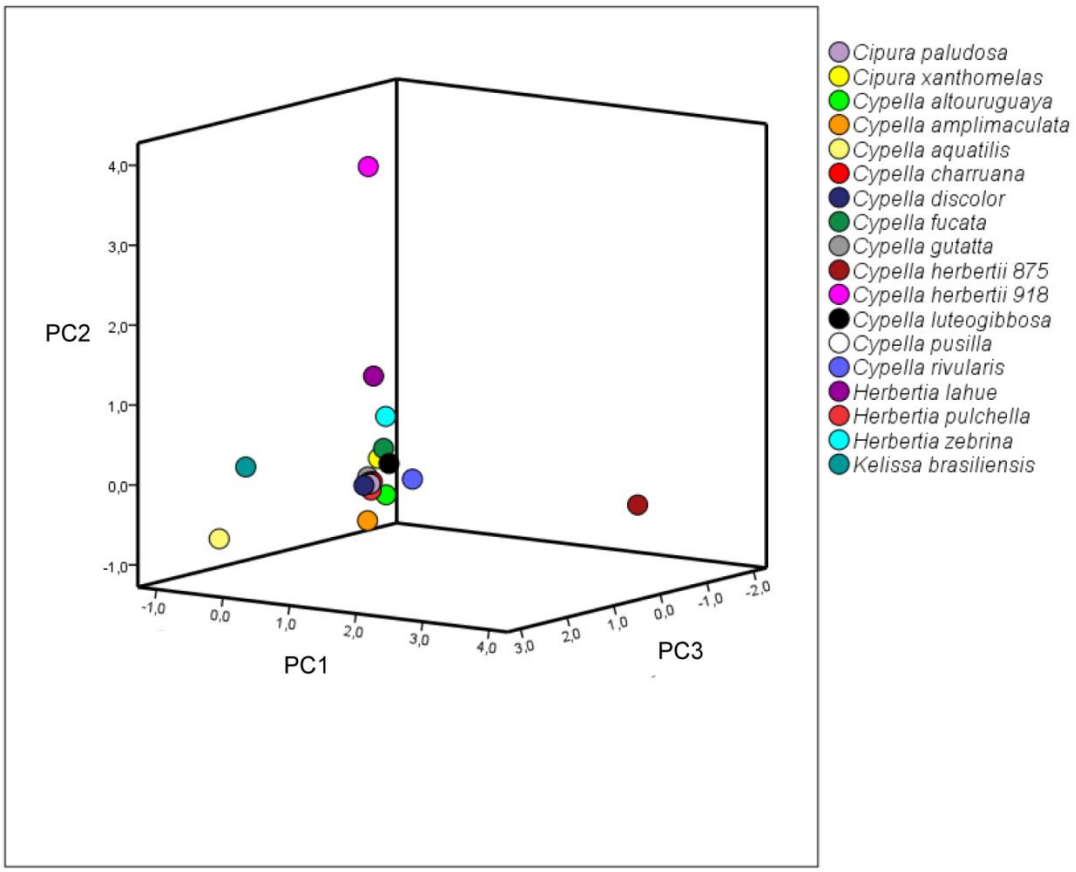

Figure 1: Principal components analysis for total free fatty acids (FFA's) and total esters of fatty acid (FAMES Fatty acids methyl ou ethyl ester). Component 1 represented 17.7% of total variance, Component 2 =13.6% and Component 3= 10.6%.

Table 1: Voucher information and geographical origin of species sampled to chemical composition of floral oil in Tigridieae.

Species	**Voucher**	**Geographical origin**
Cipura paludosa Aubl.	Chauveau & Aguiar 906C (ICN)	BR: DF, Brasília
Cipura xanthomelas Maxim. ex Klatt	Eggers & Chauveau 1009 (ICN)	BR: MT, Rosário do Oeste
Cypella altouruguaya Chauveau & L.Eggers	Eggers & Chauveau 716 (ICN)	BR: RS, Trindade do Sul
Cypella aquatilis Ravenna	Eggers & Chauveau 714 (ICN)	BR: RS, Muitos Capões
Cypella amplimaculata Chauveau & L.Eggers	Eggers & Souza-Chies 664 (MBM)	BR:RS, Porto Alegre
Cypella aquatilis Ravenna	Eggers et al. 714 (ICN)	BR: RS, Muitos Capões
Cypella charruana Deble & F.S.Alves	Pastori et al. 203 (ICN)	BR: RS, Quaraí
Cypella discolor Ravenna	Aita 75 (ICN)	BR: RS, Quaraí
Cypella fucata Ravenna	Pastori et al. 122 (ICN)	BR: RS, Alegrete
Cypella guttata Deble & F.S. Alves	Pastori et al. 204 (ICN)	BR: RS, Quaraí
Cypella herbertii (Lindl.) Herb.	Eggers et al. 875 (ICN)	BR: RS, Salto do Jacuí
Cypella herbertii (Lindl.) Herb.	Eggers et al. 918 (ICN)	BR: SC, São Joaquim
Cypella herbertii (Lindl.) Herb.	Alves 260 (ICN)	BR: SC, Caçador
Cypella luteogibbosa Deble	Eggers et al. 710 (ICN)	BR: RS, Quaraí
Cypella pabstiana Ravenna	Eggers et al. 741 (ICN)	BR: RS, Barra do Quaraí
Cypella pusilla (Link & Otto) Benth. & Hook. f. ex B.D. Jacks.	Pastori et al. 119 (ICN)	BR: RS, São Vicente do Sul
Cypella rivularis Chauveau & L.Eggers	Eggers et al. 800 (ICN)	BR: RS, Uruguaiana
Herbertia pulchella Sweet	Pastori & Chauveau 182 (ICN)	BR: RS, Pinheiro Machado
Herbertia quareimana Ravenna	Eggers & Souza-Chies 513 (ICN)	BR: RS, Quaraí
Herbertia lahue (Molina) Goldblatt	Pastori et al. 132 (ICN)	BR: RS, São Borja
Herbertia zebrina Deble	Pastori et al. 110 (ICN)	BR: RS, Amaral Ferrador
Kelissa brasiliensis (Baker) Ravenna	Pastori et al. 109 (ICN)	BR: RS, Amaral Ferrador
Onira unguiculata (Baker) Ravenna	Pastori et al. 162 (ICN)	BR: RS, Candiota

Notes: ICN = Herbarium of the Federal University of Rio Grande do Sul (Brazil); BR = Brazil;; DF = Distrito Federal; MT = Mato Grosso; RS = Rio Grande do Sul; SC = Santa Catarina.

Table 2: Total percentage of area of the derivatives of compounds of Tigridieae floral oils identified by GC/EI-MS.

	Total free fatty acids	**Total alcohols**	**Total aldehydes**	**Total ketones**	**Total esters of fatty acid**	**Total of the hydrocarbon**	**Total of monoglycerides**	**Total of not identified**
Cypella altouruguaya	55.44	-	-	-	0.04	0.37	27.95	16.20
Cypella amplimaculata	54.53	-	-	-	0.69	8.55	23.95	12.28
Cypella aquatilis	64.92	-	-	-	31.78	1.88	-	1.42
Cypella herbertii 918	23.77	-	0.23	0.04	46.10	10.35	-	19.51
Cypella discolor	61.29	-	-	-	7.08	3.52	17.08	11.03
Cypella gutatta	16.38	-	0.21	0.09	52.85	7.14	-	23.33
Cypella herbertii 875	17.06	-	0.14	0.11	28.18	2.33	-	52.18
Cypella luteogibbosa	56.69	-	-	-	16.32	6.43	15.71	4.85
Cypella rivularis	71.20	-	-	-	-	4.91	13.04	10.85
Cypella charruana	1.19	-	0.19	0.10	65.27	8.56	-	24.69
Cypella pusilla	1.40	0.19	1.70	0.68	6.92	42.74	-	46.37
Cypella fucata	75.37	-	-	-	-	1.94	16.50	6.19
Herbertia pulchella	3.87	-	-	-	85.70	4.69	-	5.74
Herbertia zebrina	72.10	-	0.05	-	-	1.79	9.41	16.65
Herbertia lahue	30.88	0.51	0.15	-	0.37	7.11	17.41	43.57
Kelissa brasiliensis	64.61	0.07	0.18	0.05	0.11	5.99	-	28.99
Cipura xanthomelas	68.33	-	-	-	-	6.95	11.21	13.51
Cipura paludosa	11.59	-	0.08	-	-	2.06	32.89	53.38

Table 3: First six components and corresponding eigenvalues value and percentage of variance.

		Initial Eigenvalues			Extraction Sums of Squared Loadings		
		Total	**% of Variance**	**Cumulative %**	**Total**	**% of Variance**	**Cumulative %**
Component	1	8.882	17.764	17.764	8.882	17.764	17.764
	2	6.810	13.620	31.384	6.810	13.620	31.384
	3	5.303	10.605	41.990	5.303	10.605	41.990
	4	4.552	9.103	51.093	4.552	9.103	51.093
	5	4.123	8.246	59.340	4.123	8.246	59.340
	6	3.853	7.707	67.046	3.853	7.707	67.046

Table 4: Principal results from total of esters of fatty acid (FAMES Fatty acids methyl ou ethyl ester) and total free fatty acids (FFA's) obtained from each species analysed.

		species																	
		C. altouruguaya	*C. amplimaculata*	*C. aquatilis*	*C. herbertii 918*	*C. discolor*	*C. gutatta*	*C. herbertii 875*	*C. luteogibbosa*	*C. rivularis*	*C. charruana*	*C. pusilla*	*C. fucata*	*Cipura xanthomelas*	*Cipura paludosa*	*H. pulchella*	*H. zebrina*	*H. lahue*	*K.brasiliensis*
Linolenic acid	.alpha.-Linolenic acid	0.03	-	-	1.51	0.02	-	-	0.07	0.11	-	-	-	-	-	-	0.05	0.36	-
	Linolenic acid	-	-	-	4.32	-	-	-	0.12	-	-	-	0.15	-	-	-	0.05	1.49	-
	Linoleic acid ethyl ester	-	-	-	-	-	-	0.15	-	-	-	-	-	-	-	-	-	-	-
	Total	0.03	-	-	5.83	0.02	-	0.15	0.19	0.11	-	-	0.15	-	-	-	0.10	1.85	-
Nonadecanoic acid	10-Nonadecenoic acid. (Z)	-	-	3.16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nonadecanoic acid	-	-	52.94	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	-	-	56.10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Docosenoic acid	13-Docosenoic acid. (Z)	0.08	0.13	-	-	0.08	-	-	0.07	-	-	-	0.08	-	0.20	-	-	0.25	-
	Docosenoic acid	2.17	9.15	-	-	2.85	-	0.02	2.42	1.37	0.21		2.81	0.83	6.62	-	3.93	7.56	0.25
	Total	2.25	9.28	-	-	2.93	-	0.02	2.49	1.37	0.21	-	2.89	0.83	6.82	-	3.93	7.81	0.25
Tetracosenoic acid	15-Tetracosenoic acid	-	45.02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Tetracosenoic acid	38.17	-	-	49.58	-	-	-	52.80	40.59	-	-	57.05	20.84	3.93	-	64.66	12.72	-
	Total	38.17	45.02	-	49.58	-	-	-	52.80	40.59	-	-	57.05	20.84	3.93	-	64.66	12.72	-
Octadecadienoic acid	9.12-Octadecadienoic acid (Z.Z)	0.02	-	0.63	0.09	0.02	-	0.31	0.07	0.06	-	-	0.09	-	-	-	0.04	0.34	0.91
	Octadecadienoic acid	-	-	6.20	13.20	0.08	3.48	0.77	0.29	0.40	-	1.14	-	-	-	1.38	0.37	0.10	22.55
	Octadecadienoic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.06
	Octadecadienoic acid. ethyl ester	-	-	-	-	-	-	0.55	-	-	-	0.84	-	-	-	-	-	-	-
	Total	0.02	-	6.83	13.29	0.10	3.48	1.63	0.36	0.46	-	1.98	0.09	-	-	1.38	0.41	0.44	23.52

		species																	
		C. altourugu aya	*C. amplimacul ata*	*C. aquati lis*	*C. herber tii 918*	*C. discol or*	*C. gutat ta*	*C. herber tii 875*	*C. luteogibb osa*	*C. rivula ris*	*C. charru ana*	*C. pusil la*	*C. fuca ta*	*Cipura xanthome las*	*Cipura paludo sa*	*H. pulchel la*	*H. zebri na*	*H. lahu e*	*K.brasilie nsis*
Octadecen oic acid	Octadecenoic acid	0.03	-	5.94	-	-	11.8 3	13.63	-	0.08	0.88	-	0.43	-	-	-	-	3.12	-
	9-Octadecenoic acid (Z)-. methyl ester	-	-	-	-	-	-	-	-	-	-	3.80	-	-	-	-	-	-	-
	Total	0.03	-	5.94	-	-	11.8 3	13.63	-	0.08	0.88	3.80	0.43	-	-	-	-	3.12	-
Hexadecen oic acid	9-Hexadecenoic acid. (Z)	-	-	-	0.26	-	0.10	0.27	-	-	-	-	0.05	-	-	-	0.11	0.30	0.06
	Hexadecenoic acid	-	-	-	0.25	-	-	-	-	-	-	-	0.05	-	-	-	0.10	-	-
	9-Hexadecenoic acid. methyl ester. (Z)-	-	-	-	-	-	-	0.03	-	-	-	-	-	-	-	-	-	-	-
	Total	-	-	-	0.51	-	0.10	0.30	-	-	-	-	0.10	-	-	-	0.21	0.30	0.06
Decanoic acid	Decanoic acid	-	-	-	-	-	-	-	-	-	-	-	0.01	-	-	-	-	-	-
heptadecan oic acid	hydroxy-heptadecanoic acid	-	-	-	-	-	-	-	-	0.04	-	-	-	-	-	-	-	-	-
Dodecanoi c acid	Dodecanoic acid	-	-	-	0.11	-	0.07	-	0.03	-	0.05	-	0.02	-	0.03	-	-	0.04	38.33
	Dodecanoic acid. nonyl ester	-	-	-	-	-	3.31	-	-	-	-	-	-	-	-	-	-	-	-
	Total	-	-	-	0.11	-	3.38	-	0.03	-	0.05	-	0.02	-	0.03	-	-	0.04	38.33
Pentadeca noic acid	Pentadecanoic acid	-	-	-	-	-	-	0.04	0.18	-	-	-	-	-	-	-	-	0.05	-
	hydroxy-pentadecanoic acid	0.03	-	1.42	-	0.06	-	1.12	0.15	0.16	-	-	-	-	-	-	0.07	-	1.15
	Total	0.03	-	1.42	-	0.06	-	1.16	0.33	0.16	-	-	-	-	-	-	0.07	0.05	1.15
Erucic acid	Erucic acid	0.04	-	-	-	-	-	-	-	0.09	-	-	-	-	-	-	-	-	-
Hexacosan oic acid	Hexacosanoic acid	-	-	-	1.12	8.22	-	-	-	-	-	-	13.7 7	44.63	-	-	-	0.43	-
	Hexacosanoic acid. TMS derivative	14.74	-	-	-	-	-	-	-	26.98	-	-	-	-	-	-	-	-	-
	Total	14.74	-	-	1.12	8.22	-	-	-	26.98	-	-	13.7	44.63	-	-	-	0.43	-

		species																	
		C. altourugu aya	*C. amplimacul ata*	*C. aquati lis*	*C. herber tii 918*	*C. discol or*	*C. gutat ta*	*C. herber tii 875*	*C. luteogibb osa*	*C. rivula ris*	*C. charru ana*	*C. pusil la*	*C. fuca ta*	*Cipura xanthome las*	*Cipura paludo sa*	*H. pulchel la*	*H. zebri na*	*H. lahu e*	*K.brasilie nsis*
Myristic acid	Myristic acid	-	0.10	-	0.28	0.04	0.07	0.06	0.04	0.05	0.09	0.14	0.09	0.12	0.08	-	0.14	0.13	0.12
	hydroxy-myristic acid	-	0.04	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	hydroxy-myristic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	-	0.14	-	0.28	0.04	0.07	0.36	0.04	0.05	0.09	0.14	0.09	0.12	0.08	-	0.14	0.13	0.12
Palmitelaidic acid	Palmitelaidic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	0.31	1.36	1.04	-	-
Palmitic Acid	Palmitic Acid	0.09	0.14	0.30	2.63	0.30	0.83	0.83	0.37	1.09	0.17	0.12	0.69	1.16	0.38	0.92	1.43	2.28	1.24
pentacosanoic acid	pentacosanoic acid	-	-	-	-	-	-	-	-	-	-	-	-	0.55	-	-	-	0.60	-
	Pentacosanoic acid. methyl ester	-	-	25.80	38.64	7.08	-	-	-	-	-	-	-	-	-	2.76	-	-	-
	Total	-	-	25.80	38.64	7.08	-	-	-	-	-	-	-	0.55	-	2.76	-	0.60	-
Stearic acid	Stearic acid	0.04	0.05	-	-	0.04	-	-	0.08	0.16	-	-	0.08	0.20	0.04	-	0.11	1.11	-
Tridecanoic acid	Tridecanoic acid	-	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-	-
	hydroxy-tridecanoic acid	-	0.17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	0.04	0.22	-	-	0.04	-	0.01	0.08	0.16	-	-	0.08	0.20	0.04	-	0.11	1.11	-
Octenoic acid	3-Octenoic acid. heptadecyl ester	-	-	-	-	-	1.33	0.38	-	-	-	-	-	-	-	-	-	-	-
Butanoic acid	Docosyl-butenoate	-	-	-	-	-	43.88	13.48	-	-	-	-	-	-	-	82.66	-	-	-
Eicosadienoic acid	Eicosadienoic acid. methyl ester	-	-	-	0.45	-	-	2.47	-	-	-	0.42	-	-	-	-	-	-	-
Ethyl 9-hexadecenoate	Ethyl 9-hexadecenoate	-	-	-	-	-	-	0.08	-	-	-	-	-	-	-	-	-	-	-
Ethyl Oleate	Ethyl Oleate	-	-	0.32	0.12	-	-	9.13	-	-	-	-	-	-	-	0.28	-	-	-

		species																	
		C. altourugua ya	*C. amplimacul ata*	*C. aquatil is*	*C. herber tii 918*	*C. discol or*	*C. gutat ta*	*C. herber tii 875*	*C. luteogibbo sa*	*C. rivular is*	*C. charrua na*	*C. pusill a*	*C. fucat a*	*Cipura xanthomel as*	*Cipura paludo sa*	*H. pulchel la*	*H. zebrin a*	*H. lahu e*	*K.brasilien sis*
Hexadecan oic acid	Hexadecanoic acid. methyl ester	0.04	-	-	-	-	-	0.07	-	-	-	1.06	-	-	-	-	-	0.31	0.05
Methyl 9-eicosenoate	Methyl 9-eicosenoate	-	-	5.66	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nonanoic acid	Nonanoic acid. eicosyl ester	-	0.69	-	6.06	-	4.33	1.54	16.32	-	10.41	-	-	-	-	-	-	-	-
Octadecano ic	Octadecanoic acid. methyl ester	-	-	-	-	-	-	-	-	-	-	0.80	-	-	-	-	-	0.06	-
	Octadecanoic acid. octadecyl ester	-	-	-	0.83	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	-	-	-	0.83	-	-	-	-	-	-	0.80	-	-	-	-	-	0.06	-

Table 5: Component matrix from PCA obtain of the chemical compounds in Tigridieae.

Component Matrix[a]							
		Component					
		1	**2**	**3**	**4**	**5**	**6**
free fattty acids							
.alpha.-Linolenic acid	C18H30O2	-.112	**.935**	.126	.078	-.049	-.026
Decanoic acid	C10H20O2	-.105	.001	.064	-.067	.003	-.132
Docosenoic acid	C22H42O2	-.351	-.125	.455	-.311	.650	.213
13-Docosenoic acid. (Z)	C22H42O2	-.277	-.062	.427	-.190	.571	.294
Dodecanoic acid	C12H24O2	.032	.125	-.512	-.078	.092	.259
Erucic acid	C19H34O2	-.149	-.141	-.008	-.114	-.107	-.595
9-Hexadecenoic acid. (Z)	C16H30O2	-.146	-.029	.059	-.065	-.079	-.164
Hexacosanoic acid	C26H52O2	-.125	.889	.078	.052	-.139	-.143
Hexacosanoic acid. TMS derivative	C23H46O2	.507	.741	.281	-.045	.208	.091
Hexadecenoic acid	C16H30O2	.002	-.047	.314	.760	.166	.408
hydroxy-heptadecanoic acid	C17H34O3	-.117	-.090	-.012	-.091	-.102	-.533
hydroxy-myristic acid	C14H28O3	-.159	-.205	.206	-.299	.483	.199
hydroxy-pentadecanoic acid	C15H30O3	.605	.033	-.732	-.082	.232	.087
hydroxy-tridecanoic acid	C13H26O3	-.159	-.205	.206	-.299	.483	.199
Linolenic acid	C18H30O2	-.107	**.939**	.158	.084	.002	.061
Myristic acid	C14H28O2	-.126	.780	.263	-.088	-.166	.285
Nonadecanoic acid	C19H38O2	.086	-.048	-.747	.060	.183	.102
10-Nonadecenoic acid. (Z)	C19H38O2	.086	-.048	-.747	.060	.183	.102
9.12-Octadecadienoic acid (Z.Z)	C18H32O2	.036	.555	-.608	-.029	.019	.221
Octadecadienoic acid (Z.Z)	C18H32O2	.056	-.203	.141	.889	-.082	.177
Octadecenoic acid	C18H34O2	.787	-.104	.030	.520	.143	.122
Palmitelaidic acid	C16H30O2	-.120	-.065	.036	.045	-.081	-.179
Palmitic Acid	C16H32O2	-.031	.820	.169	.182	.132	.020
pentacosanoic acid	C25H50O2	-.102	-.036	.037	-.042	-.085	-.125
pentacosanoic acid. TMS derivative	C22H42O2	-.086	.201	.341	.023	.402	.423
Pentadecanoic acid	C15H30O2	.116	.007	.199	.003	.154	-.041
Stearic acid	C18H36O2	-.163	.169	.381	-.025	.403	.297
Tetracosenoic acid	C24H46O2	-.159	-.205	.206	-.299	.483	.199
15-Tetracosenoic acid	C24H46O	-.306	.398	.189	-.077	-.082	-.526
Tridecanoic acid	C13H26O2	**.963**	.017	.169	-.123	.095	-.132
Esters of fatty acids							
Docosyl-butenoate	C27H52O2	.086	-.245	.053	.487	-.164	-.080
Dodecanoic acid. nonyl ester	C21H42O2	.063	-.196	.134	.885	-.084	.188
Eicosadienoic acid. methyl ester	C21H38O2	**.948**	.157	.189	-.163	-.054	-.055
Ethyl 9-hexadecenoate	C18H34O2	**.963**	.017	.169	-.123	.095	-.132
Ethyl Oleate	C20H38O2	**.967**	.023	.143	-.117	.097	-.134
Hexadecanoic acid. methyl ester	C17H34O2	.070	-.071	.169	-.331	-.593	.634
9-Hexadecenoic acid. methyl ester. (Z)-	C17H32O2	**.963**	.017	.169	-.123	.095	-.132
hydroxy-myristic acid	C14H28O3	**.963**	.017	.169	-.123	.095	-.132
Linoleic acid ethyl ester	C20H36O2	**.963**	.017	.169	-.123	.095	-.132
Methyl 9-eicosenoate	C21H40O2	.086	-.048	-.747	.060	.183	.102
Nonanoic acid. eicosyl ester	C29H58O2	-.024	.132	.123	.269	-.072	-.137
3-Octenoic acid. heptadecyl ester	C25H48O2	.033	.123	-.512	-.080	.092	.258
Octadecadienoic acid. ethyl ester	C20H36O2	.573	-.104	.169	-.352	-.575	.378
Octadecanoic acid. methyl ester	C18H36O2	.029	-.118	.111	-.328	-.702	.559
Octadecadienoic acid. ethyl ester	C20H36O2	-.074	.901	.041	.081	-.137	-.073
9-Octadecenoic acid (Z)-. methyl ester	C19H36O2	.035	-.132	.085	-.329	-.731	.526
Octadecanoic acid. octadecyl ester	C36H72O2	.330	-.187	.179	.830	-.056	.146
Pentacosanoic acid. methyl ester	C26H52O2	-.029	.723	-.396	.104	-.018	-.017
Extraction Method: Principal Component Analysis.							

a. 6 components extracted.

CAPÍTULO V

CONSIDERAÇÕES FINAIS

Os resultados da presente tese contribuíram para o esclarecimento das relações evolutivas em Tigridieae (Iridoideae: Iridaceae). Neste estudo foram utilizadas diferentes abordagens que reforçaram a necessidade de estudos integrativos para a compreensão das relações evolutivas no grupo estudado.

No Capítulo II, análises morfológicas e filogenéticas foram utilizadas para a identificação dos limites entre as linhagens estudadas. Utilizou-se abordagem multivariada que buscou identificar nos gráficos de dispersão descontinuidades entre as linhages estudadas. Através desta ferramenta foi possível identificar quatro grupos correspondentes as espécies de *Cypella aquatilis*, *C. discolor*, *C. ravenniana* e *C. pusilla*. As análises filogenéticas realizadas com a combinação de marcadores plastidiais e nucleares, evidenciram que *C. aquatilis*, *C. discolor*, *C. ravenniana* e *C. pusilla* devem ser consideradas linhagens evolutivas independentes. A comparação das duas abordagens evidenciou que *C. gloriana* não foi discriminada morfologicamete de *C. pusilla* e filogeneticamente não pode ser considerada uma linham evolutiva independente. Sendo assim, no presente capítulo é proposta a sinonimização de *C. gloriana* em *C. pusilla*. Este estudo demonstrou que, em geral, não existe uma "fórmula pronta" de metodologias que os cientistas devam utilizar para a delimitação de espécies. O mais adequado é decidir de acordo com suas hipóteses e perguntas quais são as metodologias adequadas. Quando o assunto é a delimitação de espécies é recomendado comparar os resultados de vários métodos para avaliar a congruência entre eles antes de propor decisões taxonômicas formais. Além disso, o presente trabalho demonstrou que alguns marcadores moleculares podem oferecer incongruências, principalmente no nível de espécie. Por isso é importante que análises sejam realizadas através da combinação de diferentes regiões do DNA plastidial e nuclear a fim de oferecer resultados mais robustos.

Análises estatísticas multivariadas são ferramentas pouco utilizadas pelos taxonomistas. Normalmente as descrições taxonômicas estão baseadas em medidas clássicas, com poucas populações e poucos indivíduos. Entretanto, muitas vezes as descontinuidades morfológicas entre táxons não são suficientemente importante para a descrição de novas espécies, de forma que a variação intraespecífica existente pode gerar uma "inflação" no número de espécies. Análises realizadas nesta tese demonstraram a importância da utilização de ferramentas estatísticas para a circunscrição de espécies de *Cypella*. Este tipo de análise deveria ser utilizado em trabalhos taxonômicos futuros, principalmente para Tigridieae.

As análises filogenéticas desenvolvidas no capítulo III demostraram grande valor para a compreensão da evolução de Tigridieae. As análises filogenéticas apresentadas demonstraram que, mesmo com a ampla amostragem e a utilização de oito marcadores, os principais gêneros ocorrentes na região Sul do Brasil, *Cypella* e *Calydorea,* mantiveram-se não monofiléticos. Estes resultados demonstram a necessidade de uma revisão taxonômica que contemple *Cypella, Calydorea, Catila, Herbertia, Kelissa* e *Onira*. Neste mesmo capítulo, os resultados obtidos com anatomia foliar possibilitaram a delimitação de caracteres diagnósticos para a subtribo Cipurinae (Tigridieae). Caracteres importantes como o tipo de esclerênquima e a posição foram analisados evolutivamente, e demonstraram possíveis adaptações ecológicas. Além disso, foi demonstrado que a utilização da anatomia pode contribuir muito com a taxonomia e sistemática, principalmente para a compreensão dos processos evolutivos e diversificação das espécies.

A caracterização química dos óleos florais possibilitou a compreensão da diversidade de lipídios secretados nos elaióforos de *Cypella*, *Cipura*, *Herbertia* e *Kelissa*. As variações na composição de lipídios secretados poderiam ser resultado de adaptações aos polinizadores. Os resultados obtidos nesta tese forneceram dados importantes e que podem ser utilizados em trabalhos que visem o esclarecimento das relações entre flores secretoras de óleos e abelhas especializadas. Este trabalho também forneceu uma caracterização importante para estudos futuros em biologia de polinização e hipóteses sobre a utilização destes lipídios florais pelas abelhas.

Assim, a presente tese de doutorado contribuiu com resultados importantes para a compreensão da evolução de Tigridieae e que poderão ser utilizados para a revisão taxonômica dos principais gêneros de Cipurinae. Além disso, espera-se que os resultados fornecidos também colaborem com a compreensão dos processos de diversificação da tribo.

CAPÍTULO VI (ANEXOS)

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Overlooked diversity in Brazilian *Cypella* (Iridaceae, Iridoideae): four new taxa from the Río de la Plata grasslands

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Abstract

Three new species and one subspecies of *Cypella* are described for Rio Grande do Sul (RS), Brazil: *Cypella altouruguaya* from northern RS, *C. amplimaculata* widely distributed across the state and *C. rivularis* restricted to southern RS, in grassland streams of the Pampa biome. *Cypella hauthalii* subsp. *minuticristata* is found in a central area of Rio Grande do Sul. The different taxa are described, illustrated and compared with related species. The resulting taxonomic framework shows that most of the species described for *Cypella* occur in the Río de la Plata grasslands, with various infrageneric taxa characterised by a high level of endemism, especially in the Subtropical Grasslands of Southern Brazil.

Key words: Campos eco-region, endemism, Rio Grande do Sul, Subtropical Grasslands, taxonomy

Introduction

Iridaceae is divided into seven subfamilies and comprise about 2030 species distributed among 65 to 75 genera (Goldblatt *et al.* 2008). The Iridoideae, with more than 900 species, represent one of the two major evolutionary branches of the family and make up about 44% of the species richness of the Iridaceae (Goldblatt & Manning 2008). Among the five tribes of this subfamily, the New World tribe of Tigridieae forms a monophyletic lineage of about 15 genera and 160 species (Goldblatt & Manning 2008, Chauveau *et al.* 2012). *Cypella* Herbert (1826: t. 2637), with 30 species and four subspecies accepted by the World Checklist of Iridaceae (WCI), is one of two largest genera of the tribe in South America (Goldblatt & Manning 2008, Barker 2014). The taxonomic delimitation of this genus phylogenetically closely related to *Calydorea* and *Herbertia* remains controversial (Chauveau *et al.* 2012, Deble *et al.* 2012). Indeed, among the species accepted by the WCI in *Cypella*, three species present the distinctive morphological features of *Phalocallis* Herbert (1839: t. 3710): *C. boliviana* Huaylla (2012: 297), *C. geniculata* (Klatt 1871: 517) Ravenna (1964: 53) and *C. oreophila* Spegazzini (1917: 44). The former species is considered morphologically strictly related to the type species of *Phalocallis*, *P. coelestis* (Lehmann 1826: 17) Ravenna (1977: 9) and is only distinguished by small variations of floral traits (Huaylla & Wood 2012). *Cypella geniculata* and *C. oreophila* were included by Ravenna (2009) in *Phalocallis* based on the same distinctive floral traits than the type species of the genus. Furthermore, the latest comprehensive phylogeny of Tigridieae confirmed that *Phalocallis* should be regarded as a separate genus from *Cypella* (Chauveau *et al.* 2012). The resulting circumscription of *Cypella* shows that 80% of the species and subspecies are found in the Río de la Plata grasslands (RPG), one of the most extensive biogeographic units of the grassland biome in the world (Medan *et al.* 2011). Indeed, this is the largest complex of subtropical and temperate grassland ecosystems in South America (Soriano *et al.* 1992, Miñarro & Bilenca 2008). These grasslands include the eco-regions of Pampas in North Eastern Argentina, and the Campos eco-regions in Uruguay, Northern Argentina, South East Paraguay and Southern Brazil, where most of the *Cypella* species are distributed (Di Giacomo & Krapovickas 2005, Overbeck *et al.* 2007, Paruelo *et al.* 2007). In Southern Brazil, the grassland vegetation is included in two separate biomes according to the current official classification (IBGE 2004): the Pampa and the Atlantic Forest (Overbeck *et al.* 2007). The RPG is perhaps one of the regions in the world with highest rates of land use and land cover changes related to human activities

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(Vega *et al.* 2009). Taxonomic and ecological studies are essential to understand how these changes will impact the biodiversity and to identify appropriate actions of conservation.

This study aims to describe and illustrate three new species and one new subspecies of *Cypella*, endemic to the Subtropical Grasslands of Southern Brazil (Campos eco-regions). The new taxonomic framework provided by this study is used to assess the species richness and the level of endemism of *Cypella* species in the Río de la Plata grasslands.

Description of the new taxa

The terminology used for the descriptions follows Goldblatt & Manning (2008) and Beentje (2010). Observations and measurements are based on fresh specimens.

Cypella altouruguaya Chauveau & L.Eggers, ***sp. nov.*** (Figs. 1A and 2)

Cypella altouruguaya differs from two closely related species with similar yellow flowers, C. armosa and C. pabstiana, by the cuneate proximal half of the inner tepals (vs. unguiculate). It reminds C. armosa in flower size, but differs by its wider outer tepals, slightly retuse connective apex, adaxial crests not twisted basally and longer abaxial crests. The gross morphology of C. altouruguaya comes close to C. pabstiana, which is distinguished by its smaller flower, longer and totally free filaments, longer anthers and slightly excurrent connective apex.

Type:—BRAZIL. Rio Grande do Sul: Rio Grande do Sul: Trindade do Sul, estrada Trindade do Sul - Pinhalzinho, 610 m, 03 December 2011 (fl, fr), *T.B. Guimarães & L. Dal Ri 64* (holotype, ICN!)

Perennial herb, up to (32–)39–70 cm high above the soil, underground stem up to (3.5–)4.8–8.3(–8.7) cm long. Bulb ovoid, outer cataphylls dark brown, 14–15(–16.8) × (8–)12–15 mm, prolonged in a collar up to (1.8–)3.5–5 (–5.6) cm. Basal leaves green at anthesis 1–3(–4), blades linear-attenuate, plicate, (16.8–)22.5–42(–47) × (0.15–)0.3–0.45(–0.7) cm. Flowering stem cylindrical, (28–)40–63.4 cm long, proximally foliate (one reduced cauline leaf, rarely absent), then bracteose; first internode (4.2–)10–17.2(–19.5) cm long; cauline leaf (11–)16.1–32.7(–39) × (0.15–)0.3–0.7(–0.75) cm. Synflorescence cymosely branched, branches usually 2(–3), each subtending 2–4 pedunculate inflorescences arising from the same point, peduncles (1.0–)3–10.5(–11.5) cm long. Inflorescence one-flowered (rhipidium like); spathes herbaceous, bivalved, lower valve (2–)2.4–3.9 cm long, the upper (3.5–)4–6.3(–6.7) cm long, both with narrow membranous edges. Pedicel filiform, generally shorter than the upper valve with the ovary usually partly to sometimes entirely exserted. Flowers predominantly bright yellow, 45–55 mm diameter. Tepals unequal, shortly fused proximally for 0.5 mm. Outer tepals pandurate, (32–)37–42(–46) × (21–)24–31(–33) mm; the proximal part concave, pale yellow to bright yellow, slightly translucent, broadly marked with a red-brown irregular spot at the base, the distal edge of the concave part sparsely marked by an area of yellow glandular trichomes along the central vein; the distal part reclinate, bright yellow, obovate, retuse and acuminate. Inner tepals reduced, assurgent proximally, then incurved and abruptly reclinate distally, (10–)10.5–12.5(–13) × (8.5–)9–12(–13) mm; the proximal half cuneate, not unguiculate, bright yellow, broadly streaked with red-brown; the distal half bright yellow, longitudinally depressed, except at the distal end, with a dense oblong orange-yellow area of oil-producing trichomes (elaiophore) marked with red-brown spots, the lateral sides firmly revolute, striated transversely with red-brown, the apex acute, spotted with red-brown. Filaments free, erecto-patent and abruptly incurved at the distal end, whitish to whitish-yellow, obclavate, thick, 0.8–0.9 mm wide at mid-length, striated with purple on the inflated base, rarely on the whole length, 3–3.5(–4) mm long. Anthers oblong, 6–7(–7.9) × 1.4–1.8(–2) mm, adnate to the style arms for half of the length; connective apically slightly retuse, whitish-yellow to yellow towards the distal end, 0.8–1.2(–1.5) mm wide, usually covered with a viscous and transparent secretion; locules black; pollen dark yellow-green. Ovary subclavate, (6–)7–9.5(–11) × 2–2.9(–3.1) mm. Style whitish to yellow, 5–6 (–6.2) mm long. Style arms bright yellow, conduplicate, 5–6.5(–7) mm long; crests at the apex, bright yellow, adaxial crest 2, erect, falcate inwards, (3.5–)4–6(–6.5) mm long, abaxial crest triangular, lobed, (1–)1.3–2.6(–3) mm long; stigmatic surfaces transverse, 2, on each side at the base of the abaxial crest, bright yellow, (1.1–)1.2–1.6(–1.8) mm long. Capsule obovate-truncate, (10–)11.7–16.8(–21.8) × (3–)3.5–5(–5.4) mm. Seeds obconical, triangular in adaxial view, sharply angulate, epidermis verrucose areolate, 1.3–1.5 mm long.

FIGURE 1. Habits of new species of *Cypella*. A. *C. altouruguaya* Chauveau & L.Eggers. From T.B. Guimarães & L. Dal Ri 6 (ICN!) B. *C. amplimaculata* Chauveau & L.Eggers. From *A.M. Aita 49* (ICN!), drawings by Anelise Scherer.

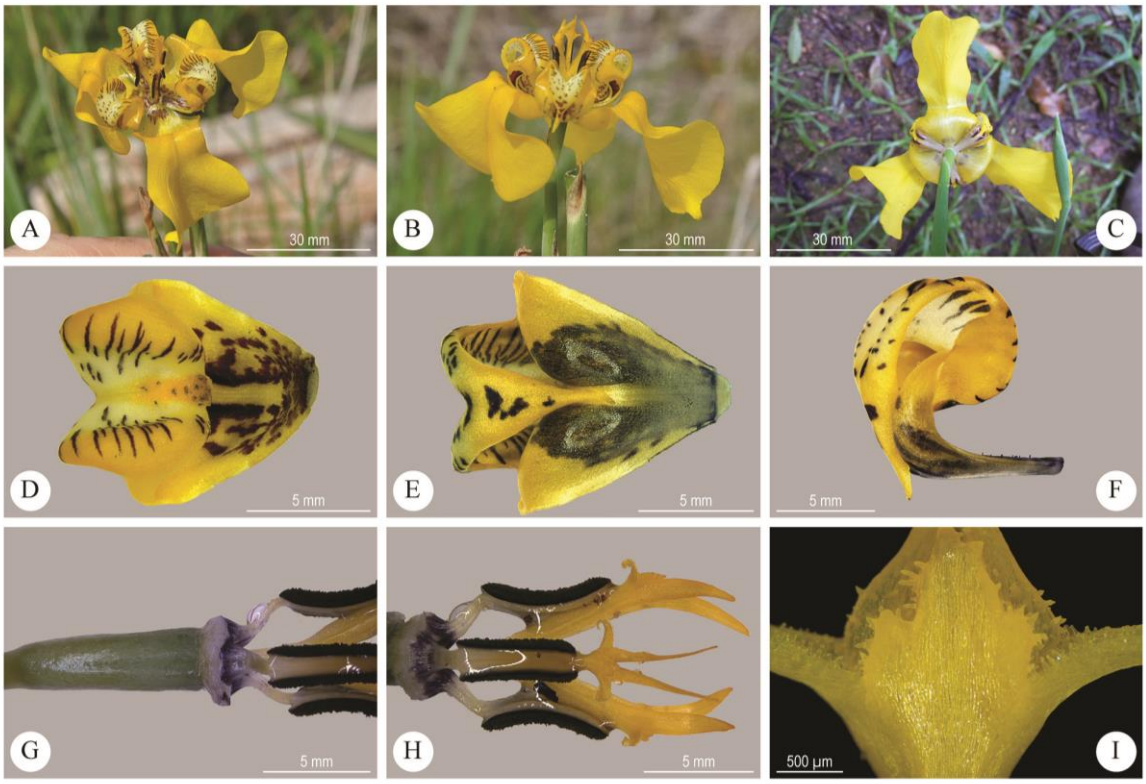

FIGURE 2. *Cypella altouruguaya* Chauveau & L.Eggers. A–C. Flower. A. apical view B. lateral view C. basal view D–F. Inner tepal D. adaxial view E. abaxial view F. lateral view G. Ovary H. Stamens and style in lateral view I. Style crests and stigmatic replicatures. From *L. Eggers & O. Chauveau 716* (ICN!).

Distribution and Habitat:—*Cypella altouruguaya* was collected in the northern part of the state of Rio Grande do Sul, Southern Brazil (Fig. 3), in herbaceous vegetation along roadsides and in contiguous grasslands. The elevation records range from 592 to 615 m. The geographical distribution of the species is markedly reduced, but the populations are dense and consist of numerous individuals. The range of the species falls within the Subtropical Highland Grasslands (Iganci *et al.* 2011), included in the Atlantic Forest biome.

Phenology:—Flowering and fruiting from August to December.

Conservation Status:—According to the IUCN Red List guidelines (IUCN 2001), the species is considered Critically Endangered (CR), with subcriteria B1 (a) and (biii): continuing decline of extent of occurrence and a decline of quality of habitat due to agricultural expansion.

Etymology:—Named after the Alto Uruguai, a physiographic area bounded by the Uruguai and Ijuí rivers where the species was encountered. This region lies in the northern part of Rio Grande do Sul.

Additional specimens examined (paratypes):—BRAZIL. Rio Grande do Sul: Trindade do Sul, estrada secundária entre Trindade do Sul e Rodeio Bonito, beira de estrada, 615 m, 18 August 2012 (fl), *L. Eggers & O. Chauveau 716* (ICN!); Trindade do Sul, estrada secundária entre Trindade do Sul e Rodeio Bonito, campo baixo, 592 m, 5 December 2013 (fl, fr), *L. Eggers & O. Chauveau 884* (MBM!).

Taxonomic relationships:—We first studied one herbarium specimen of the new species collected in 2011 and kept at ICN; thereafter, further specimens were obtained by ourselves at the type locality in August 2012. Only one plant had flowers at this time of the year, but bulbs of similar plants were collected from a large population and cultivated for identification, illustration and measurements. Additional field observations were conducted in 2013. The species is here compared with two species of *Cypella* with yellow flowers: *C. armosa* Ravenna (1981a: 20) and *C. pabstiana* Ravenna (1981a: 18). *Cypella armosa* is readily distinguished from the new species by the overall shape of the flower, marked by the laxly hanging outer tepals, and its long and twisted adaxial crests. *Cypella pabstiana* may be more easily confused with *C. altouruguaya*, and detailed observations of the stamens are needed to discriminate

between the two species. In *C. pabstiana*, the filaments are connate for more than two-thirds of their length and both filaments and anthers are distinctly shorter. The character states retained to compare and separate the different species are presented in Table 1.

TABLE 1. Morphological characters retained to compare *Cypella altouruguaya* and closely related species.

Character/Species	***C. altouruguaya***	***C. armosa****	***C. pabstiana*** **
Plant height (cm)	(32–)39–70	to 58	to 33
Flower diameter (mm)	45–55	50–65	40–45
Size of outer tepals (mm)	(32–)37–42(–46) × (21–)24–31(–33)	38–40 × 12–16	near to 30 × 26.5
Size of inner tepals (mm)	(10–)10.5–12.5(–13) × (8.5–)9–12(–13)	near to 15 long	near to 8 × 7
Inner tepal shape	proximal half cuneate, not unguiculate	narrowly unguiculate along the proximal two-thirds	proximally unguiculate on more than half
Filament length (mm)	3–3.5(–4), totally free	3.2, totally free	near to 2.5, connate for about 1.9
Anther length (mm)	6–7(–7.9)	7	near to 4.4
Connective apex	not excurrent, slightly retuse	excurrent, apiculate	slightly excurrent
Adaxial crests length (mm)	(3.5–)4–6(–6.5)	5.5–7.5	N/A
Adaxial crests shape	not twisted basally	strongly twisted basally	N/A
Abaxial crests length (mm)	(1.1–)1.2–1.6(–1.8)	near to 0.8	N/A
Geographical distribution	Southern Brazil (northern part of RS)	South Paraguay, Northeast Argentina (CC, CR, FO, MN, SF), and Southern Brazil (western border of RS)	Southern Brazil (PR)

*Data obtained from Ravenna (1981a) and the following measured specimens:—PARAGUAY. Cordillera: San Bernardino, February 1966, *P.F. Ravenna 462* (isotype, K!); ARGENTINA. Corrientes: Capital, 12 October 1967, *A. Krapovickas & C.L. Cristobal 13590* (CTES!), Santo Tomé, 16 November 1994, *M.M. Arbo et al. 6286* (CTES!); 18 November 1994, *M.M. Arbo et al. 6455* (CTES!); BRAZIL. Rio Grande do Sul: São Borja, 8 November 2012, *L. Eggers et al. 761* (ICN!).

**Data obtained from Ravenna (1981a).

Notes: N/A = not available; Provinces of Argentina: CC = Chaco, CR = Corrientes, FO = Formosa, MN = Misiones, SF = Santa Fe; States of Brazil: PR = Paraná, RS = Rio Grande do Sul.

Cypella amplimaculata Chauveau & L.Eggers, ***sp. nov.*** (Figs. 1B and 4)

Cypella amplimaculata is comparable to C. fucata and C. herbertii, two species with orange flowers; however, it is distinguished by a broad red brown central line extended longitudinally on the outer tepals and much longer style arms, the distance between the anthers being distinctly greater. The new species strongly reminds C. fucata in general aspect, but differs by a greater flower diameter, longer and erecto-patent filaments, longer anthers, and by the twisted adaxial crests. It is distinct from C. herbertii by the narrower leaf width, the shorter connate part of the filaments, the narrower width and lighter colour of the connective (vs. dark red-brown to dark violet), the longer adaxial crests and lighter colour of crests base (vs. dark red-brown to dark purple).

Type:—BRAZIL. Rio Grande do Sul: Piratini, BR 293, direção Bagé, 140 m, 25 October 2011 (fl, fr), *A.M. Aita 49* (holotype, ICN!).

Perennial herb, up to (16.5–)27–64(–70) cm high above the soil, underground stem up to (2.2–)3.5–7(–14) cm long. Bulb ovoid, outer cataphylls dark brown, 10–14(–17) × 10–13(–17) mm, prolonged in a short collar. Basal leaves green at anthesis (0–)2–3(–5), blades linear-attenuate, plicate, (8.5–)25–56.5(–65.5) × (0.15–)0.45–0.7(–0.9) cm. Flowering stem cylindrical, (11.5–)21.1–58(–64) cm long, proximally foliate (one reduced cauline leaf), then bracteose; first internode (0.4–)7.5–16.7(–24.5) cm long; cauline leaf (4.7–)12.7–24.5(–40.5) × (0.15–)0.45–0.8 cm. Synflorescence cymose, simple or 2(–3) branches, each subtending 2–5 pedunculate inflorescences arising from the same point, peduncles (3–)3.6–11.2 cm long. Inflorescence one-flowered (rhipidium like); spathes herbaceous, bivalved, lower valve 2–4 cm long, the upper (3.6–)4.1–5.6(–6.4) cm long, both with membranous edges. Pedicel filiform, generally

shorter than the upper valve with the ovary usually partly exserted. Flowers predominantly orange, 45–60 mm diameter. *Tepals* unequal, shortly fused proximally for 0.5–1 mm. Outer tepals pandurate, (27–)32–36(–39) × (19–)22–28 mm; the proximal part concave, pale yellow to orange-yellow, slightly translucent, finely purple veined mainly on the abaxial side, broadly marked with a purple spot at the base and a conspicuous purple to purplish-red central line extended longitudinally beyond the constricted region, the distal edge of the concave part sparsely marked by an area of glandular trichomes on the central line; the distal part reclinate, orange, obovate, slightly retuse and acuminate. Inner tepals reduced, assurgent proximally, then incurved and abruptly reclinate distally, 10–11(–12) × 7–9 mm; the proximal half cuneate, not unguiculate, whitish, broadly streaked with purple; the distal half orange, longitudinally depressed, except at the distal end, and white in the middle with a dense oblong orange-yellow area of oil-producing trichomes (elaiophore) marked with purple spots, the lateral sides firmly revolute, striated transversely with purple, the apex acute, spotted with purple. Filaments connate basally for 0.1–0.2 mm, porrect, whitish, obclavate, 0.5–0.6 mm wide at mid-length, striated with purple on the inflated base, rarely on the whole length, (2–)2.2–3.5(–4) mm long. Anthers oblong, (5–)5.2–6.2(–7.5) × 1.1–1.5 mm, adnate to the style arms for half of the length; connective apically slightly retuse, whitish to pale orange-yellow towards the distal end, 0.7–1 mm wide, usually covered with a viscous and transparent secretion; locules dark brown to black; pollen ochraceous. Ovary subclavate, (7–)8–9.5(–11) × 2.1–2.5(–3) mm. Style whitish to pale yellow, rarely finely striated with purple on the whole length, (4.1–)5–6(–6.8) mm long. Style arms pale yellow to orange towards the distal end, conduplicate, (4–)4.5–5(–5.5) mm long; crests at the apex, orange, adaxial crest 2, erect, longitudinally twisted at the base, slightly falcate inwards, (3.8–)4–5(–5.8) mm long, abaxial crest triangular, lobed, (0.6–)1–1.9(–2.1) mm long; stigmatic surfaces transverse, 2, on each side at the base of the abaxial crest, usually dark red-brown, (0.6–)1–1.3(–1.8) mm long. Capsule obovate-truncate, 11–20 × 4–6.5 mm. Seeds obconical, triangular in adaxial view, sharply angulate, epidermis verrucose areolate, 1.2–1.5 mm long.

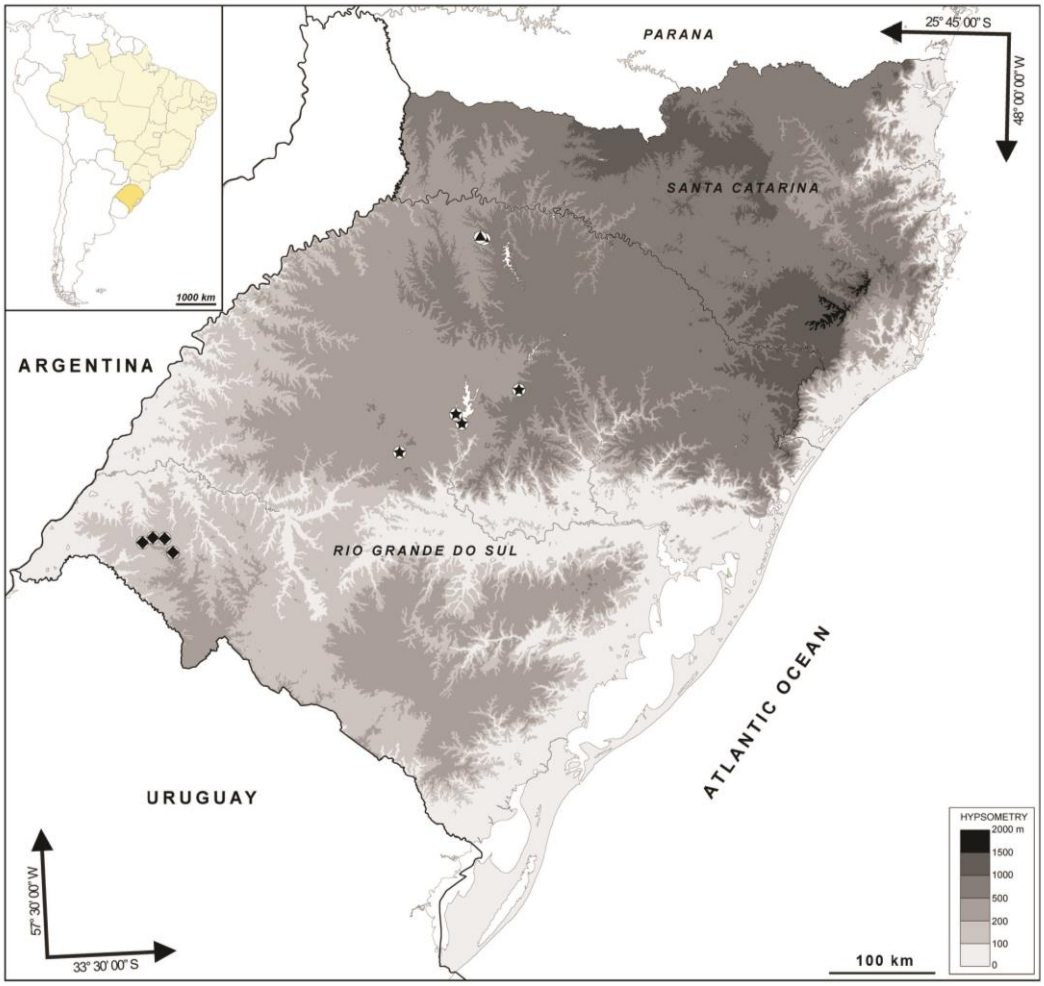

FIGURE 3. Distribution map of *Cypella altouruguaya* (triangle inside circle), *C. hauthalii* subsp. *minuticristata* (star inside circle) and *C. rivularis* (rhombus inside circle) in Southern Brazil.

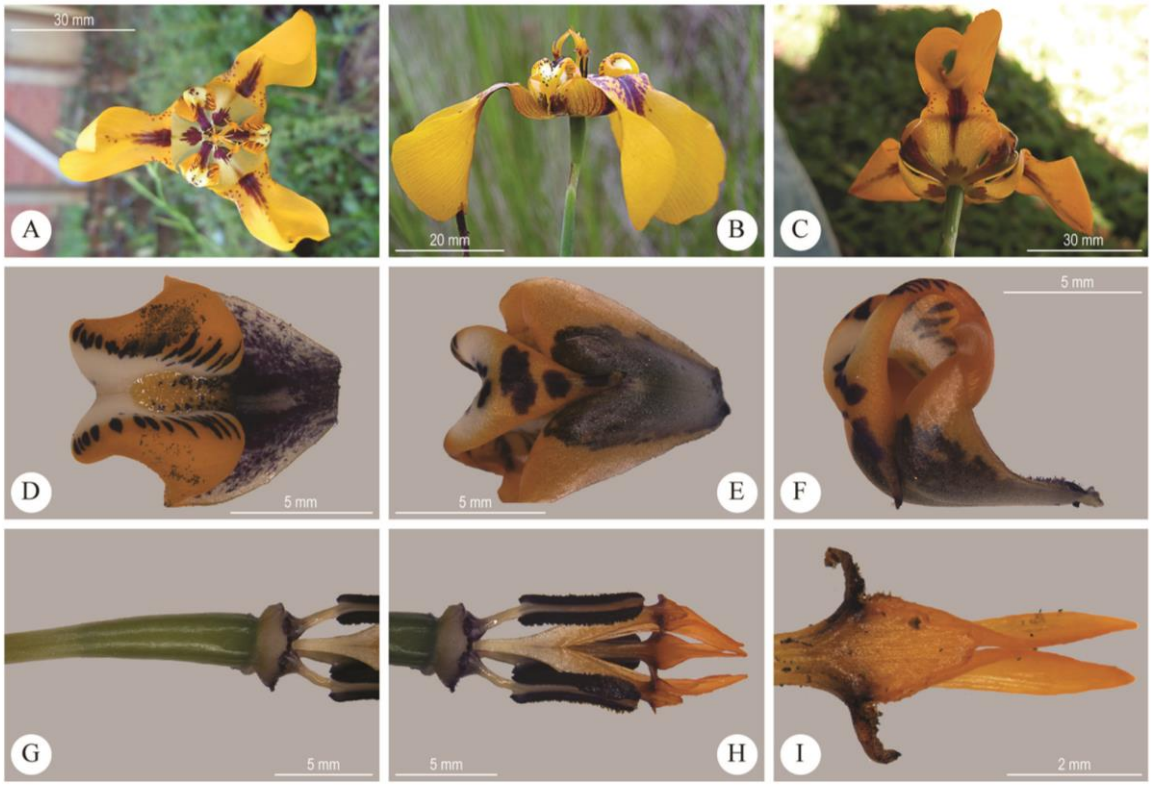

FIGURE 4. *Cypella amplimaculata* Chauveau & L.Eggers. A–C. Flower. A. apical view B. lateral view C. basal view D–F. Inner tepal D. adaxial view E. abaxial view F. lateral view G. Ovary H. Stamens and style in lateral view I. Style crests and stigmatic replicatures. From *A.M. Aita 49* (ICN!).

Distribution and Habitat:—*Cypella amplimaculata* was collected in the state of Rio Grande do Sul, Southern Brazil (Fig. 5), in grassland vegetation of low to moderate elevation (87 to 661 m). The populations usually consist of few individuals scattered in dry grasslands. The geographical distribution of the species overlaps the Pampa biome and the southern part of the Subtropical Highland Grasslands included in the Atlantic Forest biome.

Phenology:—Flowering and fruiting from September to December, March and June.

Conservation Status:—According to the IUCN Red List guidelines (IUCN 2001), the species can be considered as Nearly Threatened (NT), but may qualify for a higher threat category in the future, mainly because of decline quality or loss of habitat through substitution of natural grasslands by agricultural areas.

Etymology:—Named after the broad red-brown central line extended longitudinally on the outer tepals, since the species can be easily distinguished by this floral feature.

Additional specimens examined (paratypes):—BRAZIL. Rio Grande do Sul: Cerrito, BR 293, beira de estrada, 87 m, 18 November 2006 (fl), *L. Eggers & T.T. Souza-Chies 191* (ICN!); Capão do Leão, 92 m, 18 November 2006 (fl, fr), *L. Eggers & T.T. Souza-Chies 192* (ICN!); Livramento, Cerro do Armour, 210 m, 17 October 2009 (fl), *L. Eggers & T.T. Souza-Chies 508* (ICN!); Porto Alegre, Morro Santana, trilha para lado sul do Morro, 287 m, 23 September 2011 (fl), *L. Eggers & O. Chauveau 664* (MBM!); Porto Alegre, Morro Teresópolis, Praça Dr. Dario Rodrigues da Silva, 192 m, 05 October 2011 (fl), *T.L.S. Alves 81* (ICN!); Porto Alegre, Morro São Pedro, em campo próximo à antena, 15 December 2011 (fl, fr), *T.L.S. Alves 174* (SI!); Porto Alegre, Morro Santana, em campo recentemente queimado, face norte, 01 June 2012 (fl), *T.L.S. Alves 220* (ICN!); Viamão, Morro Santana, 286 m, 19 March 2013 (fl), *L. Eggers et al. 819* (ICN!); Júlio de Castilhos, estrada Júlio de Castilhos para Quevedos, campo pastejado, 468 m, 17 October 2013 (fl), *L. Eggers et al. 823* (ICN!); Júlio de Castilhos, estrada para baragem Kotzian, 307 m, 18 October 2013 (fl.), *L. Eggers & O. Chauveau 824* (MBM!); Fontoura Xavier, BR 386, aproximadamente Km 258, antes do Parque das Tuias, campo nativo perto de um córrego de água, 661 m, 04 December 2013 (fl, fr), *L. Eggers & O. Chauveau 883* (P!).

Taxonomic relationships:—*Cypella amplimaculata* has been collected since 2006 by ourselves, but has been erroneously neglected because of its close similarity to *C. fucata* Ravenna (1981a: 18). Most of the time, *C. amplimaculata*

is a higher plant with longer and broader basal leaves as well as larger flowers than *C. fucata*. However, these characters are not discriminant for some samples of the new species and the serious differences observed in relation to the original description of *C. fucata* have been initially attributed to a higher phenotypic plasticity. Nevertheless, further detailed observations were carried out during various field expeditions and diagnostic characters were identified to distinguish the new species from *C. fucata*. Beyond the broad central line present on the outer tepals and the length of the style arms, morphological characters such as the perigon diameter, the length of the different parts of the androecium, the way the filaments diverge from the main axis of the flower and the conformation of the adaxial crests were retained to differentiate both species. In this context, the specimens used by Marco *et al.* (2009) to study the genetic variability within *C. fucata* have been misidentified and belong definitely to *C. amplimaculata*.

The new species shares superficial similarities with *C. herbertii* (Lindley 1826: t. 949) Herbert (1826: supra t. 2599), but it can be easily distinguished by the characteristic connective and style crests base colour of the latter. Additionally, the length of adaxial crests is much shorter and the leaves are much broader in *C. herbertii*. Cross-comparisons of relevant character states between the three species are presented in Table 2.

TABLE 2. Morphological characters retained to compare *Cypella amplimaculata* and closely related species.

Character/Species	*C. amplimaculata*	*C. fucata* *	*C. herbertii* **
Plant height (cm)	(16.5–)27–64(–70)	10–35	30–100
Basal leaf length (cm)	(8.5–)25–56.5(–65.5)	8–22	16–35
Basal leaf width (mm)	(1.5–)4.5–7(–9)	0.6–3	20–25
Lower valve length (cm)	2–4	1.4–2.4	near to 1.9
Upper valve length (cm)	(3.6–)4.1–5.6(–6.4)	2.8–4.5	near to 3.8
Flower colour	orange	dull orange	orange
Flower diameter (mm)	45–60	25–33	60–70
Size of outer tepals (mm)	(27–)32–36(–39) × (19–)22–28	17–24 × 10–12	40–45 × 19–20
Size of inner tepals (mm)	10–11(–12) × 7–9	7–8 × 8–10	14 × 9
Filament length (mm)	(2–)2.2–3.5(–4), connate for 0.1–0.2	near to 1.8, connate for 0.1–0.2	near to 3.4, connate for about 1.4
Anther length (mm)	(5–)5.2–6.2(–7.5)	3.6–5	near to 5.5
Style arms length (mm)	(4–)4.5–5(–5.5)	1.2–2.5	1–1.9
Connective colour	whitish to pale orange-yellow	whitish to pale orange-yellow	dark red-brown to dark violet
Connective width (mm)	0.7–1	0.8–1.4	1.5–1.8
Adaxial crests length (mm)	(3.8–)4–5(–5.8)	3.8–5.3	1.8–2
Abaxial crests length (mm)	(0.6–)1–1.9(–2.1)	0.8–2	0.9–1
Stigmatic surfaces colour	dark red-brown	dull orange	dark red-brown to dark purple
Geographical distribution	Southern Brazil (RS)	Southern Brazil (RS, SC), Northeast Argentina (CR, ER), Uruguay	Southern Brazil, Northeast Argentina (BA, CR, ER, MN), South Paraguay, Uruguay

*Data obtained from Ravenna (1981a) and the following measured specimens:—BRAZIL. Santa Catarina: Lajes, 4 February 1963, *P.R Reitz 6579* (paratype, HBR!); Rio Grande do Sul: Pinheiro Machado, 20 November 2008, *L. Eggers & T.T. Souza-Chies 442* (ICN!); Piratini, 21 November 2008, *L. Eggers & T.T. Souza-Chies 444* (ICN!); Porto Alegre, 09 November 2010, *L. Eggers & T.T. Souza-Chies 589* (ICN!); Uruguaiana, 06 November 2012, *L. Eggers et al. 746* (ICN!); Quaraí, 22 November 2012, *L. Eggers et al. 790* (ICN!).

**Data obtained from Ravenna (1968) and the following measured specimens:—BRAZIL. Rio Grande do Sul: São Borja, 30 October 2009, *L. Eggers & T.T. Souza-Chies 547* (ICN!); Quaraí, 27 October 2011, *A.M. Aita 077* (ICN!); Aceguá, 13 November 2013, *L. Eggers et al. 866* (ICN!); URUGUAY. Flores: Cerro Colorado, 10 November 2013, *L. Eggers et al. 850* (ICN!); Cerro Largo: Melo, 13 November 2013, *L. Eggers et al. 862* (ICN!).

Notes: Provinces of Argentina: BA = Buenos Aires, CR = Corrientes, ER = Entre Ríos, MN = Misiones; States of Brazil: RS = Rio Grande do Sul, SC = Santa Catarina.

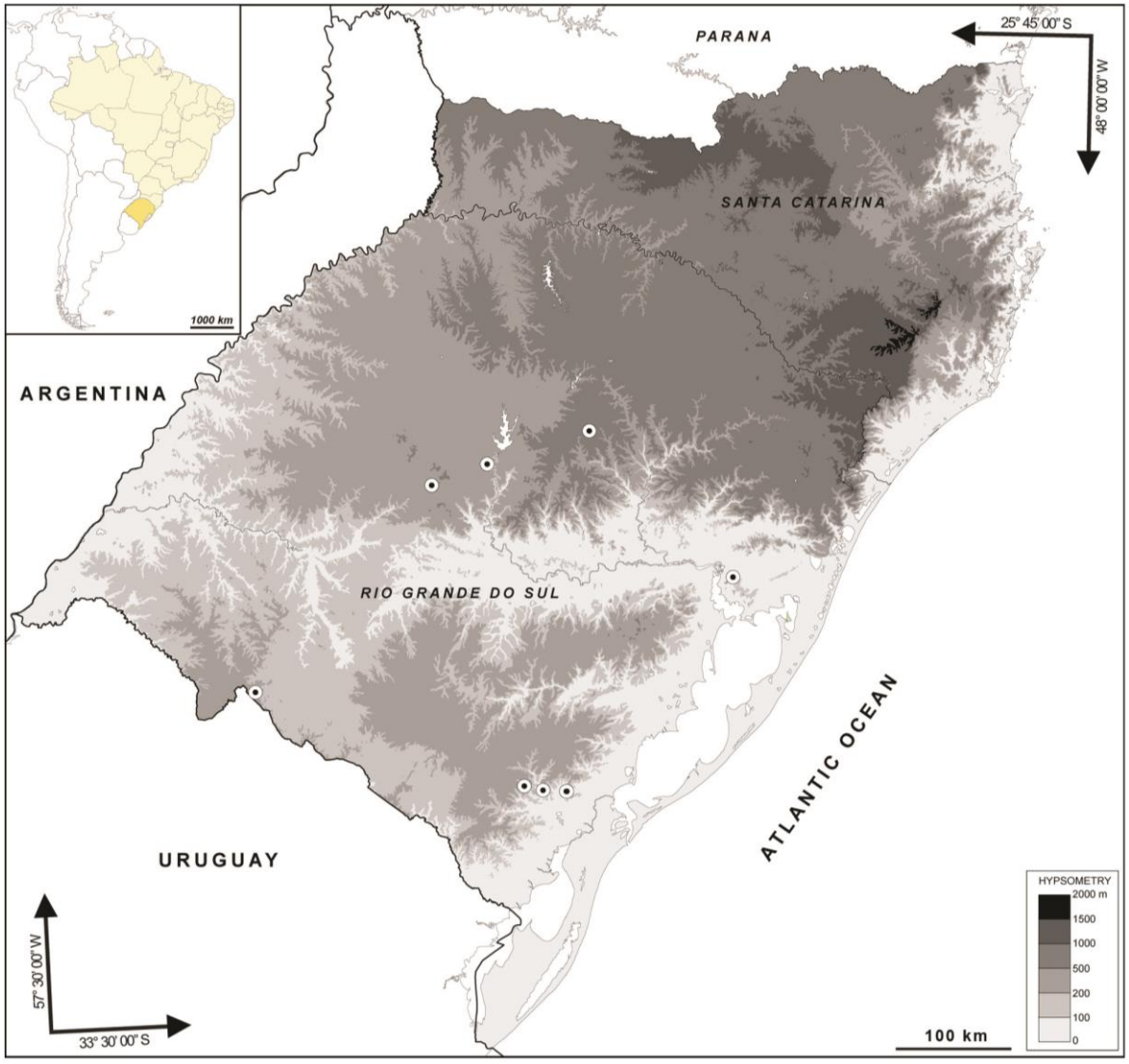

FIGURE 5. Distribution map of *Cypella amplimaculata* (circle) in Southern Brazil.

Cypella hauthalii* subsp. *minuticristata Chauveau & L.Eggers, ***subsp. nov.*** (Figs. 6 and 7)

Cypella hauthalii subsp. minuticristata reminds C. hauthalii subsp. opalina and C. hauthalii subsp. hauthalii in gross morphology, but differs from both subspecies by the much shorter or even obsolete adaxial and abaxial crests and narrower inner tepals.

Type:—BRAZIL. Rio Grande do Sul: Soledade, propriedade particular do Sr. Waldemar Freitag, 534 m, 2 November 2013 (fl, fr), *L. Eggers et al. 833* (holotype, ICN!; isotypes, MBM!, P!, SI!)

Perennial herb, up to (10–)11.4–20.6(–22.5) cm high above the soil, underground stem up to (2.9–)4.5–7.3(–9) cm long. Bulb subglobose to ovoid, outer cataphylls dark brown, (17–)19.3–24.1(–28.2) × (10.7–)13.5–20.2(–25) mm, prolonged in a short collar. Basal leaves green at anthesis (0–)1–4(–7), blades linear-attenuate, plicate, (13.9–)15.2–19.7(–22.5) × (0.3–)0.45–0.65 cm. Flowering stem cylindrical, (4.3–)5.5–12.3(–15) cm long, proximally foliate (one reduced cauline leaf), then bracteose; first internode (obsolete–)0.2–3.5(–4) cm long; cauline leaf (6.1–)8.5–12.5(–15.1) × (0.25–)0.35–0.6(–0.8) cm. Synflorescence cymosely branched, branches 2–3, each subtending 2–4 pedunculate inflorescences arising from the same point, peduncles 2.2–5.5(–6.5) cm long. Inflorescence two-flowered (rhipidium like); spathes herbaceous, bivalved, lower valve subventricose (2.3–)2.5–3.7(–3.9) cm long, the upper (3.6–)3.8–5.4(–5.6) cm long,

both with membranous edges. Pedicel filiform, generally shorter than the upper valve with the ovary usually partly exserted, the top of the ovary 1.7–4.4 mm above the top of the upper valve, but sometimes up to 2 mm below. Flowers predominantly white, subtly tinged with blue, 35–45(–50) mm diameter. Tepals unequal, conspicuously unguiculate, shortly fused proximally for 0.7–1(–1.5) mm. Outer tepals (26.9–)29–35(–38) × 20–28(–33) mm; the claw erecto-patent, attenuate towards the proximal end, marked with red-brown spots forming sometimes transversal and irregular stripes at the base, the distal edge sometimes marked in the middle with a yellow area extended longitudinally; the

FIGURE 6. Habit of *Cypella hauthalii* subsp. *minuticristata* Chauveau & L.Eggers. From *L. Eggers & O. Chauveau 728* (ICN!), drawing by Anelise Scherer.

blade flabellate, spreading, slightly apiculate to acuminate. Inner tepals reduced, conspicuously unguiculate, (13.5–)14–16(–17) × 4–5(–6) mm; the claw whitish-blue, porrect, sublorate, extending to three-fourths of the tepal length, the distal one-third slightly widened, the proximal half densely marked with red-brown spots; the blade whitish-blue, ovate, curved upward proximally, then usually reclinate at the distal end, centrally depressed with a dense ovate to cordate yellow area of oil-producing trichomes (elaiophore) in the middle, the lateral sides firmly revolute, each with a yellow area spotted with red-brown, the apex acute. Filaments free, erect, whitish, filiform, 0.2–0.25 mm wide at mid-length, densely striated with purple on the slightly inflated base, sparsely on the whole length, (5–)6–6.5 mm long. Anthers oblong, (4.6–)5–6 × (0.7–)0.9–1.3(–1.4) mm, adnate to the style arms for two-thirds to 3/4 of the length; connective apically excurrent, slightly retuse, whitish to pale yellow, (0.2–)0.4–0.8(–1) mm wide, usually covered with a viscous and transparent secretion; locules yellow to black; pollen yellow. Ovary subclavate, (5.1–)6–7.5(–8.4) × (2–)2.2–2.8(–3.1) mm. Style whitish, sometimes finely striated with purple on the whole length, (7.6–)8.4–10(–10.2) mm long. Style arms whitish to pale blue towards the distal end, conduplicate, (2.7–)3–3.5(–3.9) mm long; crests at the apex, whitish to pale blue, adaxial crest 2, erect, rounded or sometimes lobed, (obsolete–)0.1–1(–1.5) mm long, abaxial crest often absent, rounded when present, obsolete to 0.1(–0.3) mm long; stigmatic surfaces transverse, 2, on each side at the base of the abaxial crest, usually pale blue, (0.3–)0.4–0.8(–1) mm long. Capsule obovate-truncate, 6.8–7.3 × 2.8–3.1 mm. Seeds obovoid, obovate in adaxial view, slightly angulate, epidermis reticulate, 1.6–2 mm long.

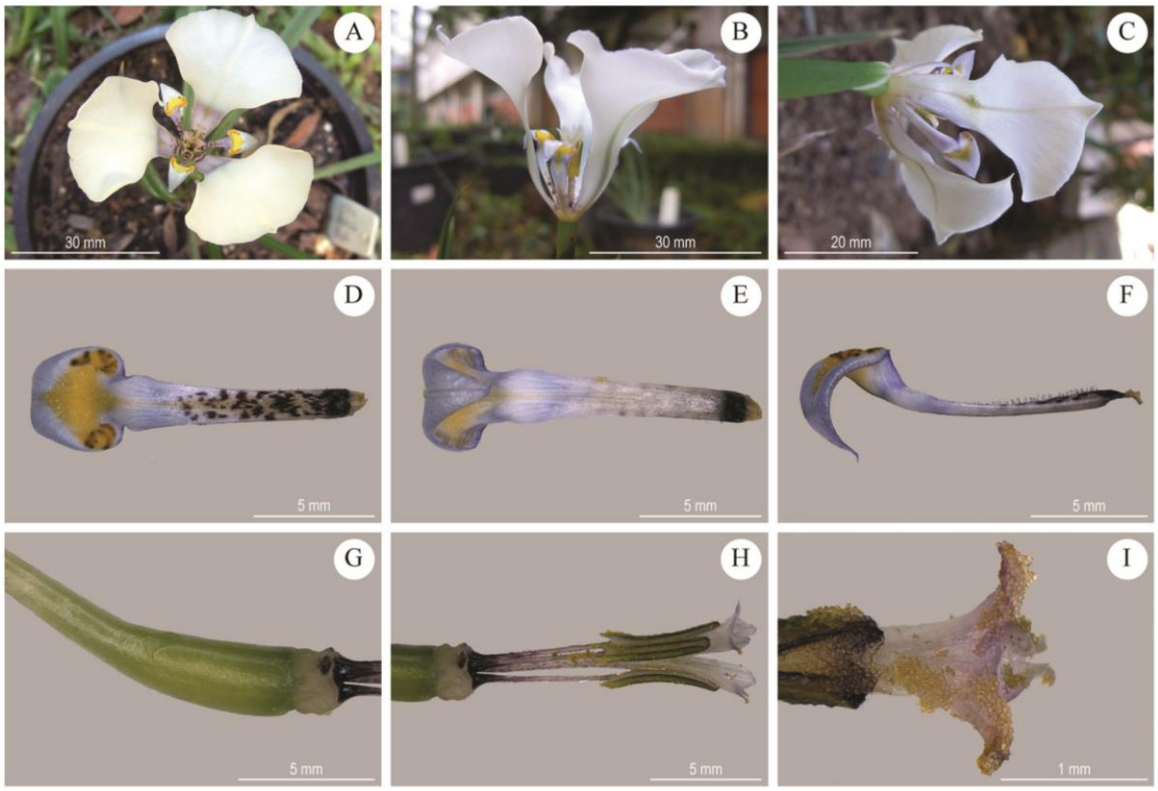

FIGURE 7. *Cypella hauthalii* subsp. *minuticristata* Chauveau & L.Eggers. A–C. Flower. A. apical view B. lateral view C. basal view D–F. Inner tepal D. adaxial view E. abaxial view F. lateral view G. Ovary H. Stamens and style in lateral view I. Style crests and stigmatic replicatures. From *L. Eggers & O. Chauveau 728* (ICN!).

Distribution and Habitat:—*Cypella hauthalii* subsp. *minuticristata* was collected in the central region of the state of Rio Grande do Sul, Southern Brazil (Fig. 3), in grassland vegetation. The elevation records range from 308 to 534 m. The species usually forms large populations. Its geographical distribution overlaps the northern limit of the Pampa biome and the southern part of the Subtropical Highland Grasslands included in the Atlantic Forest biome.

Phenology:—Flowering and fruiting from October to December.

Conservation Status:—According to the IUCN Red List guidelines (IUCN 2001), the species is considered to be Critically Endangered (CR), with subcriteria B1 (a) and (biii): decline quality or loss of habitat through substitution of natural grasslands by agricultural areas.

Etymology:—Named after the outstanding short crests of the style, when compared to the other subspecies of *Cypella hauthalii*.

Additional specimens examined (paratype):—BRAZIL. Rio Grande do Sul: Salto do Jacuí, trevo da BR 481 para baragem, 397 m, 20 October 2012, (fl), *L. Eggers et al. 727* (ICN!); Porto Alegre, planta cultivada desde 2010, proveniente de Salto do Jacuí, 18 October 2012 (fl), *L. Eggers & O. Chauveau 728* (ICN!); Salto do Jacuí, 308 m, 19 October 2013 (fl), *L. Eggers et al. 827* (ICN!). The new subspecies was also recorded from the municipality of Júlio de Castilhos (29°19'32.22"S - 53°48'44.82"W) but without specimen collection (Azambuja, B. 2012, Universidade Federal do Rio Grande do Sul, pers. comm.).

Taxonomic relationships:—This taxon is regarded as a subspecies of *Cypella hauthalii* (Kuntze 1898: 304) Foster (1950: 23) because it is strikingly similar to *Cypella hauthalii* subsp. *opalina* Ravenna (1981a: 2), another subspecies described from Northeast Argentina and western Rio Grande do Sul. The typical subspecies was transferred from *Alophia* Herbert (1840: t. 3779) and occurs in Southern Paraguay and Northeast Argentina. It differs from the other subspecies by the larger and pale lilac-blue flowers and by the long and whitish blue adaxial crests. *Cypella hauthalii* subsp. *opalina* is characterised by its white flowers, slightly tinged with yellow, and its long white adaxial crests. Both subspecies have much longer adaxial and abaxial crests than *C. hauthalii* subsp. *minuticristata* and this characteristic is easily discernible to the naked eye. Furthermore, the localities where occurrences of the new subspecies were identified suggest that its range is distinct from the distribution areas of the other subspecies. The character states retained to compare and separate the different subspecies are presented in Table 3.

TABLE 3. Morphological characters retained to compare *Cypella hauthalii* subsp. *minuticristata* and closely related species.

Character/Species	***C. hauthalii* subsp. *minuticristata***	***C. hauthalii* subsp. *opalina*** *	***C. hauthalii* subsp. *hauthalii*** **
Flower colour	white, subtly tinged with blue	White, subtly tinged with yellow	pale lilac-blue
Size of outer tepals (mm)	(26.9–)29–35(–38) × 20–28(–33)	25–34.5 × 17–23	38–43.7 × 23–24.5
Size of inner tepals (mm)	(13.5–)14–16(–17) × 4–5(–6)	14.5–16.5 × 5.2–5.9	23–24.6 × 7–8
Filament length (mm)	(5–)6–6.5	5.2–6.5	7–9
Anther length (mm)	(4.6–)5–6	5–6	6.5–7.5
Style arms length (mm)	(2.7–)3–3.5(–3.9)	3.5–4	7–7.4
Adaxial crests length (mm)	(obsolete–)0.1–1(–1.5)	3.7–4.5	4.6–4.8
Abaxial crests length (mm)	obsolete to 0.1(–0.3)	0.5–2.3	1.8–2
Stigmatic surfaces colour	whitish blue	white	whitish blue
Geographical distribution	Southern Brazil (central RS)	Northeast Argentina (CR, MN), Southern Brazil (western RS)	Southern Paraguay, Northeast Argentina (CR, MN)

*Data obtained from Ravenna (1981a) and the following measured specimens:—ARGENTINA. Corrientes: Santo Tomé, 20 September 1974, *A. Krapovickas et al. 25807* (paratype, CTES!); BRAZIL. Rio Grande do Sul: Santo Antônio das Missões, 14 October 2005, *L. Eggers & T.T. Souza-Chies 113* (ICN!); Unistalda, 30 October 2009, *L. Eggers & T.T. Souza-Chies 553* (ICN!); São Borja, 08 November 2012, *L. Eggers et al. 764* (ICN!).

**Data obtained from Kuntze (1898), Foster (1950) and the following measured specimens:—PARAGUAY. Paraguari: Ybytymi, October 1892, *R. Hauthal s.n.* (isotype, CTES!); ARGENTINA. Misiones: Posadas, 16 October 2013, *L. Eggers et al. 820* (ICN!).

Notes: Provinces of Argentina: CR = Corrientes, MN = Misiones; States of Brazil: RS = Rio Grande do Sul.

Cypella rivularis Chauveau & L.Eggers, ***sp. nov.*** (Figs. 8 and 9)

Cypella rivularis differs strongly from related species by its habitat characterised by small streams running through the pampean grasslands (vs. well drained places for C. laeta and sandy, stony soils for C. suffusa). Morphologically, it differs from C. laeta by its uniflowered spathes and longer adaxial crests. It is distinguished from C. suffusa by its wider flower diameter, longer outer and inner tepals and longer style arms.

Type:—BRAZIL. Rio Grande do Sul: Uruguaiana, BR 290, aproximadamente Km 645, campo bem preservado, embaixada, em borda de pequenos riachos, 172 m, 25 November 2013 (fl, fr), *L. Eggers et al. 869* (holotype, ICN!; isotypes, MBM!, P!).

FIGURE 8. Habit of *Cypella rivularis* Chauveau & L.Eggers. From *L. Eggers et al. 869* (ICN!), drawing by Anelise Scherer.

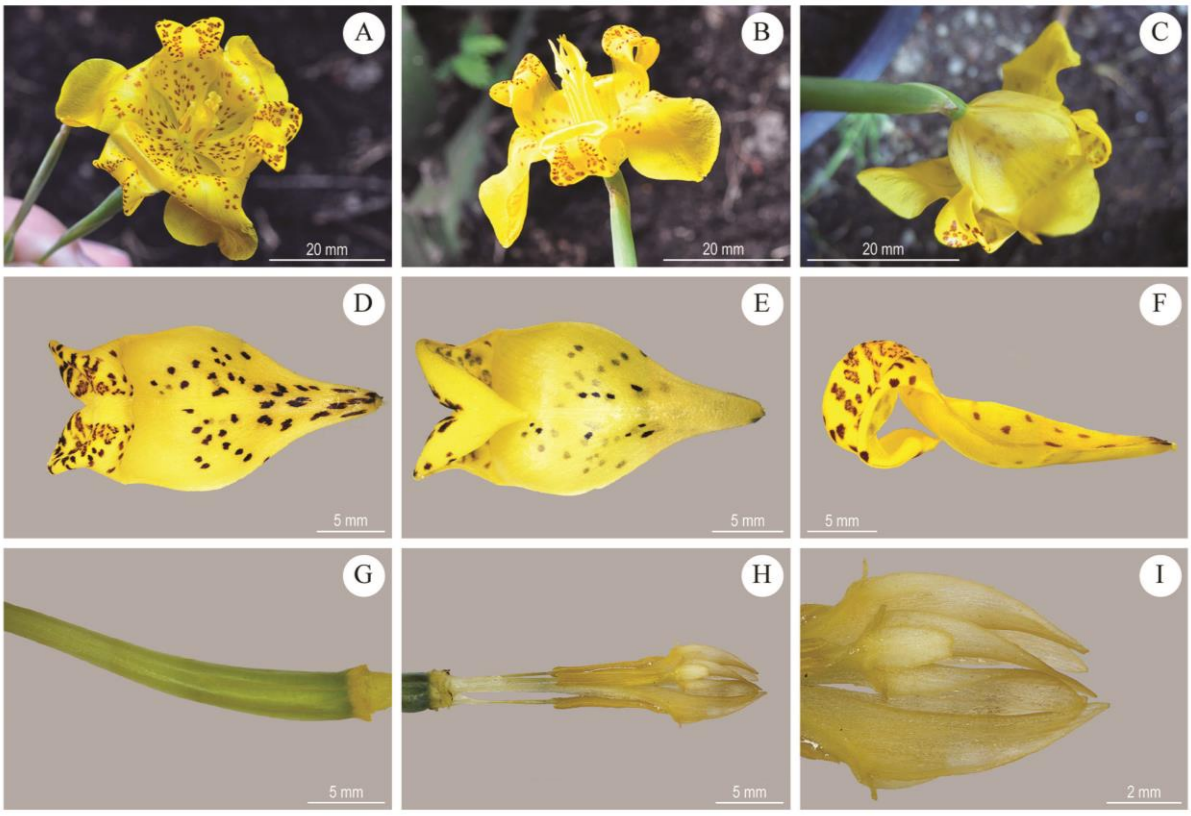

FIGURE 9. *Cypella rivularis* Chauveau & L.Eggers. A–C. Flower. A. apical view B. lateral view C. basal view D–F. Inner tepal D. adaxial view E. abaxial view F. lateral view G. Ovary H. Stamens and style in lateral view I. Style crests and stigmatic replicatures. From *L. Eggers et al. 869* (ICN!).

Perennial herb, up to (23.5–)33.5–49(–70) cm high above the soil, underground stem up to (8–)12.5–19(–22) cm long. Bulb obovoid, outer cataphylls dark brown, (28–)32.6–38(–41.4) × (18–)20.7–27.1 mm, prolonged in a long collar. Basal leaves green at anthesis (2–)4–5(–7), blades linear-attenuate, plicate, (15–)21–51(–59.5) × (0.3–)0.4–0.7(–0.75) cm. Flowering stem cylindrical, (16.8–)26.3–39(–63) cm long, proximally foliate (one reduced cauline leaf, rarely absent), then bracteose; first internode (3–)5.3–13.5(–19.5) cm long; cauline leaf (6.5–)7.2–33.7(–45.7) × 0.2–0.46(–0.6) cm. Synflorescence cymosely branched, branches 2–3(–5), each subtending 2–7 pedunculate inflorescences arising from the same point, peduncles (1.2–)1.9–6(–9) cm long. Inflorescence one-flowered (rhipidium like); spathes herbaceous, bivalved, lower valve (1.3–)1.5–2(–2.2) cm long, the upper (3.2–)3.5–4.7(–7) cm long, both with narrow membranous edges. Pedicel filiform, usually slightly shorter than the upper valve with the ovary partly to entirely exserted, the top of the ovary (3.5–)6.7–13.6 mm above the top of the upper valve. Flowers predominantly bright yellow, (46–)50–58(–61) mm diameter. Tepals unequal, shortly fused proximally for (1.2–)1.6–2.1(–2.4) mm. Outer tepals pandurate, 35–39(–42) × (17–)18–22(–25) mm; the proximal part concave, bright yellow, marked with red-brown dots scattered on the whole surface, the distal edge of the concave part devoid of trichomes; the distal part reclinate, bright yellow, obovate, slightly retuse and shortly apiculate. Inner tepals reduced, the proximal two-thirds erecto-patent and lastly curved upward, the distal one-third incurved and abruptly reclinate, 21–24(–25.5) × 9–10.5(–14) mm; the proximal part shortly unguiculate, then distinctly cuneate and slightly constricted lastly, bright yellow, marked with red-brown dots scattered usually on the whole surface; the distal part bright yellow, longitudinally depressed, except at the distal end, with a dense lanceolate yellow area of oil-producing trichomes (elaiophore), the lateral sides firmly revolute, each densely spotted with red-brown, the apex acute. Filaments 6.4–7.8(–9.3) mm long, usually connate basally for (0–)1–2(–3.2) mm, free for (5–)5.6–6.5(–7.5) mm, erect to porrect, whitish-yellow to pale yellow, filiform, 0.2–0.25 mm wide at mid-length, slightly inflated at the base. Anthers narrowly oblong, (7.6–)7.8–8.5(–9) × (1–)1.4–1.7(–2) mm, adnate to the style arms for two-thirds to three-fourths of the length; connective apically excurrent, acuminate, pale yellow, (0.7–)0.9–1.2(–1.4) mm wide, usually covered with a viscous and transparent secretion; locules pale yellow; pollen yellow. Ovary narrowly subclavate, 9–11(–12) × (2.1–)2.4–2.7(–3) mm. Style whitish-yellow to pale

yellow, (9.9–)11–15.8(–18) mm long. Style arms pale-yellow, conduplicate, (3.7–)4–4.5(–4.9) mm long; crests at the apex, pale yellow to whitish-yellow; adaxial crest 2, erect, falcate inwards, (5–)5.9–6.8(–7.5) mm long; abaxial crest ovate, obtuse, (1.2–)2.2–3.4(–3.8) mm long; stigmatic surfaces transverse, 2, on each side at the base of the abaxial crest, pale yellow, (0.21–)0.5–1(–1.3) mm long. Capsule obovate-truncate, 18.9–23.1 × 4.8–5.5 mm. Seeds irregularly obovate to conical, sharply angulate, epidermis verrucose, 2.5–4 mm long.

TABLE 4. Morphological characters retained to compare *Cypella rivularis* and closely related species.

Character/Species	*C. rivularis*	*C. laeta**	*C. suffusa***
Plant height (cm)	(23.5–)33.5–49(–70)	20–35	10–40
Bulb size (mm)	(28–)32.6–38(–41.4) × (18–)20.7–27.1	22–28 × 15–22	33–47 × 26–30
Basal leaf length (cm)	(15–)21–51(–59.5)	15–20	12–16
Basal leaf width (mm)	(3–)4–7(–7.5)	2–5	1.5–2.5
Lower valve length (cm)	(1.3–)1.5–2(–2.2)	1.4–2.2	1.2–1.9
Upper valve length (cm)	(3.2–)3.5–4.7(–7)	3.4–3.8	3–4
Flowers number/spathe	one-flowered	two-flowered	one-flowered
Flower colour	bright yellow	yellow	yellow
Flower diameter (mm)	(46–)50–58(–61)	45–51	35–37
Outer tepals size (mm)	35–39(–42) × (17–)18–22(–25)	34–37 × 18–20	22–25 × 16–20
Inner tepals size (mm)	21–24(–25.5) × 9–10.5(–14)	16–23 × 7–10.5	18 × 12
Filament length (mm)	6.4–7.8(–9.3), connate for (0–)1–2(–3.2)	near to 6	near to 6
Anther length (mm)	(7.6–)7.8–8.5(–9)	near to 7	near to 7
Style arms length (mm)	(3.7–)4–4.5(–4.9)	near to 3.6	1.5–1.9
Adaxial crests length (mm)	(5–)5.9–6.8(–7.5)	3.5–4	3–4
Abaxial crests length (mm)	(1.2–)2.2–3.4(–3.8)	near to 2	N/A
Habitat	grassland streams	well drained places	sandy, stony soils
Geographical distribution	Southern Brazil (south-west RS)	Northeast Argentina (ER, MN), western Uruguay (P)	Northeast Argentina (CR, MN)

*Data obtained from Ravenna (1981a) and the following measured specimens:—ARGENTINA. Misiones: Apóstoles, February 1907, *C. spegazzini s.n.* (paratype, LPS!); URUGUAY. Paysandú: Chapicuy, 9 November 2013, *L. Eggers et al. 843* (ICN!).

**Data obtained from Ravenna (2009) and the following measured specimens:—ARGENTINA. Misiones: Cainguás, 15 March 2000, *F. Biganzoli et al. 830* (holotype, SI!); Bonpland, October 1906, *Van de Venne s.n.* (Paratype: SI!).

Notes: N/A = not available; Provinces of Argentina: CR = Corrientes, ER = Entre Ríos, MN = Misiones; States of Brazil: RS = Rio Grande do Sul; Departments of Uruguay: P = Paysandú.

Distribution and Habitat:—*Cypella rivularis* was collected in the south-western part of the state of Rio Grande do Sul, Southern Brazil (Fig. 3), along banks and in the bed of narrow and stony grassland streams. The elevation records range from 103 to 226 m. The geographical distribution of the species is markedly reduced, but the populations are large and scattered along the small streams. The range of the species falls within the Pampa biome.

Phenology:—Flowering and fruiting from November to December.

Conservation Status:—According to the IUCN Red List guidelines (IUCN 2001), the species is considered to be Critically Endangered (CR), with subcriteria B2 (a) and (biii): continuing decline of area of occurrence and a decline of quality of habitat. The grassland streams where the species occurs are threatened by land-use changes or environmental degradations of contiguous areas.

Etymology:—Named after the specific grassland habitat of the species, which is not shared by any other member of the genus.

Additional specimens examined (paratypes):—BRAZIL. Rio Grande do Sul: Uruguaiana, estrada secundária para Santana do Livramento a partir da BR 290, campo bem preservado, em borda de pequeno riacho pedregoso, 211 m, 25 November 2013 (fl), *L. Eggers et al. 872* (ICN!); Alegrete, estrada secundária a partir da BR 290, aproximadamente Km 611, bulbos entre as pedras em borda de riacho, 103 m, 25 November 2013 (fl), *L. Eggers et al. 873* (ICN!);

Alegrete, estrada secundária a partir da BR 290, aproximadamente Km 620, campo preservado, em borda de pequeno riacho pedregoso, 226 m, 26 November 2013 (fl), *L. Eggers et al. 874* (ICN!).

Taxonomic relationships:—*Cypella rivularis* has beautiful big yellow flowers and could be superficially mistaken with *C. laeta* Ravenna (1981a: 13) and *C. suffusa* Ravenna (2009: 1) (Table 4). However, its restricted geographical range and typical habitat are so singular that it can be easily identified. Plants grow in stony soils and between rocks along and in narrow streams of the Pampa grassland. During the flowering time, populations offer dazzling sceneries with dozens of bright yellow flowers scattered along the streams. To date, *C. laeta* and *C. suffusa* were not collected in Brazil, but both species occur in the following border provinces of Argentina: Corrientes and Misiones.

FIGURE 10. Distribution of the Río de la Plata Grasslands in southern South America, between 25 and 38°S. The Pampas eco-region is indicated in clear grey and the Campos eco-region in dark grey. The map is adapted from Di Giacomo & Krapovickas (2005), Overbeck *et al.* (2007) and Paruelo *et al.* (2007).

Discussion

The description of four new taxa of *Cypella* suggests that the overall diversity of the genus has been underestimated in the Subtropical Grasslands of Southern Brazil. Actually, with the addition of the taxa described in the present study, five new species and one subspecies were discovered in Southern Brazil since the past two years (Deble *et al.* 2012) and that number accounts for one third of the taxa already known for this region. Except *C. amplimaculata*, these new taxa are considered Critically Endangered (CR) according to the IUCN Red List. The extent of occurrence of *C. altouruguaya* and *C. hauthalii* subsp. *minuticristata* is less than 100 km², while the area of occupancy of *C. luteogibbosa* Deble in Deble *et al.* (2012: 60), *C. magnicristata* Deble in Deble *et al.* (2012: 63) and *C. rivularis* is smaller than 10 km². These results testify for a high level of local endemism among the taxa recently discovered and may explain why this unexpected diversity has been ignored until now. Our observations strongly suggest that the taxa newly described range exclusively in the Campos eco-region of the Río de la Plata grasslands (Fig. 10). All species and subspecies belonging to *Cypella* are found in the RPG biogeographic unit, except *C. amambaica* Ravenna (2009: 4), *C. craterantha* Ravenna (1964: 52), *C. crenata* (Velloso 1827: t. 67) Ravenna (1965: 312), *C. elegans* Spegazzini (1917: 43) and *C. mandonii* Rusby (1896: 125). Among the taxa distributed in the RPG, 20 species and three subspecies are endemic to the Campos eco-region; *C. yatayphila* Ravenna (2009: 3), C. *herbertii* subsp. *reflexa* Ravenna (1981a: 22) and C. *herbertii* subsp. *wolffhuegelii* (Hauman in Hauman-Merck 1909: 84) Ravenna (1965: 312), are endemic to the Pampas eco-region, while *C. herbertii* subsp. *herbertii* and *C. laeta* are found exclusively in both eco-regions of the RPG. Furthermore, *C. exilis* Ravenna (1981b: 492) and *C. laxa* Ravenna (1981a: 15) are mainly distributed in the Campos eco-region, but their range area extends further to the North in nearby regions (Eggers 2014). Therefore,

more than 85% of the taxa currently described for the genus occur in the Río de la Plata grasslands, 80% are endemic to this biogeographic unit and 65% are only found in the Campos eco-region, mainly in the state of Rio Grande do Sul (Southern Brazil) where 14 species and two subspecies are presently registered. These observations suggest that the centre of diversity of *Cypella* is located in the Río de la Plata grasslands, mostly in the subtropical Campos grasslands, and that local endemism is not uncommon at the infrageneric level.

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