
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(BIOLOGIA VEGETAL)

MICROMORFOLOGIA E ANATOMIA FLORAL DAS SEÇÕES
NEOTROPICAIS DE *Bulbophyllum* THOUARS (ORCHIDACEAE,
ASPARAGALES): CONSIDERAÇÕES TAXONÔMICAS E EVOLUTIVAS

ELAINE LOPES PEREIRA NUNES

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Biologia Vegetal).

Setembro - 2014

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Orientadora: Profa. Dra. Alessandra Ike Coan

Coorientador: Prof. Dr. Eric de Camargo Smidt

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581.4
N972m Nunes, Elaine Lopes Pereira
 Micromorfologia e anatomia floral das seções neotropicais de
 Bulbophyllum Thouars (Orchidaceae, Asparagales) : considerações
 taxonômicas e evolutivas / Elaine Lopes Pereira Nunes. - Rio Claro, 2014
 252 f. : il., figs., tabs.

 Tese (doutorado) - Universidade Estadual Paulista, Instituto de
 Biociências de Rio Claro
 Orientador: Alessandra Ike Coan
 Coorientador: Eric de Camargo Smidt

 1. Anatomia vegetal. 1. Anatomia floral de Orchidaceae. 3.
 Dendrobieae. 4. Epidendroideae. 5. Labelo. 6. Nectário. 6. Osmóforos. I.
 Título.

Ficha Catalográfica elaborada pela STATI - Biblioteca da UNESP
Campus de Rio Claro/SP

CERTIFICADO DE APROVAÇÃO

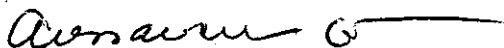
TÍTULO: MICROMORFOLOGIA E ANATOMIA FLORAL DAS SEÇÕES NEOTROPICAIS DE *Bulbophyllum THOUARS* (ORCHIDACEAE, ASPARAGALES): CONSIDERAÇÕES TAXONÔMICAS E EVOLUTIVAS

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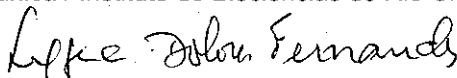
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Data da realização: 26 de setembro de 2014.

Agradecimentos

A Universidade Estadual Paulista “Júlio de Mesquita Filho” (UNESP, campus de Rio Claro), seu corpo docente e técnico-administrativo, por me receber de braços abertos e permitir a realização de um sonho.

A Universidade Federal do Paraná (onde me graduei, me tornei mestre e servidora), seu corpo docente e técnico-administrativo, pela formação inicial e licença de três anos que permitiu que eu concluísse o tão almejado doutorado.

A Ruhr-Universität Bochum, seu corpo docente, técnico-administrativo e discente, pela infraestrutura disponibilizada, todo o auxílio e prestatividade.

À FAPESP pelas bolsas concedidas (de doutorado regular e BEPE - bolsa estágio em pesquisa no exterior), sem as quais esse trabalho não poderia ter sido desenvolvido.

A Dra. Alessandra Ike Coan, pela confiança, pelos ensinamentos, por toda a paciência e pelas broncas mais que merecidas. Mas, sou grata principalmente pela orientação de forma tão presente, por vezes assumindo papel de psicóloga e amiga, mas sem nunca suprimir minhas ideias e tirar a identidade do trabalho.

Ao Dr. Eric C. Smidt, pela coorientação, confiança, discussões e por me apresentar aos “Bulbophyllums”.

Ao Dr. Thomas Stützel, por me receber no “Lehrstuhl für Evolution und Biodiversität der Pflanzen (Ruhr-Universität Bochum)”, por sua supervisão tão dedicada e aconselhamento.

Ao grupo de colegas de pós e amigos carinhosamente apelidado de “in flames” (Kaire Nardi, Luís Felipe Daibes, Mariana Maciel, Naiara Lopes, Paula Maldonado e Thales Henrique Dias) por serem os melhores amigos “do Brasil e do Paraná”, pelo companheirismo, risadas e por serem minha família em Rio Claro.

Aos colegas de laboratório (Ângela, Arthur, Blanca, Kaire, Letícia, Mariana, Mayra, Paula, Renata, Rita, Shirley, Thales, Yuri) pela convivência amigável e divertida, além da troca de experiências e aprendizados.

A Paula Maldonado, pela parceria incrível no capítulo de *B. sect. Micranthae*, além de toda a ajuda com pranchas, formatação (além de me ajudar a manter a sanidade) nos relatórios e fim da tese.

Ao Luís Felipe, meu vizinho, melhor amigo e irmão do coração por tudo (TUDO MESMO! A lista é muito grande e por vezes impublicável). Amo-te!

Aos demais amigos e colegas da pós, companheiros de “salinha”, sujinhos e afins (Elizabeth - Beth, Bruna, Diogo, Eliana, Maria Gabriela - Gabi, Milene, Natália Costa, Nathália Miranda,

Talita, Vítor), o meu muito obrigada por todos os momentos de alegria, devaneios, descontração, companheirismo, carinho e por aguentarem essa curitibana metida.

A Missiani Ciochetta de Mello, colega na Ruhr que, além de companheira de laboratório, viagens e festas nos meus quatro meses de Alemanha, se tornou uma grande amiga.

A Eliana e Milene, em especial pelo apoio durante o período na Alemanha. A amizade e presença de vocês (ainda que separadas por horas de trem) fizeram a estadia bem mais agradável.

A minha família por ter me dado as ferramentas para realizar meus sonhos, por ter me apoiado durante a vida toda e compreendido as minhas muitas ausências.

A todas as pessoas legais que conheci na cidade de Rio Claro e que fizeram com que eu deixasse de detestá-la e passasse a amá-la.

Ao “quarteto fantástico” (Jenifer, Jocieli e Makeli), por estarem sempre presentes independente da distância. Um obrigada especial à Makeli, por ter me levado pra Botânica na graduação e, por ter me acolhido com tanto carinho em Piracicaba na transição entre Curitiba e Rio Claro.

Aos amigos de Curitiba, que compreenderam minha ausência e torceram pelo meu sucesso.

Agradeço também a todos que passaram pela minha vida ao longo destes quatro anos, enriquecendo-a e me ensinando algo. “Aqueles que passam por nós não vão sós, não nos deixam sós. Deixam um pouco de si, levam um pouco de nós”.

Ao Lucas - last but not least – por te se juntado a minha caminhada num momento tão importante, por toda a compreensão enquanto eu “sofria de tese”, mas principalmente por todos os sorrisos e pelo amor.

[...]

Nobody said it was easy

It's such a shame for us to part

Nobody said it was easy

No one ever said it would be this hard

Oh, take me back to the start

I was just guessing at numbers and figures

Pulling the puzzles apart

Questions of science, science and progress

Do not speak as loud as my heart

[...]

(The Scientist – Coldplay)

RESUMO

Bulbophyllum, o maior gênero de Orchidaceae, possui distribuição pantropical mas sua maior diversidade concentra-se no sudeste Asiático. Na região Neotropical, o gênero é representado por 60 espécies, sendo que 49 delas ocorrem no Brasil. A maior diversidade de espécies neotropicais encontra-se no Sudeste brasileiro, nas regiões de contato entre o Cerrado e a Floresta Atlântica. Após o advento de filogenias moleculares, auxiliadas por dados morfológicos, foram reconhecidas e recircunscritas seis seções monofiléticas e neotropicais de *Bulbophyllum*: *Bulbophyllaria*, *Furvescens*, *Napelli*, *Micranthae*, *Didactyle* e *Xiphizusa*. Até o momento, informações sobre anatomia e micromorfologia florais para espécies neotropicais do gênero estão disponíveis apenas para seis espécies e tratam apenas do labelo. Além disso, informações sobre a polinização do grupo foram reportadas para apenas oito espécies. Considerando a recente recircunscrição do grupo, escassez de estudos sobre características florais e importância destas para a polinização em Orchidaceae, o objetivo deste trabalho foi analisar a micromorfologia e anatomia florais de 30 espécies neotropicais, bem como de sete espécies não-neotropicais e uma de *Dendrobium*, para: auxiliar na caracterização floral de cada seção, na diferenciação de espécies dentro das seções e para melhorar a compreensão do grupo como um todo. De forma geral, as flores das espécies das seções neotropicais de *Bulbophyllum* apresentam sépalas livres, com tricomas glandulares na face abaxial; pétalas com um feixe vascular colateral; labelo com epiderme estriada, com sulco na face adaxial, geralmente secretor, e uma quilha com estômatos na face abaxial; antera com epiderme papilosa e quatro polínias; presença de viscidio originado da desintegração das células do rostelo; ovário 6-lobado e com um feixe vascular por lobo. Cada seção apresenta características anatômicas e micromorfológicas florais comuns e outras que são variáveis e que são discutidas em cada capítulo, bem como suas implicações sistemáticas e para a polinização. Para diferenciar entre espécies de uma mesma seção, ornamentação de paredes periclinais externas e/ou cutícula das peças florais, número de feixes vasculares nas regiões medianas das peças florais e presença de tecidos secretores podem ser considerados características úteis. Na busca de possíveis sinapomorfias florais para o grupo Neotropical e para cada seção, apenas a presença de duas polínias em *B. sect. Napelli* e a presença de uma cavidade secretora no hipoquilo além de idioblastos com paredes espessadas no epiquilo em *B. sect. Didactyle* foram identificadas.

Palavras-chave: *Bulbophyllinae*, *Dendrobieae*, *Epidendroideae*, labelo, nectário, osmóforo.

ABSTRACT

Bulbophyllum, the largest genus within Orchidaceae, is pantropical but its greatest diversity is found in the Asian Southeast. In the Neotropics, the genus comprises 60 species, being 49 of them found in Brazil. The greatest diversity of Neotropical species is in Brazilian Southeast, in transitional areas between the Atlantic Rainforest and Cerrado Vegetation. After the advent of molecular phylogenetic trees, and analysis of morphological data, six monophyletic Neotropical *Bulbophyllum* sections were recognised and recircumscribed: *Bulbophyllaria*, *Furvescens*, *Napelli*, *Micranthae*, *Didactyle* e *Xiphizusa*. However, information regarding floral anatomy and micromorphology for Neotropical species are available only for the labellum of six species. In addition, information on the pollination biology is available only to eight species. Considering the recent recircumscription of this group, and the paucity of information regarding floral features and their importance to pollination in Orchidaceae, this work aimed to analyse the floral anatomy and micromorphology of 30 Neotropical species, seven non-Neotropical species, and one *Dendrobium* species, in order to: characterise the flower of each section, help in species differentiation within section, and get a better understanding of the Neotropical group. The flowers of the Neotropical group can be characterised by free sepals, with sunken glandular trichomes on their abaxial surface; petals with a single collateral vascular bundle; labellum with a striate epidermis, with an adaxial sulcus that is usually secretory, and an abaxial keel with stomata; a papillose anther with four pollinia; a viscidium deriving from rostellar cells; and a 6-lobed ovary with one vascular bundle per lobe. Each section has common floral features and variable ones, which are discussed in each chapter with considerations regarding systematic and pollination implications. In general, the ornamentation of the outer periclinal walls and/or cuticle, number of vascular bundle in the median portion and presence and localization of secretory tissues are useful to differentiate species within sections. Only the presence of two pollinia in *B.* sect. *Napelli*, besides the presence of a secretory cavity in the hypochile, and idioblasts with helical wall thickenings in the epichile in *B.* sect. *Didactyle* were identified as possible infrageneric synapomorphies.

Keywords: *Bulbophyllinae*, Dendrobieae, Epidendroideae, labellum, nectary, osmophore.

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Introdução Geral

Orchidaceae pode ser considerada a maior família vegetal, com cerca de 25.500 espécies, e ocupa todos os ecossistemas terrestres (Dressler, 1993; Govaerts *et al.*, 2014).

Apesar de sua conhecida diversidade floral, as flores de Orchidaceae, segundo Dressler (1993), podem ser assim caracterizadas: flores precedidas por uma bráctea, hermafroditas, zigomórficas e ressupinadas durante o desenvolvimento (invertidas 180° de sua posição adaxial original); com três tépalas (ou também chamadas de sépalas) externas petalóides e imbricadas, conadas ou livres; três tépalas internas (ou pétalas) livres, variavelmente coloridas, sendo a mediana diferenciada em labelo; androceu composto por três ou menos estames, adnatos ao estilete e estigma pelos filetes, formando uma estrutura denominada de coluna ou ginostêmio; grãos de pólen agrupados em massas chamadas polínias; ovário tricarpelar, com placentação parietal, ou menos frequentemente axial, e ínfero.

Além das características supracitadas, vale ressaltar que estilete e estigma são bastante modificados, com uma porção deste último, não receptiva, chamada rostelo; frequentemente, o rostelo forma uma espécie de almofada viscosa, o viscidio, que, ligada à polínia, adere ao polinizador (Dressler, 1993). O ovário é geralmente pouco diferenciado durante o florescimento, terminando o desenvolvimento somente após a polinização; apresenta numerosos óvulos com nucelo de parede delgada (Dressler, 1993). Néctar, em geral, está ausente, mas pode ser produzido em esporas no labelo, ápices das sépalas ou nectários septais (Dressler, 1993).

Tradicionalmente, Orchidaceae era dividida em cinco subfamílias: Apostasioideae, Cypripedioideae, Spiranthoideae, Orchidoideae e Epidendroideae (Dressler, 1993). Entretanto, mais recentemente, Spiranthoideae foi inserida entre as Orchidoideae, e uma nova subfamília – Vanilloideae – foi proposta com base em dados moleculares (Chase *et al.*, 2003; Kocyan *et al.*, 2008). Apesar de mudanças na compreensão dos relacionamentos dentro da família, propiciadas por uma série de trabalhos nas últimas duas décadas (por exemplo, Chase *et al.*, 2003, 2005; van den Berg *et al.*, 2005), Epidendroideae se mantém como a maior subfamília em número de espécies, com cerca de 650 gêneros e 18.000 espécies (Cribb & Chase, 2005).

Dentre as Epidendroideae, a tribo pantropical Dendrobieae contém apenas dois gêneros (*Dendrobium* Sw. e *Bulbophyllum* Thouars), que juntos contêm cerca de 3650 espécies (Schuiteman, 2014). *Bulbophyllum*, com cerca de 2200 espécies (Gravendeel *et al.*, 2014), é considerado o segundo maior gênero de angiospermas (Frodin, 2004). Gravendeel *et al.*

(2003), através de dados moleculares, sugeriram que *Bulbophyllum* originou-se na Ásia e que as espécies africanas e neotropicais formam agrupamentos monofiléticos e irmãos do clado asiático. Tal hipótese é corroborada pela distribuição quase exclusivamente asiática do gênero-irmão de *Bulbophyllum*, *Dendrobium*.

Bulbophyllum possui distribuição pantropical, mas sua maior diversidade concentra-se no sudeste Asiático, ocorrendo também na Australásia e trópicos americanos (Vermeulen, 1987, 1993; Dressler, 1993; Siegerist, 2001; Gravendeel *et al.*, 2014). Na região Neotropical, o gênero é representado por 60 espécies (Smidt *et al.*, 2011), sendo 49 delas no Brasil (Barros *et al.*, 2014). A maior diversidade de espécies neotropicais encontra-se no Sudeste brasileiro, nas regiões de contato entre o Cerrado e a Floresta Atlântica (Smidt *et al.*, 2007).

O tratamento histórico do grupo foi realizado por Pabst & Dungs (1975, 1977) onde o gênero foi dividido em cinco seções e seis alianças informais de espécies, com base em caracteres morfológicos florais: seção *Didactyle*, aliança *B. glutinosum*; seção *Xiphizusa*, aliança *B. plumosum*; seção *Bolbophyllaria*, alianças *B. pachyrhachis* e *B. micropetalum*; seção *Micrantha*, aliança *B. micranthum*; e seção *Napellii*, aliança *B. napellii*.

Smidt *et al.* (2011) elaboraram filogenias baseadas em dados moleculares (Figs 1, 2), de sequências do espaçador nuclear ITS e de dois espaçadores de plastídeo (*psbA-trnH* e *trnS-trnG*). Com base nestas filogenias e em dados morfológicos, foram reconhecidas seis seções monofiléticas e neotropicais de *Bulbophyllum*: *Bulbophyllaria*, *Furvescens*, *Napelli*, *Micranthae*, *Didactyle* e *Xiphizusa*. Destas, *Furvescens* foi proposta pela primeira vez e as demais, já descritas por outros autores, foram recircunscritas, tornando-se monofiléticas (Smidt *et al.*, 2011).

Segundo o tratamento de Smidt *et al.* (2011), as seções podem ser caracterizadas da seguinte forma:

- *B. sect. Bulbophyllaria* (Rchb.f.) Griseb.: possui três espécies distribuídas em Cuba, Colômbia, Costa Rica, El Salvador, Guatemala, Honduras, México, Nicarágua, Panamá, República Dominicana e Venezuela. Compreende espécies com pseudobulbos bifoliados; inflorescências com raque carnosa e flores arranjadas espiraladamente; sépalas totalmente livres; pétalas eretas; pé da coluna com ápice inteiro e mais curto a coluna.
- *B. sect. Furvescens* E.C.Smidt, Borba and van den Berg: apresenta cinco espécies distribuídas no Brasil, Equador, Guiana, Guiana Francesa, México, Peru, Suriname e Venezuela. O pseudobulbo é unifoliado; as inflorescências possuem raque carnosa e flores dispostas espiraladamente; sépalas livres; pétalas eretas; pé da coluna menor

que esta e com ápice inteiro.

- *B. sect. Napelli* Rchb.f.: compreende 12 espécies distribuídas na Argentina, Brasil e Venezuela. A inflorescência possui raque delgada com flores disticamente arranjadas; sépalas livres; pétalas eretas; pé da coluna mais longo que esta e com ápice inteiro.

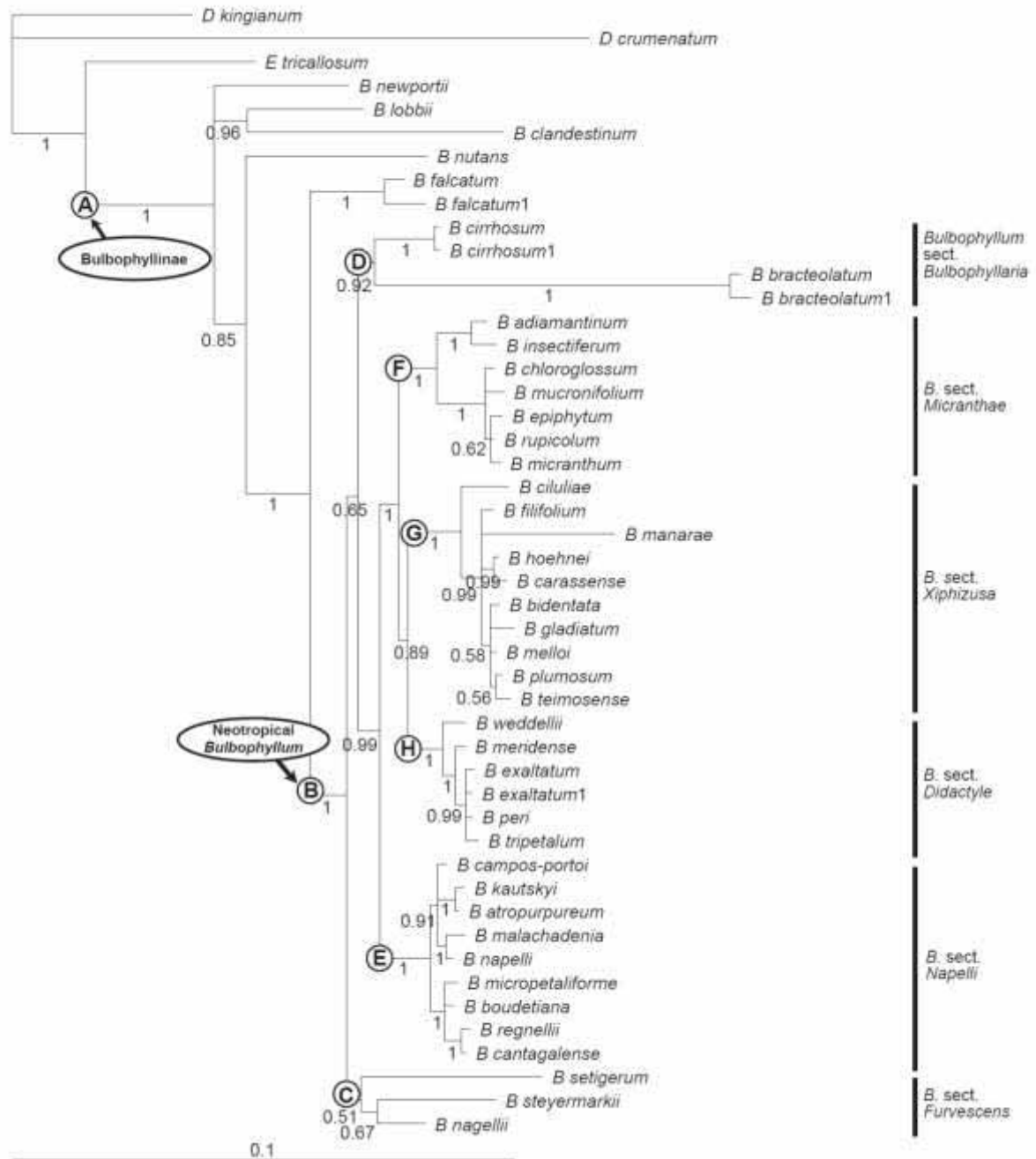


Figura 1. Árvore de consenso de maioria de *Bulbophyllum* Neotropical, derivada da análise molecular Bayesiana combinada de dados nuclear e de plastídeo (extraída de Smidt *et al.*, 2011).

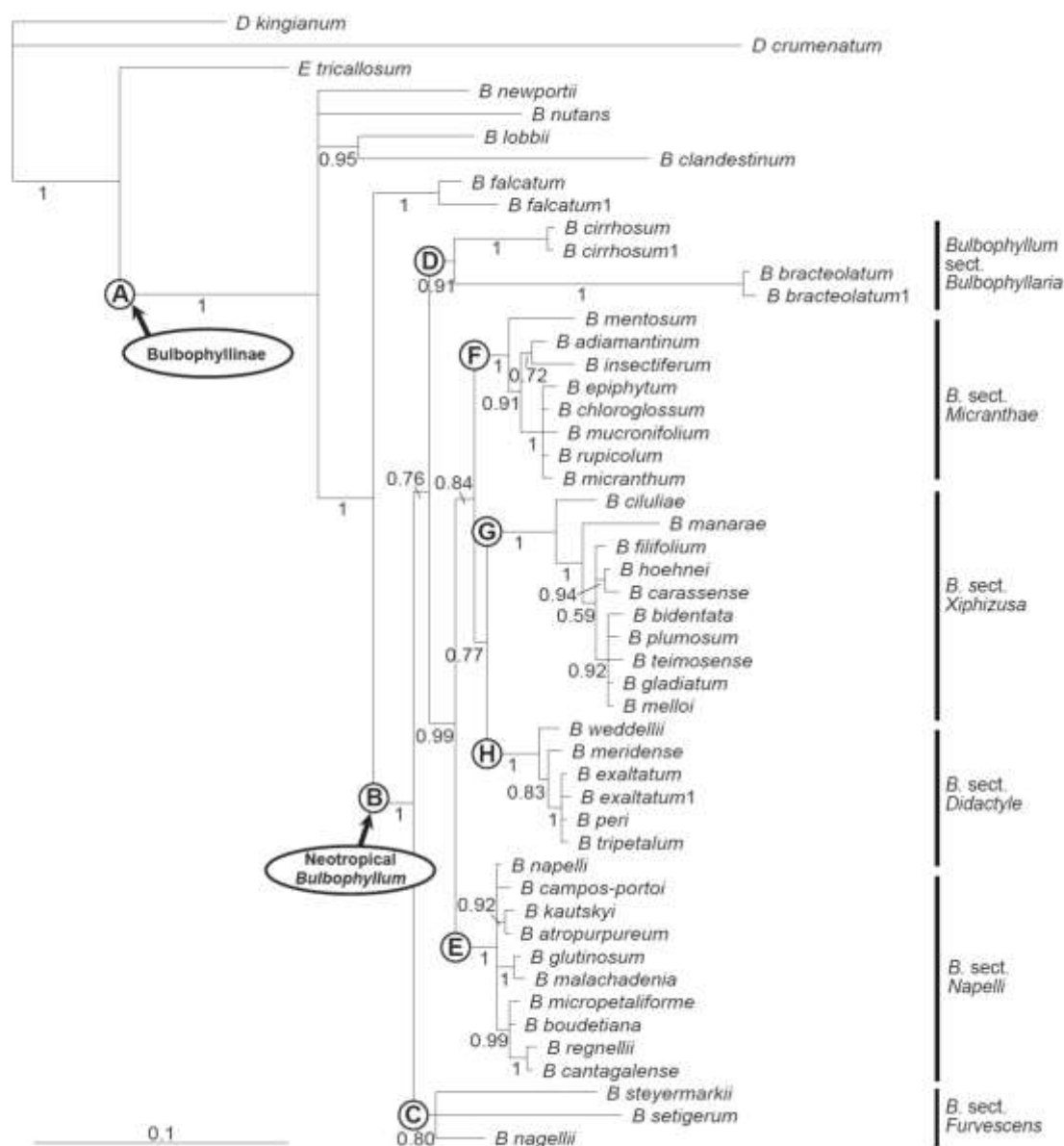


Figura 2. Árvore de consenso de maioria de *Bulbophyllum* Neotropical, derivada da análise molecular Bayesiana a partir de dados do nrITS (extraída de Smidt *et al.*, 2011 – Electronic Supplement).

- *B. sect. Micranthae* Barb.Rodr.: compreende doze espécies distribuídas na Bolívia e Brasil. A inflorescência possui raque delgada com flores distribuídas espiraladamente; sépalas livres; pétalas eretas; pé da coluna mais curto com esta e com ápice inteiro.
- *B. sect. Didactyle* (Lindl.) Cogn.: apresenta sete espécies distribuídas na Bolívia, Brasil, Colômbia, Equador, Guiana, Paraguai, Peru e Venezuela. A inflorescência tem raque delgada e flores distribuídas dísticamente; sépalas livres; pétalas patentes; pé da

coluna mais curto do que esta e com ápice bilobado.

- *B. sect. Xiphizusa* Rchb.f.: corresponde a 23 espécies distribuídas na Bolívia, Brasil, Colômbia, Jamaica, México, Paraguai, Peru e Venezuela. A inflorescência tem raque delgada com flores distribuídas disticamente; sépalas laterais totalmente conadas, formando um sinsépalo; pétalas eretas; pé da coluna mais curto do que esta e com ápice inteiro.

A morfologia floral é bastante variada em *Bulbophyllum* e hipóteses apontam para o papel de pressões ambientais e especificidade de agentes polinizadores nesta variação (Borba & Semir, 1998; Verola, 2002; Teixeira *et al.*, 2004). Verola (2002), ao estudar sete espécies neotropicais do gênero, relacionou a grande variação morfológica floral à polinização por diferentes famílias de Diptera. Este mesmo autor também sugeriu que as diferentes formas de polinização descritas podem estar relacionadas à ocupação de diferentes ambientes, com uma sequência de adaptações na transição de grupos que ocupavam mata fechada e irradiaram até áreas abertas, como os campos rupestres.

Os estudos morfológicos e anatômicos florais em *Bulbophyllum* são restritos, tendo sido reportadas apenas a morfologia do ginostêmio de *B. ecornutum* (Rasmussen, 1985), espécie asiática, e a anatomia do labelo de seis espécies brasileiras, *B. epiphytum*, *B. glutinosum*, *B. regnellii*, *B. involutum*, *B. ipanemense*, *B. weddellii*, e de uma espécie asiática, *B. rothschildianum* (Teixeira *et al.*, 2004).

Segundo Smidt *et al.* (2013), os dados anatômicos, tanto vegetativos como reprodutivos, podem contribuir para a elaboração de hipóteses filogenéticas mais robustas em *Bulbophyllum*, muitas vezes com mais suporte e resolução que aqueles moleculares, tal como evidenciado no estudo anatômico foliar de *B. sect. Micranthae*.

Considerando a recente recircunscrição de *Bulbophyllum* neotropicais, a escassez de estudos sobre características florais e a importância destas para a polinização em Orchidaceae, o objetivo deste trabalho foi analisar a micromorfologia e anatomia florais de 30 espécies neotropicais, bem como de sete espécies não-neotropicais e uma de *Dendrobium*, para: auxiliar na caracterização floral de cada seção, na diferenciação de espécies dentro das seções e para melhorar a compreensão do grupo como um todo.

Nesse contexto, a tese foi estruturada em seis capítulos, redigidos na forma de artigos científicos, sendo cinco deles tratando dos aspectos micromorfológicos e anatômicos florais das seções Neotropicais de *Bulbophyllum* e um, final, buscando entender a evolução de alguns caracteres florais, combinando dados morfológicos, anatômicos e moleculares.

Assim, os objetivos específicos da presente tese foram:

- Capítulo I: Descrever a micromorfologia e anatomia florais de *Bulbophyllum* sect. *Didactyle*, identificando características potenciais para a distinção específica e também diagnóstica para a seção.
- Capítulo II: Responder às seguintes questões: a) quais as características micromorfológicas e anatômicas florais são comuns aos representantes de *B.* sect. *Napelli*? b) quais destas características distinguem as espécies na seção? e c) há características que podem estar relacionadas ao hábito e habitat dos representantes da seção?
- Capítulo III: Descrever a micromorfologia e anatomia florais de *B.* sect. *Micranthae*, respondendo às seguintes questões: a) quais as características micromorfológicas e anatômicas florais são comuns aos representantes de *B.* sect. *Micranthae*? b) quais destas características distinguem as espécies na seção? e c) há características relacionadas ao hábito, habitat e polinização nos representantes desta seção?
- Capítulo IV: Responder às seguintes questões: a) quais as características micromorfológicas e anatômicas florais são diagnósticas dos representantes de *B.* sect. *Xiphizusa*? b) quais destas características distinguem as espécies na seção? e c) há nectários e/ou osmóforos nas flores dos representantes desta seção?
- Capítulo V: Descrever a micromorfologia e anatomia florais de duas espécies de *B.* sect. *Furvescens* e de uma espécie de *B.* sect. *Bulbophyllaria*, respondendo às seguintes questões: a) quais características micromorfológicas e anatômicas florais são comuns às três espécies estudadas? b) quais destas características são comuns às espécies de *B.* sect. *Furvescens* e, assim, auxiliam na caracterização desta seção recentemente proposta? e c) há características que distinguem espécies pertencentes a cada uma dessas seções?
- Capítulo VI: Levantar e avaliar caracteres florais que possam ser considerados sinapomorfias florais dos clados neotropicais de *Bulbophyllum*, e b) discutir o significado de alguns desses caracteres e sua relação com o ambiente e polinização.

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CAPÍTULO I

What do floral anatomy and micromorphology tell us about Neotropical *Bulbophyllum* sect. *Didactyle* (Orchidaceae: Bulbophyllinae)?

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(Submetido ao periódico *Botanical Journal of the Linnean Society* em 21/10/2013, revisado em 17/02/2014, aceito para publicação em 12/04/2014)

(Artigo publicado vide Apêndice 1)

ABSTRACT

Bulbophyllum sect. *Didactyle* comprises seven species, but distinction between these is often problematic. These species are pollinated by Milichiidae flies and air currents move the hinged labellum and press the pollinator against the gynostemium. The labellum structure is considered homogeneous and conservative for genus. Therefore, the floral anatomy and micromorphology of *B.* sect. *Didactyle* were studied in order to identify characters useful for distinguishing the species. All species have sunken glandular trichomes on the abaxial surface of the sepals (possible osmophores) and a trilobed labellum, clothed with trichomes, together with a secretory cavity in the callus that is bound by scale-like papillae. Of the ca. 100 characters assessed, 25 varied between species, and each pair of species differed by at least four character states, mainly occurring on the labellum. The data presented allows for distinction between species and corroborates their grouping within *B.* sect. *Didactyle* as previously proposed. The presence of osmophores and nectary on the labellum is confirmed, although their structure is more diverse than anticipated. Moreover, structural differences between *B. weddellii* and the core of the section might indeed be result of the odour-mediated attraction of pollinators rather than flower morphology and thus phylogeny.

ADDITIONAL KEYWORDS: *Bulbophyllum exaltatum* – *B. involutum* – *B. meridense* – *B. perii* – *B. popayanense* – *B. tripetalum* – *B. weddellii* – myophilous – nectary – osmophore

INTRODUCTION

Bulbophyllum Thouars comprises ca. 1870 species (WCSP, 2013) and is the second largest genus within the Angiosperms (Frodin, 2004). Its distribution is Panropical, occurring in Australasia and the American tropics, but its greatest diversity is concentrated in Southeastern Asia (Vermeulen, 1987, 1993; Dressler, 1993; Siegerist, 2001). In the Neotropics, the genus is represented by 62 species (Smidt *et al.*, 2011) with a distribution ranging from Northern Mexico to Southern Brazil (Smidt *et al.*, 2007). The greatest diversity of Neotropical *Bulbophyllum* is found in Southeastern Brazil in contact areas between the Atlantic Rainforest and Cerrado vegetation (Smidt *et al.*, 2007).

Based on molecular data, six monophyletic sections were recognised within Neotropical *Bulbophyllum*: *B.* sect. *Bulbophyllaria* (Rchb.f.) Griseb., *B.* sect. *Furvescens* E.C. Smidt, Borba & Van den Berg, *B.* sect. *Napelli* Rchb.f., *B.* sect. *Micranthae* Barb.Rodr.,

B. sect. *Didactyle* (Lindl.) Cogn., and *B.* sect. *Xiphizusa* (Rchb.f.) Cogn. (Smidt *et al.*, 2011). Of these sections, *B.* sect. *Furvescens* was first proposed by Smidt *et al.* (2011), while the remaining sections were re-circumscribed to become monophyletic (Smidt *et al.*, 2011).

Species of *Bulbophyllum* sect. *Didactyle* are mainly found in the Cerrado vegetation (Smidt *et al.*, 2011), growing directly on rocks and exposed to sunlight (Smidt, 2007). This section is characterised by unifoliate pseudobulbs, a racemose inflorescence with thin rachis and distichously arranged flowers showing free lateral sepals, patent petals, and a gynostemium foot with a bilobed apex that is shorter than the gynostemium length (Smidt *et al.*, 2011). The current circumscription of the section includes seven species based on the molecular phylogenetic analysis of five species and on the similar morphology of the other two (Smidt *et al.*, 2011).

This section shows the greatest problems in specific delineation due to its highly variable morphology and this has resulted in the naming of many geographically restricted “species” (Smidt *et al.*, 2011), especially within the *B. exaltatum* species complex (Ribeiro *et al.* 2008), with its broad distribution in the highland areas of South America and the presence of natural hybrids (Borba & Semir, 1998a; Mancinelli & Smidt, 2012).

The floral morphology of Neotropical *Bulbophyllum* is quite diverse, and some authors have suggested that this may be driven by environmental pressures and pollinator specificity (Borba & Semir, 1998b; Verola, 2002; Teixeira, Borba & Semir, 2004). The reproductive biology of three species from *Bulbophyllum* sect. *Didactyle* (*B. weddellii* (Lindl.) Rchb.f., *B. involutum* Borba, Semir & F. Barros, and *B. exaltatum* Lindl. syn. *B. ipanemense* Hoehne), is relatively well known. The main pollinators are Milichiidae flies, which show some specificity in pollinating *Bulbophyllum* species, although the main pollinator of one species might be the secondary of the other (Sazima, 1978; Borba & Semir, 1998b; Silva *et al.*, 1999; Azevedo, Borba & van den Berg, 2006). The pollination mechanism displayed by these species utilizes air currents press the fly against the gynostemium (Sazima, 1978; Borba & Semir, 1998b), and it has been suggested that floral odour is the main cue in attracting these pollinators (Borba & Semir, 1998b; Silva *et al.*, 1999).

Studies of flower morphology and anatomy of *Bulbophyllum* are scarce. Teixeira, Borba & Semir (2004) studied the labellum anatomy of six Brazilian and one Asian species of *Bulbophyllum* having osmophores on their adaxial surface, and suggested that the labellum structure is homogeneous and highly conservative for the genus. Other than this paper, the only other report on the flower morphology for the genus is a study of the gynostemium of the Asian *B. ecornutum* J.J.Sm. (Rasmussen, 1985).

Therefore, the aims of the present study are to describe the floral micromorphology and anatomy of *Bulbophyllum* sect. *Didactyle* and to identify characters useful both for distinguishing between the species and for circumscribing and diagnosing the entire section.

MATERIALS AND METHODS

Samples for this study were collected in the field or obtained from botanical gardens; all seven species currently assigned to *Bulbophyllum* sect. *Didactyle* were sampled (Table 1). Mature flowers were fixed in various fixatives chosen for their availability and suitability for use on field trips: FAA 50 (Johansen, 1940), 1% glutaraldehyde and 4% formaldehyde in phosphate buffer 0.1 M, pH 7.2 (McDowell & Trump, 1976) or 2% glutaraldehyde and 2% formaldehyde in phosphate buffer 0.1 M, pH 7.2 (Karnovsky, 1965). Micromorphological and anatomical characters were assessed using conventional means, such as light (LM) and scanning electron microscopy (SEM). At least four flowers from different specimens were analysed for each species, whenever possible, sourced from different localities (Table 1).

The samples for LM analysis were embedded in historresin (Leica or Technovit), according to the instructions of the suppliers, and sectioned at 5 to 8µm using disposable blades. Sections were stained with 0.05% Toluidine Blue O in 0.1 M sodium phosphate buffer pH 6.8 (O'Brien, Feder & McCully, 1965) for general analysis. The following histochemical tests were performed on resin-embedded sections: Coomassie Brilliant Blue for proteins (Southworth, 1973), Calcofluor White MR2 for cellulose (Hughes & McCully, 1975), and the Periodic Acid-Schiff reaction (PAS) for total insoluble polysaccharides (Feder & O'Brien, 1968).

Fresh material was hand-sectioned and was tested with iodine/potassium iodide solution (IKI) for starch (Johansen, 1940), Sudan III for lipids (O'Brien & McCully, 1981), Fehling's solutions A and B for reducing sugars (Purvis, Collier & Walls, 1964), Neutral Red under UV excitation for total lipids (Kirk, 1970), Ruthenium Red for pectic acids/mucilage (Johansen, 1940), ferric chloride for phenolic compounds (Johansen, 1940), and acidified phloroglucinol for lignin (Sass, 1951). Entire fresh flowers were immersed in Neutral Red for osmophore identification and distribution (Kearns & Inouye, 1993).

Photomicrography was achieved by means of a Leica DMLB microscope coupled with a digital camera and using the LAS (Leica Application Suite V3.3.0) software.

For SEM analysis, the samples were transferred from 70% ethanol to formaldehyde-dimethylacetal for 16 to 24h (Gerstberger & Leins, 1978), further dehydrated by critical-point

drying (CPD 030, Balzers), and sputter-coated with gold (SCD 050, Bal-Tec). SEM observations were made using a Zeiss DSM 950 and images were obtained using Digital Image Processing Software 2.2 (DIPS-Leipzig).

RESULTS

The flowers of *Bulbophyllum* sect. *Didactyle* studied have free sepals (Fig. 1A-B), reduced petals (Fig. 1A), and a movable labellum that is divided into three lobes (Fig. 1C, D). The median lobe is the largest (Fig. 1C, dashed line) and is divided into two parts: the hypochile (the proximal and fleshy part) (Fig. 1C-D) and the epichile (the distal, less fleshy part) (Fig. 1C-D). On the hypochile, there is a callus with a secretory cavity (Fig. 1D, arrow). The lateral lobes are smaller and ear-shaped (Fig. 1C-D, solid line). The gynostemium has a bilobed foot, two long stelidia (Fig. 1A, E-P), and two ventral teeth (Fig. 1A, J-P).

The dorsal sepal has on its adaxial surface conspicuous striate wall ornamentation (Fig. 2A), except for *B. popayanense* and *B. meridense* (Fig. 2B); the striae are very pronounced in *B. perii* and *B. tripetalum* (Fig. 2C). In frontal view, the epidermal cells are transversely elongate (Fig. 2B-C), except for *B. weddellii*, where the cells are more isodiametric (Fig. 2A). In transverse sections, the outer periclinal wall is flat (Fig. 2D). On the abaxial surface, the cuticle ornamentation is irregular (Fig. 2E), and the outer periclinal wall flat (Fig. 2D), but usually striate (Fig. 2F). All species have abaxial stomata (Fig. 2E, arrow) and sunken multicellular glandular trichomes that react positively to an *in vivo* test for osmophore localisation and faintly for phenolic compounds and pectic acids (Fig. 2E-arrowhead, G).

The margin of the dorsal sepal is smooth (Fig. 2H) except in *B. popayanense*, where it is papillose (Fig. 2I); in transverse sections of all species, the margin consists of both epidermal and mesophyll cells (Fig. 2H). The mesophyll is homogeneous (Fig. 2D, J) with regular parenchyma and shows two types of idioblasts, the first with cellulosic, helical wall thickenings (Fig. 2D, K-M) and the other with raphides (Fig. 2J, L, M). In transverse section, the central third of the dorsal sepal of most species contains three collateral vascular bundles (Fig. 2J), one of which is sometimes indistinct; *B. weddellii* and *B. popayanense* have five vascular bundles (Fig. 2M, M1-5), of which two are much reduced (Fig. 2M2, M5).

The lateral sepals show obvious striate ornamentation on their adaxial surface (Fig. 2N) except for *B. weddellii*, where it is inconspicuous (Fig. 2O); the striae are particularly conspicuous in *B. tripetalum*. In frontal view, the adaxial epidermal cells (Fig. 2N-O) and the

outer periclinal wall in transverse sections (Fig. 2P) are similar to those of the dorsal sepal. On the abaxial surface, the ornamentation is also irregular (Fig. Q), but the outer periclinal wall, in transverse section, is slightly convex (Fig. 2P). On this surface, all species show stomata (Fig. 2Q, arrow) and multicellular glandular trichomes (Figs. 2Q – arrowhead, 3A-B). The margin and mesophyll of the lateral sepals are similar to those of the dorsal sepal (Figs. 2J, M, P, 3B), usually with three collateral vascular bundles, but there are five in *B. weddellii* (Fig. 3C) and six in *B. popayanense* (Fig. 3D).

The petals show striate ornamentation on the adaxial surface (Fig. 3E-F). In frontal view, the epidermal cells are more or less isodiametric (Fig. 3E) except for *B. weddellii*, where they are longitudinally elongate (Fig. 3F). The outer periclinal wall, in transverse section, is flat in most species (Fig. 3G), but there are also papillose cells in *B. popayanense*, *B. exaltatum* and *B. involutum* (Fig. 3E, H, I). The ornamentation, cell shape, and periclinal wall of abaxial epidermal cells are similar to those of their adaxial counterparts. The margin has unicellular trichomes (Fig. 3H) except for *B. weddellii* (Fig. 3I), *B. perii*, and *B. tripetalum*. The mesophyll is homogeneous, with regular parenchyma and idioblasts with raphides and cellulosic helical wall thickenings (Fig. 3G, J). There is a single, central collateral vascular bundle (Fig. 3G, J).

The epichile shows striate ornamentation on its adaxial periclinal walls (Fig. 4A). Most epidermal cells are trichomes (Fig. 4B) except for *B. weddellii*, which has only longitudinally elongate cells (Fig. 4C); in *B. perii*, the trichomes are shorter and more regular in size than in the other species (Fig. 4D). Both abaxial and adaxial surfaces are similar (Fig. 4E). The margin bears larger trichomes with globose bases (Fig. 4B – arrows, F) except for *B. perii*, whose trichomes are similar to those found on the surface of the epichile (Fig. 4D), and *B. weddellii*, where they are lacking. The mesophyll is homogeneous, formed of regular parenchyma (Fig. 4B), with very large intercellular spaces in *B. weddellii* (Fig. 4G), and containing idioblasts with raphides and cellulosic, helical wall thickenings (Fig. 4B, raphides not clearly visible).

The hypochile has a callus on its adaxial surface, which has scale-like papillose epidermal cells with striate ornamentation (Fig. 4H). These cells give positive reaction for lipids and form the boundary of the secretory cavity (Fig. 4I). The secretory cavity, in transverse sections, varies in size and shape between species (Fig. 4J-P) and is less conspicuous in *B. weddellii* (Fig. 4J, arrow). The epidermis usually has papillose cells (Fig. 4Q), especially at the base of the cavity (except for *B. popayanense* and *B. involutum*, which have short trichomes – Fig. 4R). Together with the epidermis, three to five subepidermal

secretory layers (Fig. 4J-R) contain elevated levels of cytoplasmic proteins, as revealed by histochemical tests. At the callus periphery, the epidermis is similar to that of the epichile (Fig. 4D, E, J-P). The abaxial epidermis of the hypochile comprises either only cells that have convex walls (Fig. 4S), papillose (Fig. 4T), or a combination of both convex walls and trichomes (Fig. 4U). The mesophyll is similar to that of the epichile, but reacts positively for starch and lipids (Fig. 4J-P).

The lateral lobes of the labellum usually have trichomes on their adaxial surface (Fig. 4V), and papillae on the abaxial surface (Fig. 5A), whereas long trichomes occur at the margins and these test positively for lipids (Fig. 4U, 5A). The exceptions are *B. perii*, where papillae clothe the entire surface of the lateral lobes (Fig. 5B), and *B. tripetalum*, which has flattened cells on its abaxial surface (Fig. 5C). The mesophyll is similar to that of the remainder of the labellum, but without vascularisation (Fig. 5D).

The gynostemium lacks conspicuous ornamentation (Fig. 5E), and the epidermal cells, in frontal view, are longitudinally elongate (Fig. 5E). The mesophyll is homogeneous, with regular parenchyma containing idioblasts with raphides and cellulosic, helical wall thickenings (Fig. 5F). The gynostemium foot usually lacks conspicuous ornamentation (Fig. 5G), but it is striate in *B. exaltatum* and *B. tripetalum* (Fig. 5H). Its cells are longitudinally elongate from the frontal view (Fig. 5G-H).

The anther is papillose and its irregularly shaped cells have striate ornamentation (Fig. 5I). The pollinarium is composed of four pollinia (with smooth tetrads, the central two pollinia being smaller than the outer pollinia - Fig. 5J), and a viscidium formed by the disorganization of rostellar cells (Fig. 5K).

The stigma is longitudinally elongate (Fig. 5L), except in *B. weddellii*, which has a wider and isodiametric stigma (Fig. 5M); it comprises long, narrow cells embedded, but loosely arranged, in a mucilaginous matrix (Fig. 5K-M). The ovary is a complex, six-lobed structure, whose lobes are arranged in two alternating whorls of three. The three outer lobes occur along the same radius as the sepals and the three inner lobes occur along the same radius as the petals and carpels (Fig. 5N). In transverse section, the outer epidermal cells of the ovary appear columnar in shape, with convex outer periclinal walls (Fig. 5O). The inner epidermal cells are elliptic cells with convex outer walls (Fig. 5P). The ovary mesophyll is homogeneous and is formed of regular parenchyma containing idioblasts with raphides, together with others having cellulosic, helical wall thickenings (Fig. 5N-O). Each lobe of the ovary contains a single collateral vascular bundle (Fig. 5N-O). Histochemical tests were performed on all species, and the results are summarised in the Table 2. A summary of those

characters that allow distinction between species is presented in the Table 3.

DISCUSSION

In the present study, it was found that several floral micromorphological and anatomical characters were shared by most of the species of *Bulbophyllum* sect. *Didactyle*. The distinguishing features first described for the section were the presence of multicellular glandular trichomes on the abaxial surface of the sepals; reduced petals with smooth or indumented margins, with a single collateral vascular bundle; a trilobed labellum, mostly clothed with trichomes, with a callus having a secretory cavity bound by scale-like papillae; a pollinarium formed of four pollinia with smooth tetrads; and mesophyll with both crystalliferous and thickened idioblasts in all floral parts.

These data display better characterization and congruence when those species considered representatives of *Bulbophyllum* sect. *Didactyle*, are grouped according to Smidt *et al.* (2011). Although these authors used molecular techniques to study only five species, they speculated, on the basis of gross morphology, that the remaining two species also belong in this section. Prior to that, the most recent treatment of the group was that of Pabst & Dungs (1975) who considered only four species currently assigned to *B.* sect. *Didactyle*, together with several species of *B.* sect. *Napelli* Rchb.f. and a single species of *B.* sect. *Xiphizusa* E.C.Smidt, Borba & van den Berg.

Orchidaceae displays a great diversity of labellar osmophores (Stern, Curry & Pridgeon, 1987; Vogel, 1990; Ascensão *et al.*, 2005; Wiemer *et al.*, 2009; Pansarin, Castro & Sazima, 2009; Lumaga *et al.*, 2012). The labellum of all species of *B.* sect. *Didactyle* studied here bear epidermal cells that, on the basis of their structure and cellular content, can be identified as osmophores. Similar data were obtained by Teixeira, Borba & Semir (2004) for representatives of the same section and the present results both confirm and build upon their findings.

An interesting feature first described here for *Bulbophyllum* flowers is the presence of sunken multicellular glandular trichomes on the abaxial surface of the sepals. Despite several attempts to discover their contents, histochemical tests performed on fresh flowers were not conclusive, but some cellular components showed a strong affinity for Neutral Red, whereas others stained weakly for phenolic compounds and pectic acids (Table 2). Although there is anatomical evidence to consider these trichomes to be osmophores, the other evidence is lacking (Stern, Curry & Whitten, 1986). However, this is not entirely true since, based on the

behaviour of the pollinators of three out of the seven species assigned to this section, there is indeed evidence that these structures are osmophores. For example, according to Borba & Semir (1998b), pollinating flies are more frequently attracted to (and alight on) the sepals than to other parts of the perianth. From here, they pass to the labellum and feed on the nectar. The presence of two possible types of trichome on two different floral parts indicates the importance of floral odour in attracting pollinators to these species. If confirmed, this is only the second report of this phenomenon in Orchidaceae. The first report of this kind is, to our knowledge, that of Stpicyńska (1993), who investigated *Cymbidium tracyanum* L.Castle. However, osmophores occur at the tips of the petals and labellum in that species.

Somewhat similar sunken trichomes have also been recorded for the vegetative organs of Maxillarinae and Lycastinae (Stern, Judd & Carlsward, 2004), Pleurothallidinae (Pridgeon 1981; Pridgeon, 1982), and Dendrobiinae (Morris, Stern & Judd, 1996) and on the flower (tepals and labellum) of a single species of *Maxillaria* Ruiz & Pav. (Stpicyńska & Davies, 2009). These floral hairs are thought to secrete resin. Mayer, Cardoso-Gustavson & Appezzato-da-Glória (2011) also found similar trichomes on both the vegetative and reproductive organs of *Cyrtorchilum flexuosum* Kunth (syn. *Oncidium flexuosum* (Kunth) Lindl.). These hairs secreted a mixture of mucilage, lipophilic compounds and proteins and were identified as colleters. Similar histochemical analyses should now be performed in order to determine the composition of the secretory products of the glandular sepal trichomes of this section of *Bulbophyllum*. The secretory cavity of the labellar callus was also present in all species and, based on anatomical and histochemical evidence, is here interpreted to be a nectary. This agrees with the findings of Teixeira, Borba & Semir (2004) for certain species of Neotropical *Bulbophyllum*. One interesting discovery was that the secretory cavity of *B. weddellii*, sister-species to the core of the section, was notably smaller than that of other species, which might indicate a tendency within this lineage to develop increasing amounts of nectariferous tissue during the course of evolution. Pollination in *B. weddellii*, *B. exaltatum*, and *B. involutum* is, to a degree, dependent on air currents (Borba & Semir, 1998b), but the presence of nectar also appears to be important for this type of pollination mechanism to work, as has been suggested elsewhere (Borba & Semir, 1998b; Teixeira, Borba & Semir, 2004). We speculate that there is a trade-off of highly adapted floral morphology in wind-assisted species, where wind is involved both in the attraction of the pollinator and in pressing it against the gynostemium, as occurs in *B. weddellii* (pendulous inflorescence, several flowers open simultaneously, labellum is petaloid and spatulate) for simpler flowers that represent the core of the section (erect inflorescence, few flowers open simultaneously, sepals

smaller and the labellum more fleshy) that are capable of producing more nectar. This resulting in flies being attracted to the flower for longer periods of time.

Other characters that help better distinction between species were also present (Table 3). Of all the species of *B. sect. Didactyle* investigated, *B. weddellii* showed 11 exclusive character states, mostly on the labellum. *Bulbophyllum perii* was second in terms of number of exclusive character states, with six character states occurring exclusively on the labellum. *Bulbophyllum popayanense* and *B. tripetalum* showed four exclusive character states, whereas *B. involutum* and *B. exaltatum* showed only one, and *B. meridense* showed none. Despite the paucity of exclusive character states present in some species, other character states differed between similar species, thus allowing distinction between the latter. *Bulbophyllum exaltatum* and *B. involutum* can be distinguished on the basis of four characters (secretory cavity length, secretory cavity shape in transverse section, number of vascular bundles in the labellum hypochile, and gynostemium foot surface ornamentation), while *B. meridense* and *B. tripetalum* differ in seven characters (ornamentation of the adaxial surface of the dorsal sepal, ornamentation of the adaxial surface of the lateral sepal, type of petal margin cell, secretory cavity length, secretory cavity shape in transverse section, cell type of abaxial surface of the lateral lobes, and gynostemium foot surface ornamentation).

Moreover, *B. weddellii* also exhibits different floral morphological characters, such as falcate petals, a long spatula-like labellum with a small callus, and a small secretory cavity labellum, which is why it was formerly assigned to *Bulbophyllum sect. Xiphizusa* by Pabst & Dungs (1975). However, despite these floral differences, *B. weddellii* also shares many characters with other species of the section (this study), shares the same pollinators as *B. involutum* (Borba & Semir, 1998b), and a similar floral odour to that of *B. involutum* (Silva *et al.*, 1999), to which it is closely related (Azevedo *et al.*, 2006). More recently, *B. weddellii* was placed in *B. sect. Didactyle* as sister group to the remaining six species, and *B. sect. Xiphizusa* section was placed as sister group to *B. sect. Didactyle* (Smidt *et al.*, 2011). As reported in other members of Orchidaceae (van der Pijl & Dodson, 1966; Hills, Williams & Dodson, 1972; Schiestl, Peakall & Mant, 2003; Schiestl & Ayasse, 2002; Mant, Peakall & Schiestl, 2005; Xu *et al.*, 2011) and other plant families (Proctor, Yeo & Lack, 1996), floral odour plays a critical role both in attracting pollinators and pollinator specificity. As suggested by Borba & Semir (1998b), this may be the most important signal for the attraction of a pollinator by *B. weddellii*, *B. involutum*, and *B. exaltatum*. Attraction primarily by floral odours may allow for less rigid variation in floral morphology, micromorphology, and anatomy and this, in turn, probably explains the differences shown here between the flowers

of *B. weddellii* and *B. involutum* (despite these species sharing the same two pollinators) and similarities between *B. involutum* and *B. exaltatum* (despite being pollinated by different species and possessing different floral odours - Borba & Semir, 1998b; Silva *et al.*, 1999).

Information on floral morphology and anatomy in Neotropical species of *Bulbophyllum* are scarce, and the variation found in this study was greater than anticipated, especially with regard to the labellum micromorphology, which had previously been studied only for a small number of species (Teixeira, Borba & Semir, 2004). We also found differences between the species that they studied, including greater distinction between the labellar surfaces of each taxon. For example, in *B. weddellii*, the abaxial surface of the hypochile possessed both convex-walled cells and short trichomes (referred to as papillae), whereas on the lateral lobes, the papillae were restricted to the abaxial surface, the adaxial surface having trichomes, with much larger trichomes at the margins (referred to only as the presence of papillae and trichomes). Only trichomes were found on the epichile of *B. involutum* and *B. exaltatum*, whereas that of *B. weddellii* was glabrous (previous authors reported papillae and trichomes for *B. involutum* and a smooth surface for *B. exaltatum* and *B. weddellii*, referring to this part of the labellum as the ‘limb’). These differences may solely be due to differences in terminology, especially since distinction between trichomes and papillae is somewhat subjective, and this may be exacerbated by the fact that papillae often mature to form trichomes. Moreover, a degree of intraspecific variation is to be expected. Although previous authors considered the organization of the epidermal surface to be homogeneous and conservative in *Bulbophyllum* (Teixeira, Borba & Semir, 2004), the present study, merely by expanding taxon sampling within *B. sect. Didactyle*, demonstrated considerable variation and it is anticipated that even greater variation will follow the expansion of sampling to include other species of Neotropical *Bulbophyllum*.

ACKNOWLEDGEMENTS

We thank São Paulo Research Foundation (FAPESP) for the Doctorate fellowship (Process 2011/11374-3) and for the Research Internship Abroad Fellowship (BEPE) (Process 2012/03433-2) to E.L.P.N. and National Council of Technological and Scientific Development (CNPq) (Process 475212/2011-8 and 306498/2012-0) to A.I.C. We are also very grateful to Sabine Adler (Ruhr Universität Bochum) for all the support and assistance with SEM and the anonymous reviewers who greatly helped to improve the manuscript.

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Table 1. List of species studied and collection data.

Species	Collection data
1. <i>B. exaltatum</i> Lindl.	BHCB (BHMH 479: Itabirito, Minas Gerais State, BR) HRCB (E.L.P. Nunes 13: Resende, Rio de Janeiro State, BR) HRCB (A.I. Coan <i>et al.</i> 195: São Gonçalo do Rio das Pedras, Minas Gerais State, BR)
2. <i>B. involutum</i> Borba, Semir & F.Barros	BHCB (MHNJB 918: Ouro Branco, Minas Gerais State, BR) HRCB (E.L.P. Nunes 12: Rio Pardo de Minas, Minas Gerais State, BR) HRCB (A.I. Coan <i>et al.</i> 134: São Gonçalo do Rio das Pedras, Minas Gerais State, BR)
3. <i>B. meridense</i> Rchb.f.	HRCB (E.L.P. Nunes 11: Caraguatatuba, São Paulo State, BR)
4. <i>B. perii</i> Schltr.	UPCB (W.S. Mancinelli 1143: Tibagi, Paraná State, BR)
5. <i>B. popayanense</i> Kraenzl.	HB (W. Sweiden 737: El Tambo, Cauca State, COL)
6. <i>B. tripetalum</i> Lindl.	UPCB (W.S. Mancinelli 1141: Tibagi, Paraná State, BR)
7. <i>B. weddellii</i> (Lindl.) Rchb.f.	HUEFS (C. Azevedo 188: Mucugê, Bahia State, BR) HRCB (A.I. Coan <i>et al.</i> 136: São Gonçalo do Rio das Pedras, Minas Gerais State, BR)

BHCB: Herbário Universidade Federal de Minas Gerais; BHMH: Museu de História Natural e Jardim Botânico da Universidade Federal de Minas Gerais; HB: Herbarium Bradeanum; HRCB: Herbário Rioclarense, Universidade Estadual Paulista; UPCB: Herbário Universidade Federal do Paraná; HUEFS: Herbário Universidade Estadual de Feira de Santana.

Table 2. List of histochemical tests performed on species of *Bulbophyllum* sect. *Didactyle* with common results and reaction sites.

Reagent	Reaction	Location
Coomassie Brilliant Blue	Proteins stain blue.	Labellum secretory epithelium
Calcofluor White MR2	Cellulosic cell walls fluoresce bright blue	Idioblasts with helical wall thickenings; epidermis striate wall
PAS	Polysaccharides stain purplish red	Cell walls, labellum mesophyll starch
IKI	Starch stains blue-black	Labellum mesophyll starch
Neutral Red <i>in vivo</i>	Lipids/volatile compounds stain red	Sepal glandular trichomes; larger trichomes of the labellum margin and lateral lobes
Neutral Red under UV light	Lipids fluoresce greenish blue	Larger trichomes of the labellum margin and lateral lobes, callus papillae
Sudan III	Lipids stain orange to red	Larger trichomes of the labellum margin and lateral lobes; small drops in the mesophyll cells
Fehling's solutions A and B	Aldehyde group (reducing sugars) presence is indicated by a red precipitate	Labellum secretory cavity cells - inconclusive
Ruthenium Red	Pectic acids stain pink to red.	Sepal glandular trichomes
Ferric chloride	Phenolic compounds stain dark gray or blue-black	Sepal glandular trichomes
Acidified phloroglucinol	Lignin stains red	Secondary walls of xylem elements

Table 3. List of variable characters between species of *Bulbophyllum* sect. *Didactyle*.

Character	<i>B. weddellii</i>	<i>B. popayanense</i>	<i>B. involutum</i>	<i>B. exaltatum</i>	<i>B. meridense</i>	<i>B. tripetalum</i>	<i>B. perii</i>
Dorsal Sepal	Surface ornamentation	striate	inconspicuous	striate	striate	heavily striate	heavily striate
	Ad Cell shape in frontal view	± isodiametric	transverse	transverse	transverse	transverse	transverse
	Margin type	smooth	elongate	elongate	elongate	elongate	elongate
	Mesophyll Bundles	5	papillose	smooth	smooth	smooth	smooth
Lateral Sepal	Surface ornamentation	inconspicuous	striate	striate	striate	heavily striate	striate
	Ad Cell shape in frontal view	irregular	transverse	transverse	transverse	transverse	transverse
	Margin type	smooth	elongate	elongate	elongate	elongate	elongate
	Mesophyll Bundles	5	papillose	smooth	smooth	smooth	smooth
Petal	Cell shape in frontal view	longitudinally elongate	± isodiametric	± isodiametric	± isodiametric	± isodiametric	± isodiametric
	Ad + Ab Outer periclinal wall shape	flat	flat + papillose	flat + papillose	flat + papillose	flat	flat
	Margin cell type	flat	unicellular trichome	unicellular trichome	unicellular trichome	unicellular trichome	flat

	Cell type	longitudinally						
Labellum median lobe (epichile)	Ad + Ab	elongate	unicellular	unicellular	unicellular	unicellular	unicellular	unicellular
	Trichome size	-	long	long	long	long	long	short
	Trichomes	absent	larger	larger	larger	larger	larger	similar to the remaining
	Mesophyll	Parenchyma	loose	compact	compact	compact	compact	compact
Labellum median lobe (hypochile)	type	inconspicuous	conspicuous	conspicuous	conspicuous	conspicuous	conspicuous	conspicuous
	Secretory	1/7 of callus	1/2 of callus size	almost 1/2 of callus size	1/3 of callus size	less than 1/2 of callus size	longer than 1/2 of callus size	
	cavity length	size						
	Secretory cavity shape in TS	irregular	ovate	oblong-elliptic		lanceolate	subulate	
	Cell type	convex + short trichomes	convex	convex	convex	convex		papillae
	Vascular Bundles	5	3	3	5	3	3	3
Labellum lateral lobe	Ad	Cell type	trichome	trichome	trichome	trichome	trichome	papillae
	Margin	Cell type	larger trichomes	larger trichomes	larger trichomes	larger trichomes	larger trichomes	papillae
	Ab	Cell type	papillae	trichome	papillae	papillae	flat	papillae
Gynostemium	Foot	Surface ornamentation	inconspicuous	inconspicuous	striate	inconspicuous	striate	inconspicuous
	Stigma	Shape	almost circular	longitudinally elongate	longitudinally elongate	longitudinally elongate	longitudinally elongate	longitudinally elongate

Character states in bold are exclusive to that species.

Figure 1. Floral morphology of *Bulbophyllum* section *Didactyle*. A, B, E–I, Flower in frontal view. J–P, Flower in side view. A, *Bulbophyllum exaltatum*. B, *Bulbophyllum weddellii* (arrows indicate flies visiting the flower). C, Labellum morphology of *B. weddellii* (middle lobe marked with a broken line and lateral lobes with a full line). D, Labellum morphology of *B. involutum* (middle lobe marked with a broken line and lateral lobes with a full line; arrow indicates the secretory cavity of the callus). E, *Bulbophyllum popayanense* (dorsal sepal in orange, lateral sepals in pink, petals in blue, middle lobe of the labellum in darker green and lateral lobes in light green, anther in yellow). F, *Bulbophyllum involutum*. G, Flower of *B. tripetalum*. H, *Bulbophyllum meridense*. I, *Bulbophyllum perii*. J, *Bulbophyllum weddellii*. K, *Bulbophyllum popayanense*. L, *Bulbophyllum involutum*. M, *Bulbophyllum exaltatum*. N, *Bulbophyllum tripetalum*. O, *Bulbophyllum meridense*. P, *Bulbophyllum perii*. DS, dorsal sepal; eq, labellum epichile; hq, labellum hypochile; L, labellum; LS, lateral sepal; P, petal; st, stolidium; te, teeth. Scale bars, 2 mm.

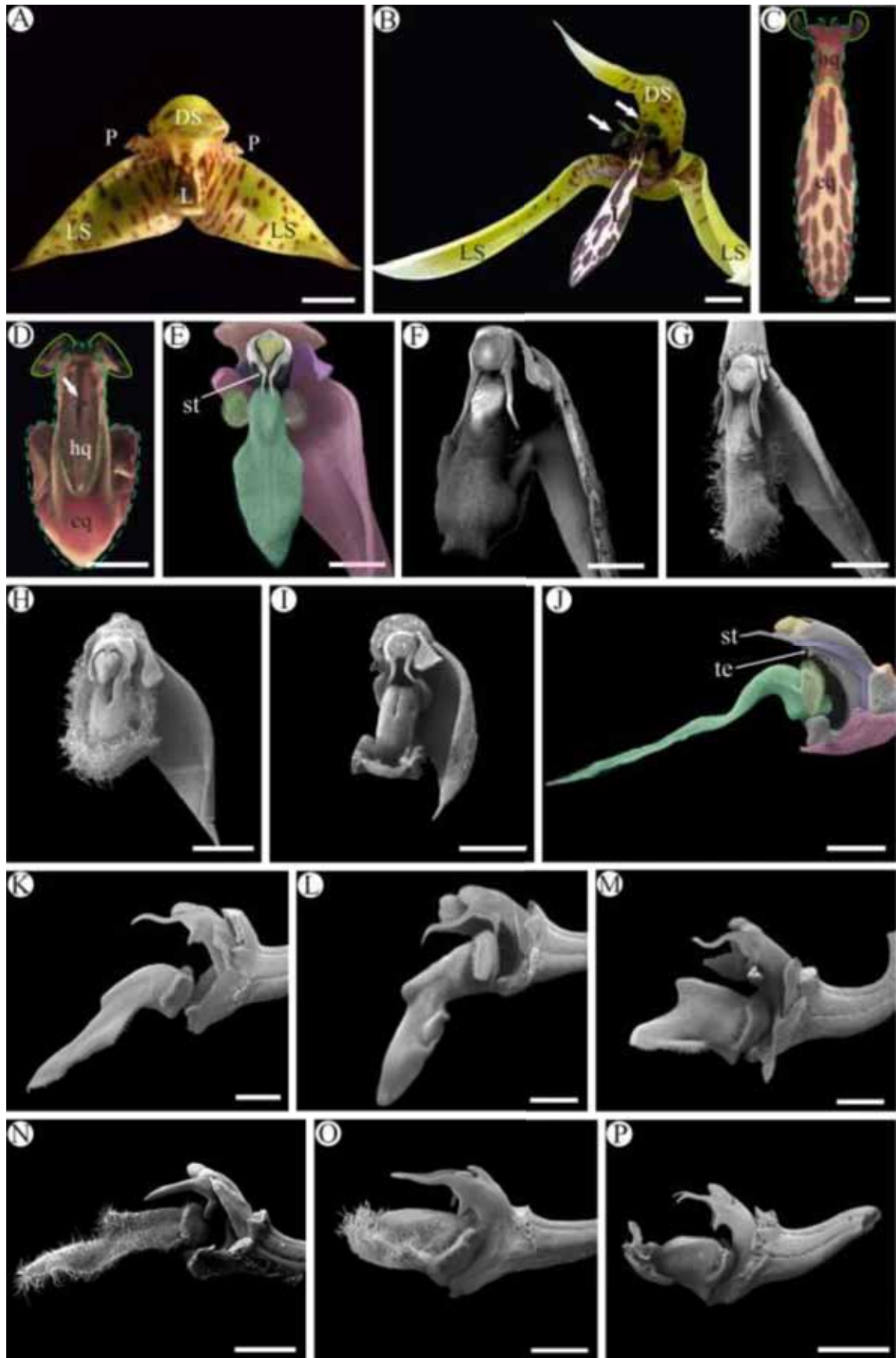


Figure 2. Micromorphology and anatomy of the sepals of *Bulbophyllum* section *Didactyle*. A–C, I, N, O, Adaxial surface. E, Q, Abaxial surface. D, F–H, J–M, P, Transverse section. A, *Bulbophyllum weddellii*. B, *Bulbophyllum meridense*. C, *Bulbophyllum tripetalum*. D, *Bulbophyllum exaltatum*. E, *Bulbophyllum involutum*. F, *Bulbophyllum weddellii*. G, *Bulbophyllum involutum*. H, *Bulbophyllum tripetalum*. I, *Bulbophyllum popayanense*. J, *Bulbophyllum involutum*. K, *Bulbophyllum perii*. L, *Bulbophyllum weddellii*. M, *Bulbophyllum popayanense*. N, *Bulbophyllum popayanense*. O–Q, *Bulbophyllum weddellii*. hi, idioblast with helical wall thickenings; ri, idioblast with raphides; vb, vascular bundle. Scale bars: 40 μm (A–C, K, N, O); 50 μm (D, L); 100 μm (E, M1–5, P, Q); 20 μm (F); 500 μm (G, J, M); 250 μm (H); 200 μm (I).

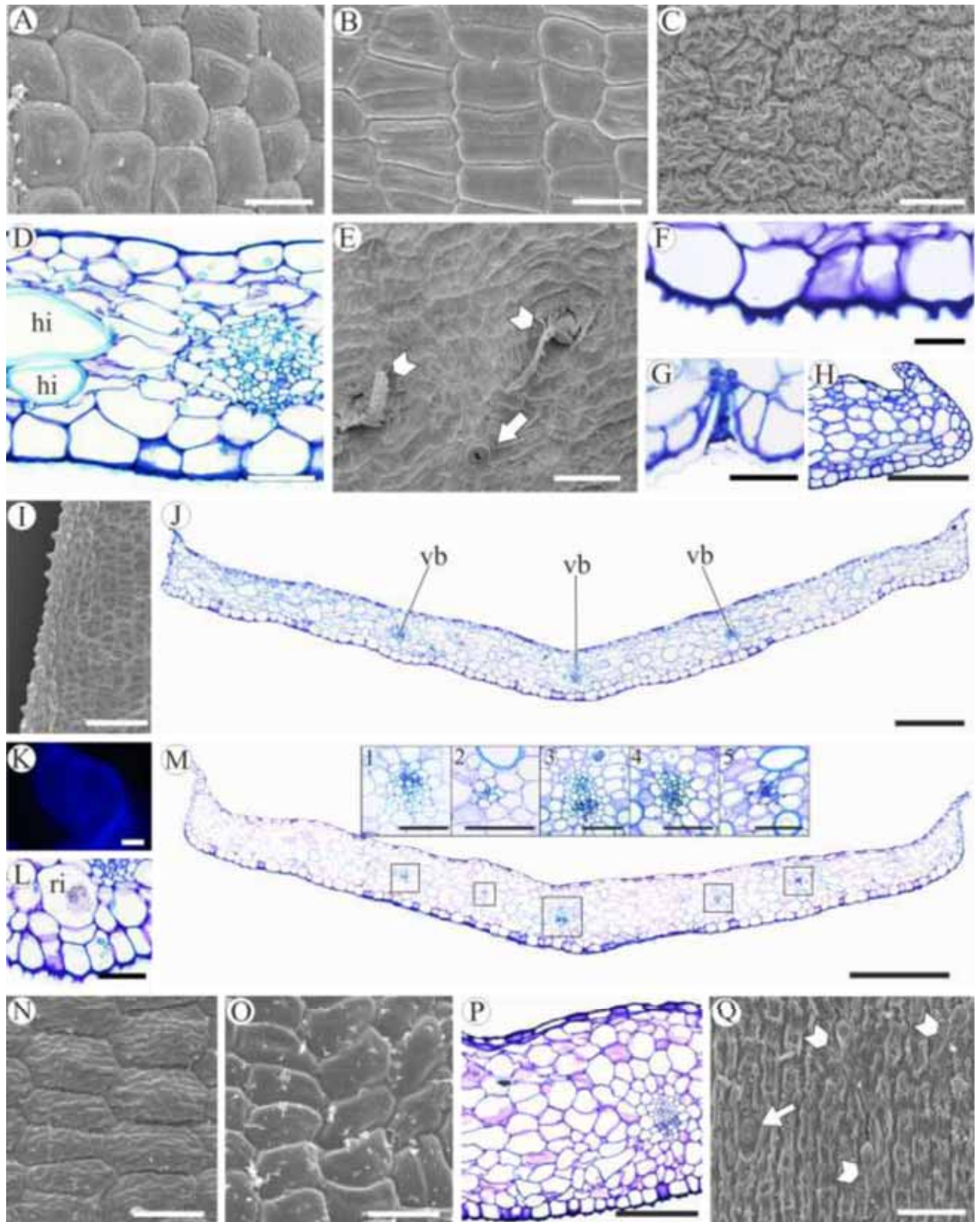


Figure 3. Micromorphology and anatomy of the lateral sepal and petals of *Bulbophyllum* section *Didactyle*. A, Scanning electron microscopy (SEM) detail of the abaxial surface of the lateral sepal of *B. weddellii* showing a glandular trichome. B, Light microscopy (LM) detail of a glandular trichome of *B. exaltatum*. C, Transverse section (TS) of the lateral sepal of *B. weddellii*. D, TS of the lateral sepal of *B. popayanense*. E, Adaxial surface of the petal of *B. involutum*. F, Adaxial surface of the petal of *B. weddellii*. G, TS of the petal of *B. meridense*. H, Detail of the margin of the petal of *B. meridense*. I, Detail of the margin of the petal of *B. weddellii*. J, TS of the petal of *B. popayanense*. hi, idioblast with helical wall thickenings; ri, idioblast with raphides; vb, vascular bundle. Scale bars: 40 μm (A, E, F); 20 μm (B); 200 μm (C, D); 100 μm (C1–5, D1–6, G, H, J); 60 μm (I).

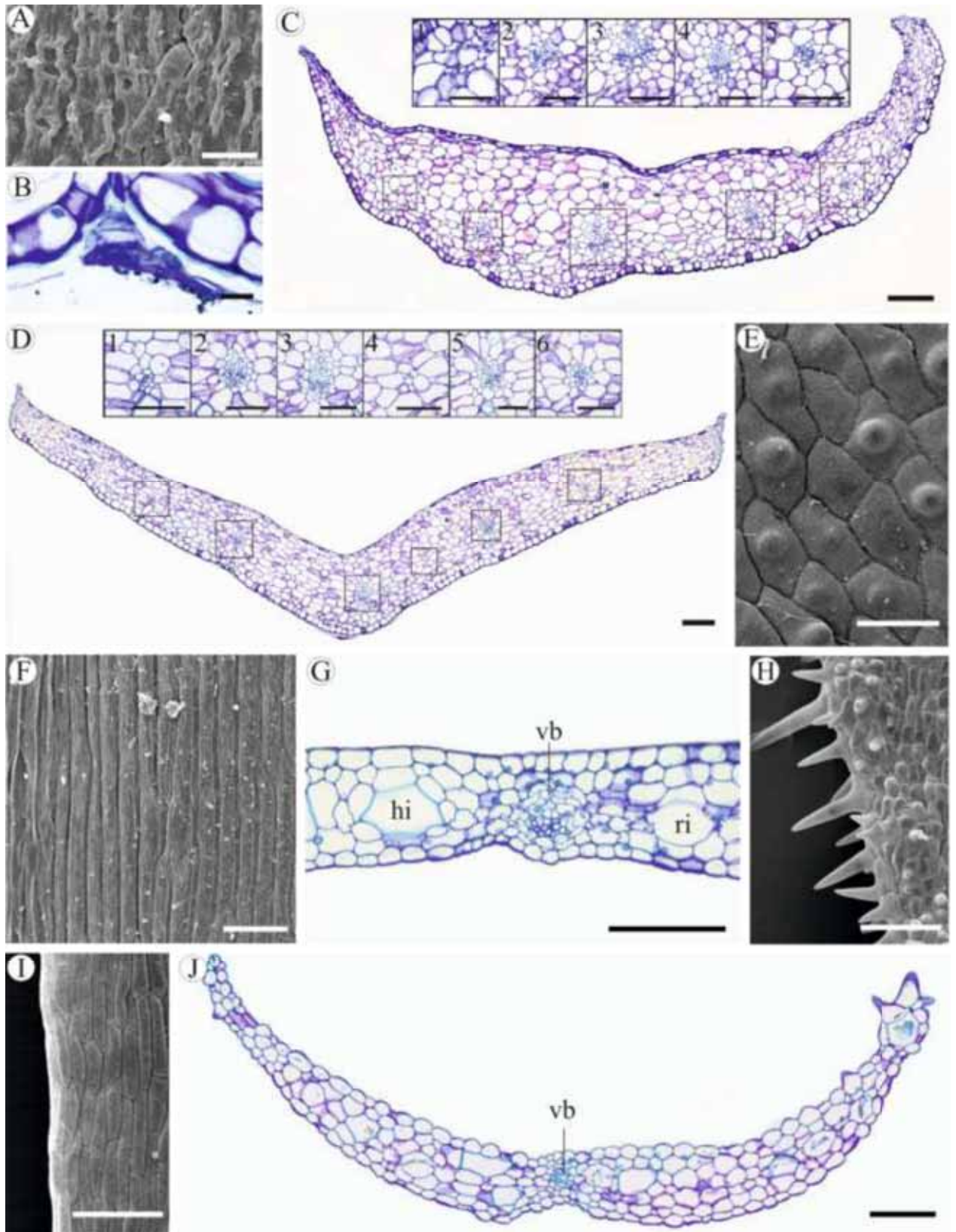


Figure 4. Micromorphology and anatomy of the labellum of *Bulbophyllum* section *Didactyle*. A, Adaxial surface of the epichile of *B. popayanense*. B, Transverse section (TS) of the epichile of *B. tripetalum*. C, Adaxial surface of the epichile of *B. weddellii*. D, Adaxial surface of the epichile of *B. perii*. E, Abaxial surface of the epichile of *B. exaltatum*. F, Detail of the margin trichomes of the epichile of *B. involutum*. G, TS of the epichile of *B. weddellii*. H, Adaxial surface of the labellum hypochile (callus) of *B. popayanense*. I, Detail of the callus adaxial surface showing the secretory cavity opening of *B. popayanense*. J, TS of the callus of *B. weddellii*. K, TS of the callus of *B. popayanense*. L, TS of the callus of *B. involutum*. M, TS of the callus of *B. exaltatum*. N, TS of the callus of *B. meridense*. O, TS of the callus of *B. tripetalum*. P, TS of the callus of *B. perii*. Q, Detail of the secretory cavity cells of *B. meridense*. R, Detail of the secretory cavity cells of *B. involutum*. S, Abaxial surface of the hypochile of *B. involutum*. T, Abaxial surface of the hypochile of *B. perii*. U, Abaxial surface of the hypochile of *B. weddellii*. V, Lateral lobe adaxial surface of *B. involutum*. vb, vascular bundle. Scale bars: 40 μm (A, E, H, S, T); 100 μm (B, D, F, I, V); 20 μm (C, G, Q, R); 500 μm (J–P); 200 μm (U).

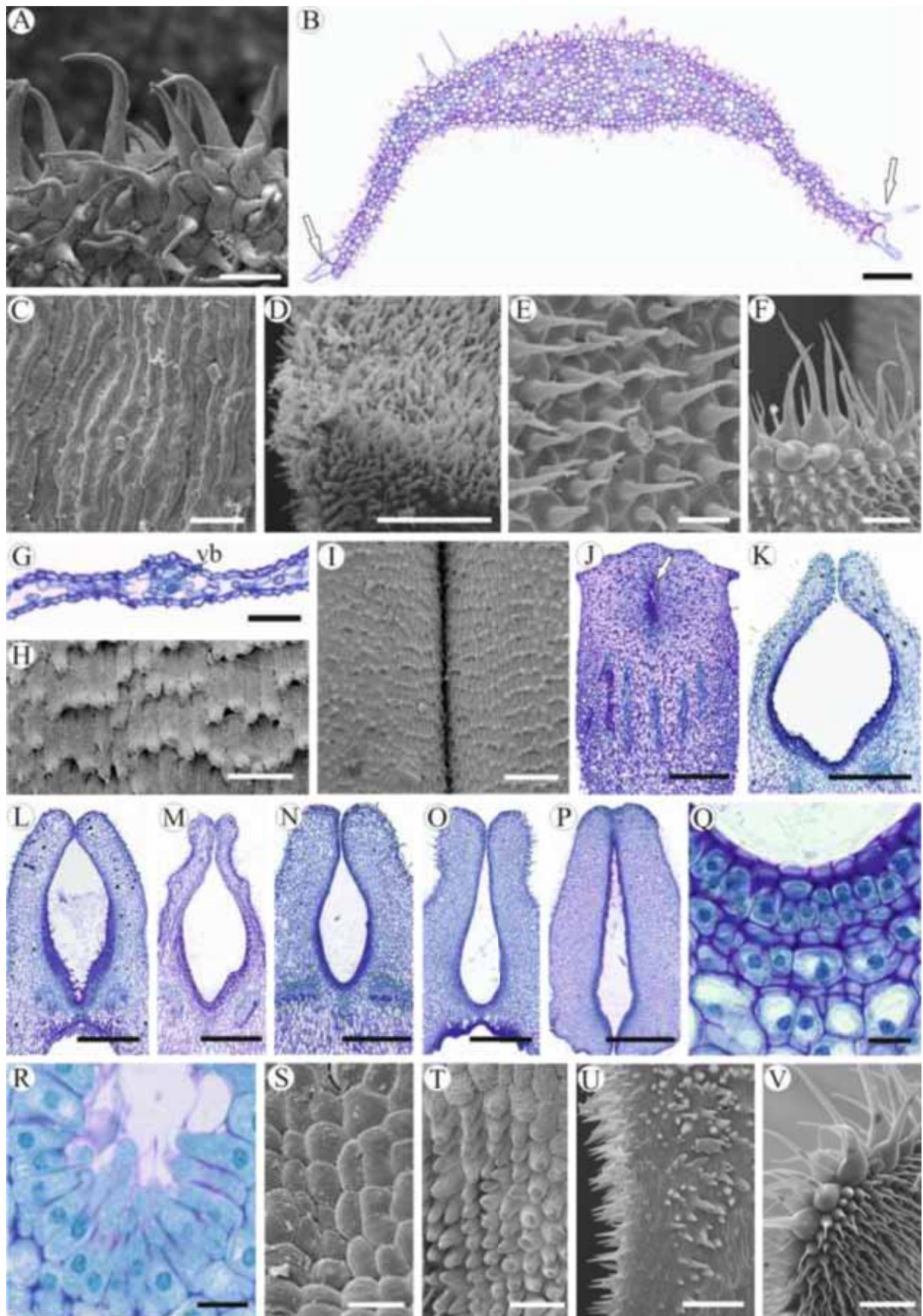
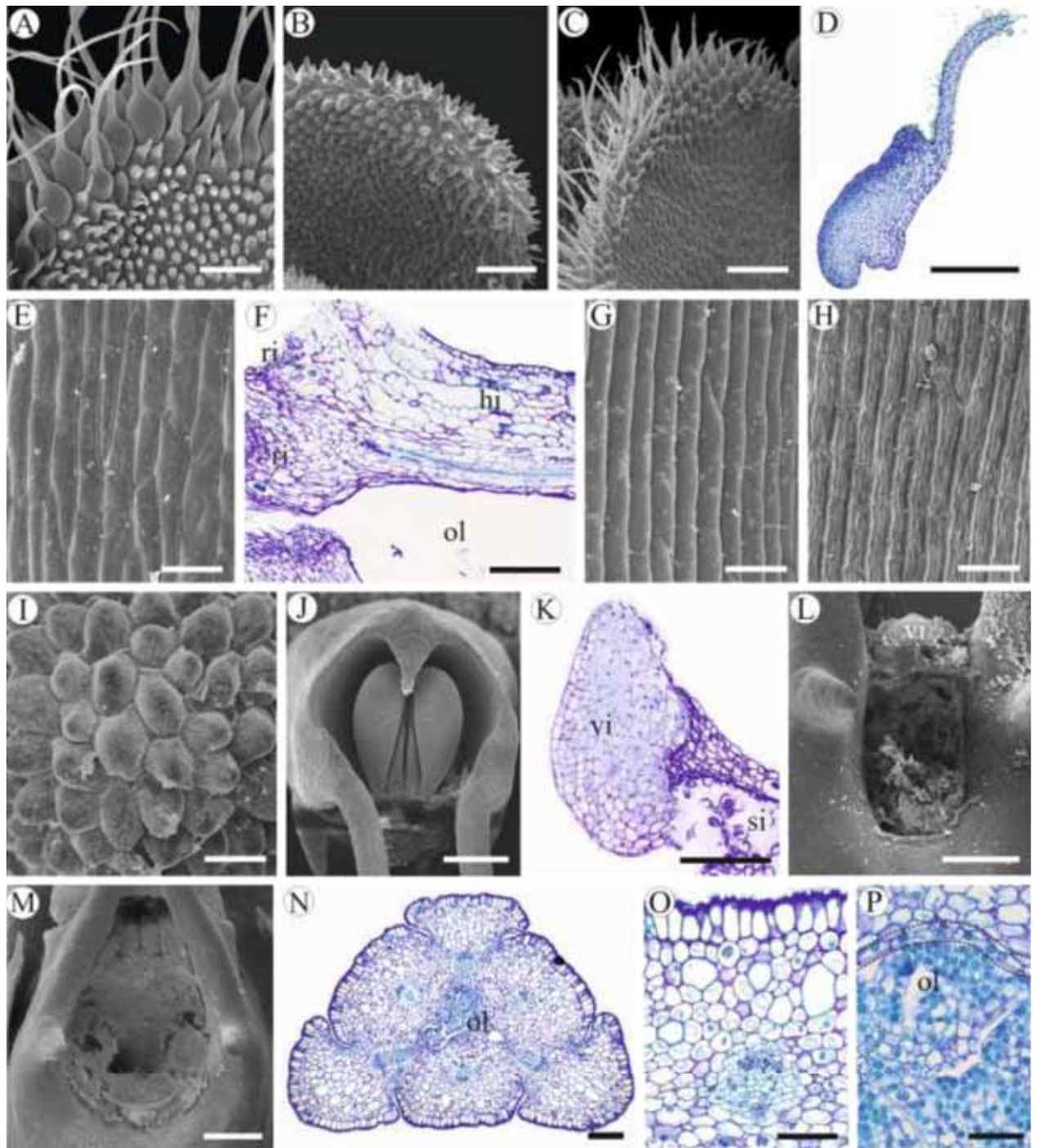


Figure 5. Micromorphology and anatomy of the labellum lateral lobes and gynostemium. A, Abaxial surface of the lateral lobe of *B. meridense*. B, Adaxial surface of the lateral lobe of *B. perii*. C, Abaxial surface of the lateral lobe of *B. tripetalum*. D, Transverse section (TS) of the lateral lobe of *B. meridense*. E, Gynostemium surface of *B. meridense*. F, Longitudinal section (LS) of the gynostemium of *B. exaltatum*. G, Gynostemium foot surface of *B. weddellii*. H, Gynostemium foot surface of *B. tripetalum*. I, Anther surface of *B. perii*. J, Frontal view of the gynostemium without the anther cap of *B. exaltatum*. K, LS of the developing viscidium of *B. involutum*. L, Detail of the longitudinally elongate stigma of *B. popayanense*. M, Detail of the broad stigma of *B. weddellii*. N, TS of the ovary of *B. involutum*. O, Detail of the ovary of *B. involutum* showing its outer epidermis. P, Detail of the ovary locule of *B. involutum* showing its inner epidermis (broken lines). hi, idioblast with helical wall thickenings; ol, ovary locule; ri, idioblast with raphides; S, sepal; si, stigma; vi, viscidium. Scale bars: 100 μm (A, B, O); 200 μm (C, K, N); 500 μm (D, F); 40 μm (E, G–I); 400 μm (J, L, M); 50 μm (P).



CAPÍTULO II

**Comparative floral micromorphology and anatomy of species of
Bulbophyllum sect. *Napelli* (Orchidaceae), a Neotropical section widely
distributed in forestal habitats**

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(Submetido ao periódico *Botanical Journal of the Linnean Society* em 14/07/2014)

ABSTRACT

Bulbophyllum sect. *Napelli*, recently circumscribed based on molecular analyses, comprises 12 species. These species occur as epiphytes in the Brazilian Atlantic Rain Forest and in gallery forests in the Cerrado vegetation, unlike the other Neotropical sections of *Bulbophyllum*, which are more variable in habit and habitat. To identify diagnostic characteristics that are representative of this section and to verify whether there are characteristics that are related to their habit and habitat, the floral micromorphology and anatomy were studied using conventional techniques. *B.* sect. *Napelli* is characterised by sepals with sunken glandular trichomes and stomata on the abaxial surface; reduced petals with one vascular bundle (sometimes lacking); an entire labellum with a secretory sulcus on the adaxial surface and a keel with stomata or glandular trichomes on the abaxial surface; two pollinia; and crystalliferous idioblasts in all of the floral parts. Our data distinguish between species and show congruence with the present circumscription of *B.* sect. *Napelli*. An unusual epidermal surface with unicellular trichomes was found in the labellum of three closely related species and seems to work as an osmophore. A relationship between striate surfaces, iridescence and pollinator attraction related to the epiphytic habit and forest habitat is suggested.

ADDITIONAL KEYWORDS. *Bulbophyllum atropurpureum* – *B. campos-portoi* – *B. granulatum* – *B. malachadenia* – *B. regnellii* – cell wall striation – nectary – petal reduction osmophore – unusual epidermal surface.

INTRODUCTION

The genus *Bulbophyllum* Thouars is represented by 62 species in the Neotropics that were recently distributed into six monophyletic sections, based mostly on molecular data: *B.* sect. *Bulbophyllaria* (Rchb.f.) Griseb., *B.* sect. *Furvescens* E.C. Smidt, Borba & Van den Berg, *B.* sect. *Napelli* Rchb.f., *B.* sect. *Micranthae* Barb.Rodr., *B.* sect. *Didactyle* (Lindl.) Cogn., and *B.* sect. *Xiphizusa* (Rchb.f.) Cogn. (Smidt *et al.*, 2011). Most species can occur both as epiphytes or lithophytes in open and woodland areas of the Cerrado, Atlantic Rain Forest, Andean vegetation and Central America Forest (Smidt *et al.*, 2007, 2011, 2014).

Bulbophyllum sect. *Napelli* currently comprises 12 species that mostly occur in the Brazilian Atlantic Rain Forest and also in gallery forests in the Cerrado vegetation, except for

one species with a disjunct distribution in Venezuelan forest – *B. dunstervillei* Garay & Dunst. (Smidt *et al.*, 2011). This section differs from the other neotropical sections of *Bulbophyllum* by its almost exclusively epiphytic habit and also its forest habitat (Smidt *et al.*, 2011, 2014). The flowers of *B. sect. Napelli* are characterised by the presence of a pedicel, the distichal arrangement of the flowers in the inflorescence axis, lateral sepals with attenuate to caudate apices, usually reduced petals, an entire labellum that is usually fleshy and glabrous, a column with teeth and a foot longer than the column itself (Smidt *et al.*, 2011, 2014).

There are few studies on the floral anatomy of neotropical *Bulbophyllum*. Recently, Nunes *et al.* (2014), studying the floral micromorphology and anatomy of *B. sect. Didactyle*, found that this approach is useful for differentiating between species and have also demonstrated congruence with the proposed circumscription of Smidt *et al.* (2011). In addition to this study, the only report on the floral anatomy of neotropical *Bulbophyllum* is that of Teixeira, Borba & Semir (2004) investigating the labellum anatomy of six Brazilian and one Asian species of *Bulbophyllum*. This study includes two species belonging to *B. sect. Napelli* that have osmophores on their adaxial labellum surface and, despite the presence of nectar, no nectariferous tissue was found.

The floral morphology of neotropical *Bulbophyllum* is quite diverse, and some authors have suggested environmental pressures and pollinator specificity as possible drivers for such variation (Borba & Semir, 1998; Verola, 2002; Teixeira *et al.*, 2004). The reproductive biology of two species belonging to *B. sect. Napelli* was studied by Verola (2002), who found that *B. glutinosum* Cogn. is pollinated by female flies of a Tachinidae species, whereas *B. regnellii* Rchb.f. was not pollinated during the study. The reported pollination mechanism is similar to that of *B. macranthum* Lindl. as described by Ridley (1890): the fly lands on the labellum, which is displaced by the pollinator weight, and as the fly moves towards the labellum base, the balance point of the latter is surpassed and returns to its original position, causing the pollinarium removal by the fly.

Therefore, the aim of this study was to answer the following questions: a) which micromorphological and anatomical floral features are common to the representatives of *B. sect. Napelli*; b) which micromorphological and anatomical floral features distinguish between the species of this section; and c) are there characteristics that may be related to the habit and habitat of the representatives of this section?

MATERIAL AND METHODS

The mature flowers of six species that are currently assigned to *Bulbophyllum* sect. *Napelli* were collected in the field or obtained from the Botanical Gardens (Table 1). The samples were fixed in FAA 50 (3.7% v/v formaldehyde, 50% ethanol, 5% acetic acid; Johansen, 1940) or 2.5% glutaraldehyde and 2% formaldehyde in phosphate buffer 0.1 M, pH 7.2 (modified from Karnovsky, 1965).

The micromorphological and anatomical characteristics were assessed using scanning electron (SEM) and light (LM) microscopy. For each species, at least four flowers from different specimens were analysed, whenever possible, and sourced from different localities (Table 1).

The samples for SEM analysis were transferred from 70% ethanol to formaldehyde-dimethyl acetal for dehydration for 16 to 24 h (Gerstberger & Leins, 1978), further critical-point-dried (CPD 030 - Bal-Tec AG, Balzers, Liechtenstein), and sputter-coated with gold (SCD 050 - Bal-Tec AG, Balzers, Liechtenstein). SEM observations were made using a Zeiss DSM 950 (Carl Zeiss, Jena, Germany), and images were obtained using Digital Image Processing Software 2.2 (DIPS-Leipzig, Germany).

For LM analysis, the samples were dehydrated in an ethanol series, embedded in historesin Leica (Leica Microsystems GmbH, Heidelberg, Germany) or Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim, Germany) according to the instructions of the suppliers and sectioned at 5 to 8 μm on a rotary microtome (RM 2065 -Leica Microsystems GmbH, Wetzlar, Germany) using disposable blades. The sections were stained with 0.05% Toluidine Blue O in 0.1 M sodium phosphate buffer pH 6.8 (O'Brien, Feder & McCully, 1965) for general analysis. The following histochemical tests were performed on resin-embedded sections: iodine/potassium iodide solution (IKI) for starch (Johansen, 1940), Ruthenium Red for pectic acids/mucilage (Johansen, 1940), Coomassie Brilliant Blue for proteins (Southworth, 1973), Calcofluor White MR2 for cellulose (Hughes & McCully, 1975), and Periodic Acid-Schiff reaction (PAS) for total insoluble polysaccharides (Feder & O'Brien, 1968).

The material that was fixed in Karnovsky's fixative was washed in phosphate buffer, hand-sectioned and tested with ferric chloride for phenolic compounds (Johansen, 1940), Sudan III for lipids (O'Brien & McCully, 1981), Neutral Red under UV excitation for total lipids (Kirk, 1970), and acidified phloroglucinol for lignin (Sass, 1951). Entire fresh flowers

were immersed in Neutral Red for osmophore identification and distribution (Kearns & Inouye, 1993). The appropriate controls were run simultaneously whenever applicable.

Photomicrography was achieved using a Leica DMLB microscope that was coupled with a digital camera using the LAS (Leica Application Suite V3.3.0) software.

RESULTS

The flowers of the studied species of *Bulbophyllum* sect. *Napelli* can be characterised by free sepals (Fig. 1A-D, lateral sepals in red and dorsal in pink - some of the sepals were partially or totally removed to improve visualisation) with an attenuate to caudate apex; reduced petals (Fig. 1A-D, G-J; in purple), except for *B. napelli* and *B. regnellii* (Fig. 1E, F); entire labellum, tongue-shaped and glabrous (Fig. 1A-L, in green); gynostemium with inconspicuous stelidia and short or long teeth (Fig. 1A-L, in orange); long column foot (Fig. 1G-L, in orange); and short anthers (Fig. 1A-J, L, in yellow). The labellum usually shows a fleshy part on its abaxial surface called the keel (Fig 1G-L).

The dorsal sepal shows an ornamented adaxial wall surface that is striate (Fig. 2A), irregular (Fig. 2B) or granulose (Fig. 2C). In the frontal view, the epidermal cells are transversely elongated (Fig. 2D), longitudinally elongated (Fig. 2A) or irregular (Fig. 2C). In the transverse section, the outer periclinal wall is mostly convex (Fig. 2E), but straight in *B. napelli* and *B. atropurpureum* (Fig. 2F). At the abaxial surface, the ornamentation is longitudinally striate (Fig. 2G) or irregular (Fig. 2H). The cell shape is not clearly visible due to the cuticle pattern. In the transverse section, the outer periclinal wall is straight or slightly convex (Fig. 2E, F). On this surface, sunken pluricellular glandular trichomes are present in all of the studied species (Fig. 2G-J, arrows).

The margin of the dorsal sepal is smooth (Fig. 2J), except for that of *B. atropurpureum*, which is papillose (Fig. 2K); in the transverse section, the margin is formed by epidermal and mesophyll cells (Fig. 2L). The mesophyll is homogeneous with regular parenchyma and two types of idioblasts: one with raphides and the other with cellulosic helical wall thickenings (Fig. 2E, F, L-O). The number of collateral vascular bundles varies between one (Fig. 2O), three (Fig. 3A), four (Fig. 3B) or five (Fig. 3C).

The lateral sepals on the adaxial surface either show a striate ornamentation (Fig. 3D) or lack a conspicuous ornamentation (Fig. 3E). The epidermal cells, in the frontal view are similar to those of the dorsal sepal. In the transverse section, the outer periclinal wall is mostly straight (Fig. 3F). At the abaxial surface, the surface ornamentation is similar to that

of the dorsal sepal. The outer periclinal wall in the transverse section is either straight (Fig. 3F) or convex (Fig. 3G). Sunken pluricellular glandular trichomes are also present at the abaxial surface of the lateral sepal. The margin, mesophyll and vascular bundles are of the same type as for the dorsal sepal, but the number of bundles varies between two (Fig. 3G), three (Fig. 3H) and seven (Fig. 4A).

The petals show a striate wall pattern and irregularly shaped cells on both the adaxial and abaxial surfaces (Fig. 4B, C). The outer periclinal walls are usually convex in the transverse section (Fig. 4D, E). The margin is usually papillose (Fig. 4F) and formed by epidermal and mesophyll cells in the transverse section (Fig. 4D, G). In the transverse section, the mesophyll is homogeneous, with regular parenchyma and idioblasts with raphides (Fig. 4D, E, G); idioblasts with cellulosic helical wall thickenings may be present or absent. There is a single collateral vascular bundle (Fig. 4D, E, G), except for *B. granulorum* and *B. atropurpureum* (Fig. 4G), which show reduced and non-vascularised petals.

The labellum, on its distal and middle third, shows a striate ornamentation at the adaxial surface (Fig. 4H). The epidermal cells are isodiametric but of a distinct shape and arrangement according to the species. In *B. granulorum* (Fig. 4H, I), *B. atropurpureum* (Fig. 4J) and *B. campos-portoi* (Fig. 5A), the epidermis shows an extra layer of trichomes in which the cells eventually connect to each other through “bridges” but leaving considerable intercellular spaces. In *B. atropurpureum* (Fig. 4J) and *B. campos-portoi* (Fig. 5A), the linking of such trichomes produces a reticulated pattern. In *B. malachadenia*, *B. napelli* and *B. regnellii*, the epidermal cells are papillose (Fig. 5B) and, in transverse sections, show an intermediary pattern between *B. campos-portoi*/*B. atropurpureum* and *B. granulorum* (Fig. 5C).

At the abaxial surface of the distal and middle third of the labellum, the ornamentation is also striate (Fig. 5D), except for *B. napelli*, in which it is inconspicuous (Fig. 5E). In the frontal view, the epidermal cells are either longitudinally elongated (Fig. 5F) or irregularly shaped (Fig. 5D, E). The outer periclinal walls are convex, or there is a combination of papillose and convex-walled cells (Fig. 5D-I). At those regions, the stomata were observed in some species (Fig. 5D, E, arrows). The mesophyll is homogeneous, with regular parenchyma and idioblasts with raphides (Fig. 5G-I). The vascular bundles of the median third are collateral and vary between five (Fig. 5G), seven (Fig. 5H) and nine (Fig. 5I).

The adaxial surface of the proximal third of the labellum shows similar epidermal cells and surfaces as those of the other parts. However, this region shows a well-developed

and secretory sulcus (Figs. 1A-F, 5J, K). On this sulcus, a small amount of exudate was observed in at least three species in the field (*B. campos-portoi*, *B. atropurpureum* and *B. malachadenia*). The sulcus is mostly shallow (Fig. 5J), except for that of *B. malachadenia* (Figs. 5K, 6A). In the frontal view, the epidermal cells of the sulcus are either isodiametric (Fig. 6A) or irregularly shaped (Fig. 6B); the outer periclinal wall of the epidermal cells is mostly convex (Fig. 6A), although it is papillose in *B. campos-portoi* (Fig. 6C).

The abaxial surface of the proximal third of the labellum shows a conspicuous keel (Fig. 1G-K), except for *B. napelli*, in which it is inconspicuous (Fig. 1L). In the frontal view, the epidermal cells are longitudinally elongated (Fig. 6D), isodiametric or irregularly shaped (Fig. 6E); stomata are present in all species (Fig. 6D, E); and pluricellular glandular trichomes are seen only in *B. campos-portoi* and *B. atropurpureum* (Fig. 6E, F). The mesophyll and vascular bundles are similar to that described for the other parts of the labellum, but the bundles vary between five (Fig. 5J, K) and seven (Fig. 6G).

The gynostemium in the frontal view is covered by longitudinally elongated cells, mostly with striate ornamentation (Fig. 6H), except for *B. malachadenia*, in which they are smooth (Fig. 6I). The mesophyll is homogeneous with regular parenchyma and idioblasts with raphides or with cellulosic helical wall thickenings (Fig. 6J). The column foot shows striate ornamentation and longitudinally elongated cells (Fig. 6K, L). Its epidermal cells show convex outer periclinal walls (Fig. 6K), except for *B. atropurpureum* (Fig. 6L), in which they are papillose. The mesophyll is similar to that of the gynostemium (Fig. 6J).

The anther shows isodiametric epidermal cells with striate ornamentation (Fig. 7A) and papillose outer periclinal walls (Fig. 7A). The pollinarium is composed of only two pollinia (Fig. 7B, C), but in *B. atropurpureum*, eventually two more reduced pollinia were found (Fig. 7D). In addition to the pollinia, the pollinarium is composed by a viscidium that originated from the disintegration of part of the rostellum (Figs. 6J, 7E, F).

The sigma is wide (Fig. 7F), except for *B. malachadenia*, in which it is longitudinally elongated (Fig. 7E); the stigmatic cavity is filled with elongated cells and a mucilaginous matrix (Figs. 6J, 7E, F, K). The ovary is a more complex six-lobed structure whose three outermost lobes derive from the sepals (Fig. 7G). In the transverse section, the outer epidermis of the ovary shows columnar cells with a convex outer periclinal wall (Fig. 7G, H); the inner epidermis shows elliptic cells with a convex outer periclinal wall (Fig. 7H). The ovary mesophyll is homogeneous with regular parenchyma and idioblasts with raphides and idioblasts with cellulosic helical wall thickenings (Figs. 6J, 7G, H); there is a single collateral vascular bundle per lobe (Fig. 7G, H).

Iridescence was observed on the floral surfaces of fresh flowers of *B. campos-portoi* and *B. malachadenia* when dissecting them under the stereomicroscope; in *B. atropurpureum* and *B. granulosum*, iridescence was also observed but only after critical-point drying. In neither the aforementioned cases, documentation through digital photography gave adequate results.

A survey of the most relevant characteristics that are variable between the species is presented in Table 3.

DISCUSSION

In this study, several floral micromorphological and anatomical characteristics were described that are shared by most of the studied species of *Bulbophyllum* sect. *Napelli*. The distinguishing features were the presence of multicellular glandular trichomes and stomata on the abaxial surface of the sepals; reduced petals with papillose margins and a single collateral vascular bundle that is sometimes lacking; an entire labellum with a striate surface, papillose epidermis or with unicellular trichomes and a secretory sulcus on the adaxial side, in addition to a keel on the abaxial surface; two pollinia with smooth tetrads; and mesophyll with both crystalliferous and thickened idioblasts in all of the floral parts.

Our findings better characterise *B.* sect. *Napelli* as grouped according to Smidt *et al.* (2011), even though we have studied only six species, and these authors have used molecular markers to study nine species. Prior to these studies, the most recent treatment of the group was that of Pabst & Dungs (1975), in which the species that are currently assigned to *B.* sect. *Napelli* were scattered among the *B.* sect. *Bulbophyllaria* - *B. micropetaliforme* alliance, *B.* sect. *Didactyle* - *B. glutinosum* alliance, and *B.* sect. *Micrantha* - *B. micranthum* alliance. In addition, we found 25 characteristics whose states vary between species, and are thus useful for distinguishing between them (see Table 3).

An interesting feature of four of the studied species is a petal reduction that leads in two species to a lack of vascular bundles. Such a reduction is reported for most species of this section, with the exception of *B. regnellii* and *B. napelli* (Smidt *et al.*, 2011). Showy petals are part of the visual signs causing effective pollination, and specific elaborations of petals are often triggered by the kind of pollinators that evolved with the flowers (Ronse de Craene, 2010); this applies especially for the labellum in Orchidaceae. However, the reduction of the other two petals is not uncommon in Orchidaceae and is reported for other *Bulbophyllum* (Vermeulen, 1982) and several Pleurothallidinae genera (Karremans *et al.*, 2013; Kolanowska

2013), being associated with the dominant petaloidy of the sepals in *Stelis* and other allied genera (Karremans *et al.*, 2013). As the petals of the species of *Bulbophyllum* sect. *Napelli* are mostly reduced, the floral display is composed mainly of the sepals and maybe by the labellum. In the sepal, petal and labellum, most of the studied species show epidermal cells with ornamented outer walls, commonly with some kind of striation, as shown by SEM, transverse sections and histochemical tests.

Although such striation patterns are usually believed to be due to the cuticle deposition process and cell wall growing pressures (Kourounioti *et al.*, 2013), in *B.* sect. *Napelli*, we demonstrated that these patterns are primarily formed by the cell wall itself. However, we believe that those striation patternings may work as diffraction gratings. Such structures create iridescence, changing the hue of a surface with varying observation angles, at least in ten Angiosperm families, and may act as cues for pollinators, as shown by experiments with bees (Whitney *et al.*, 2009). Sometimes, iridescence is visible to human eyes only when the pigment colour is separated from the petal structure (Whitney *et al.*, 2009), and, in the case of species of *B.* sect. *Napelli*, iridescence was observed when preparing samples for SEM, after the critical-point drying and consequent removal of pigments. In addition to the presence of striation on the flower surface, convex or papillose walled epidermal and idioblasts containing raphides cells were common in the studied species. These cells are believed to act as lenses that focus light into pigment vacuoles and enhance colour saturation (Kay, Daoud & Stirton, 1981; Gorton & Vogelmann, 1996; Whitney *et al.*, 2011), and crystals may have a light-gathering and reflection function (Franceschi & Horner, 1980; Franceschi, 2001). Therefore, it is reasonable to suggest that these cells might, together with the striate flower surfaces, be involved in pollinator attraction in *B.* sect. *Napelli*, most likely related to the epiphytic and forest habitats. Further evidence is provided by *B.* sect. *Didactyle* (Nunes *et al.*, 2014) and *B.* sect. *Micranthae* (Nunes *et al.*, in prep.), which show more variability in those characteristics as well in their habit and habitat. Still regarding iridescence, although it has been correlated in leaves with shaded habitats in some plants (Graham, Lee & Norstog, 1993; Feild, Franks & Sage, 2003), to our knowledge, no information is available regarding flowers or on the relationship of this iridescence to fly pollination.

Two of the species that were studied here do not exhibit petal reduction (or perhaps show a secondary increase in size): *B. napelli* and *B. regnellii*. It is interesting to note that Verola (2002) reported that *B. regnellii* emitted a sweet cinnamon scent and was only visited by a species of Halictidae (Hymenoptera), although pollination did not take place; *B.*

glutinosum (closely related to *B. campos-portoi* studied here), however, emitted an unpleasant plant decomposing scent and was pollinated by female Tachinidae flies in a mechanism that is similar to that described by Ridley (1890) for the Asian *B. macranthum*. Considering the different morphology between this pair of species (reduced petals and prominent column teeth in *B. glutinosum* – closely related to the *B. campos-portoi* studied here – and enlarged petals and reduced teeth in *B. regnellii*), we might speculate a trade-off in the function of petals and column teeth in *B. regnellii* and *B. napelli*, most likely related to a change in pollinator and in the mechanical support that is needed by the flower to accommodate the movement of the pollinator inside it and cause pollinarium removal by the insect.

In three of the studied species (*B. campos-portoi*, *B. atropurpureum* and *B. granulatum*), the labellum epidermis shows a layer in which the cells connect to each other but leave considerable intercellular spaces (in *B. campos-portoi* and *B. atropurpureum*). Because the interlocking of epidermal cells provides mechanical strength and acts as a barrier that was almost totally impermeable to water and protected against herbivory (Glover, 2000), it seems unlikely that this external layer is the only component of the epidermis. Thus, we have chosen to interpret the cells of the external layer as unicellular trichomes that eventually form “bridges” (in *B. atropurpureum* and *B. campos-portoi*) that give rise to a reticulate pattern to the labellum surface. These trichomes apparently function as osmophores based on the following evidence: usually unpleasant odour during collection (personal observation), positive reactions to neutral red tests *in vivo* and under UV light and Sudan III, as well as the presence of a dense cytoplasm and a very large nucleus. A somewhat similar epidermal arrangement, although without bridges between trichomes, was observed at least in the orchid species *Cyclopogon elatus* (Wiemer *et al.*, 2009). Developmental studies are advisable to elucidate the epidermal formation in species of *B. sect. Napelli*, especially to understand the formation of the bridges between trichomes and their phylogenetic significance because they occur in closely related species (Smidt *et al.*, 2011).

Most of the species that were studied here showed a well-differentiated sulcus at the adaxial proximal portion of the labellum. The secretory nature of the sulcus is revealed by histochemical tests, a dense cytoplasm and a prominent nucleus of their cells and the presence of nectar observed during the collection, allowing us to interpret the sulcus as a nectary. Previously, the presence of nectar has been reported near the labellum base for *B. glutinosum* and *B. regnellii* (Verola, 2002; Teixeira *et al.*, 2004), but no histological evidence for a nectary was found (Teixeira *et al.*, 2004). We believe that there is enough evidence to

consider the labellum sulcus of the studied species, including *B. regnellii*, as a nectary, but for *B. glutinosum* that remains to be verified.

An interesting characteristic of all of the studied species is the presence of only two pollinia in the mature pollinarium, identified for the first time in this report, whereas the basic number for *Bulbophyllum* and Orchidaceae is four - in agreement with the four pollen sacs that are usually found in Angiosperms (Dressler, 1993). This character is a promising apomorphy of this group but the other neotropical sections of the genus must be verified. Secondary reduction or an increase in the number of pollinia is common and widespread within Orchidaceae (Dressler, 1993), including Pleurothallidinae, which are also predominantly fly pollinated (Dressler, 1993). According to Freudenstein & Rasmussen (1996), within the Epidendroideae there are two archesporial zones that only by septation result in four or eight pollinia per anther, while the lack of septation gives rise to two pollinia. For *B. sect. Napelli*, the ontogenetic process leading to two pollinia remains unclear, and because *B. atropurpureum* occasionally showed two additional central reduced pollinia, the early fusion of four pollen sacs cannot be ruled out.

ACKNOWLEDGEMENTS

This work was supported by São Paulo Research Foundation (FAPESP) [2011/11374-3 and 2012/03433-2 to E.L.P.N.] and National Council of Technological and Scientific Development (CNPq) [475212/2011-8 and 306498/2012-0 to A.I.C.]. We thank Sabine Adler (Ruhr Universität Bochum) for all the support and assistance with SEM.

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Table 1. List of studied species and collection/collector data.

Species	Collection Data
<i>Bulbophyllum atropurpureum</i> Barb.Rodr.	SP 12014 (V.L. Gil, M. Sakane & P. Brólio s.n.: Santo André, São Paulo State, Brazil).
<i>B. campos-portoi</i> Brade	SP 6027 (P. Brólio s.n.: Nova Friburgo, Rio de Janeiro State, Brazil). SP 6028 (P. Brólio s.n.: Nova Friburgo, Rio de Janeiro State, Brazil).
<i>B. granulosum</i> Barb.Rodr.	UPCB 67312 (W.S. Mancinelli 1012: Joinville, Santa Catarina State, Brazil).
<i>B. malachadenia</i> Cogn.	SP 2691 (P. Brólio s.n.: Bauru, São Paulo State, Brazil). SP 10225 (M.B. da Silva s.n.: Ilha do Cardoso, São Paulo State, Brazil). UPCB 72468 (W.S. Mancinelli 1396: Adrianópolis, Paraná State, Brazil).
<i>B. napelli</i> Lindl.	UPCB 72452 (W.S. Mancinelli 1367: Campo Alegre, Santa Catarina State, Brazil). E.L.P. Nunes 10: São Miguel Arcanjo, São Paulo State, Brazil.
<i>B. regnellii</i> Rchb.f.	E.C. Smidt 791: Caldas, Minas Gerais State, Brazil.
SP: living collection from the State Orchidarium of Instituto de Botânica de São Paulo; UPCB: Herbarium of Universidade Federal do Paraná.	

Table 2. List of the histochemical tests that were performed on species of *Bulbophyllum* sect. *Napelli* with common results and reaction sites.

Reagent	Reaction	Location
Acidified phloroglucinol	Lignin stains red	Secondary walls of xylem elements
Calcofluor White MR2	Cellulosic cell walls fluoresce bright blue	Idioblasts with helical wall thickenings; epidermis striate wall
Coomassie Brilliant Blue	Proteins stains blue	Cytoplasm of the secretory epithelium of the labellum
Ferric chloride	Phenolic compounds stain dark gray or blue-black	Sepal glandular trichomes - faint
IKI	Starch stains blue-black	Labellum mesophyll starch
Neutral Red <i>in vivo</i>	Lipids/volatile compounds stain red	Sepal glandular trichomes; adaxial surface of the labellum
Neutral Red under UV light	Lipids fluoresce greenish blue	Callus papillae; adaxial surface of the labellum; cuticle
PAS	Polysaccharides stain purplish red	Cell walls; labellum mesophyll starch
Ruthenium Red	Pectic acids stain pink to red.	Sepal glandular trichomes - faint
Sudan III	Lipids stain orange to red	Labellum small drops in the mesophyll cells; cuticle

Table 3. List of the variable characteristics between species of *Bulbophyllum* sect. *Napelli*. States of a characteristic that are exclusive to a single species are marked in bold. Ad: adaxial surface, Ab: abaxial surface, aw: anticlinal walls, pw: periclinal walls.

Floral part	Character	<i>B. malachadenia</i>	<i>B. napelli</i>	<i>B. campos-portoi</i>	<i>B. granulatum</i>	<i>B. atropurpureum</i>	<i>B. regnellii</i>
Dorsal Sepal	Surface ornamentation on pw	longitudinally striate	irregular	granulose	striate	irregular	striate
	Cell shape front view	longitudinally elongated	transversely elongated	irregular	transversely elongated	irregular	transversely elongated
	Surface ornamentation on pw	longitudinally striate	striate	irregular	irregular	irregular	irregular
	Margin cell type	smooth	smooth	smooth	smooth	papillose	smooth
Lateral Sepal	Meso-phyll Vascular Bundles (middle third)	5	4	3	1	3	3
	Surface ornamentation on aw	present	absent	present	present	present	present
	Cell shape front view	transversely elongated	irregular	isodiametric	irregular	irregular	longitudinally elongated
	Meso-phyll Vascular Bundles (middle third)	7	3	3	2	3	2
Petal	Surface ornamentation on aw	present	absent	present	present	present	present
	Outer periclinal wall shape	convex	flat	convex	convex	convex	convex
	Outer periclinal wall shape	convex	flat	convex	convex	convex	convex

Labellum: middle third	Meso- phyll	Vascular Bundles	1	1	1	0	0	1
	Surface ornamentation on pw		striate	smooth	striate	striate	striate	striate
	Surface ornamentation on aw		present	absent	present	present	present	present
Labellum: proximal third	Meso- phyll	Vascular Bundles	7	9	5	7	5	5
	Secretory sulcus shape		deep	shallow	shallow	shallow	shallow	shallow
	Ad	Secretory sulcus periclinal wall	convex	convex	papillose	convex	convex	convex
Gynostem- ium	Surface ornamentation on pw		striate	smooth	striate	striate	striate	striate
	Ab	Surface ornamentation on aw	present	absent	present	present	present	present
	Keel		very conspicuous	inconspicuous	conspicuous	conspicuous	conspicuous	conspicuous
Column Foot	Epider- mis	Surface ornamentation on pw	smooth	striate	striate	striate	striate	striate
	Teeth		elongate	short	short	short	short	inconspicuous
	Outer periclinal wall		convex	convex	convex	convex	papillose	convex
Anther	Epider- mis	Outer periclinal wall	convex	papillose	papillose	papillose	papillose	papillose
Carpel	Stigma	Shape	longitudinally	transversely	transversely	transversely	transversely	transversely
			elongated	elongated	elongated	elongated	elongated	elongated

FIGURES

Figure 1. General floral morphology of the studied species of *Bulbophyllum* sect. *Napelli* in frontal (A-F) and lateral view (G-L). A, G) *B. malachadenia*; B, H) *B. campos-portoi*; C, I) *B. atropurpureum*; D, J) *B. granulosum*; E, K) *B. regnellii*; F, L) *B. napelli*. cf: column foot, ke: keel, st: stelidia, te: teeth. Scale bars =1 mm.

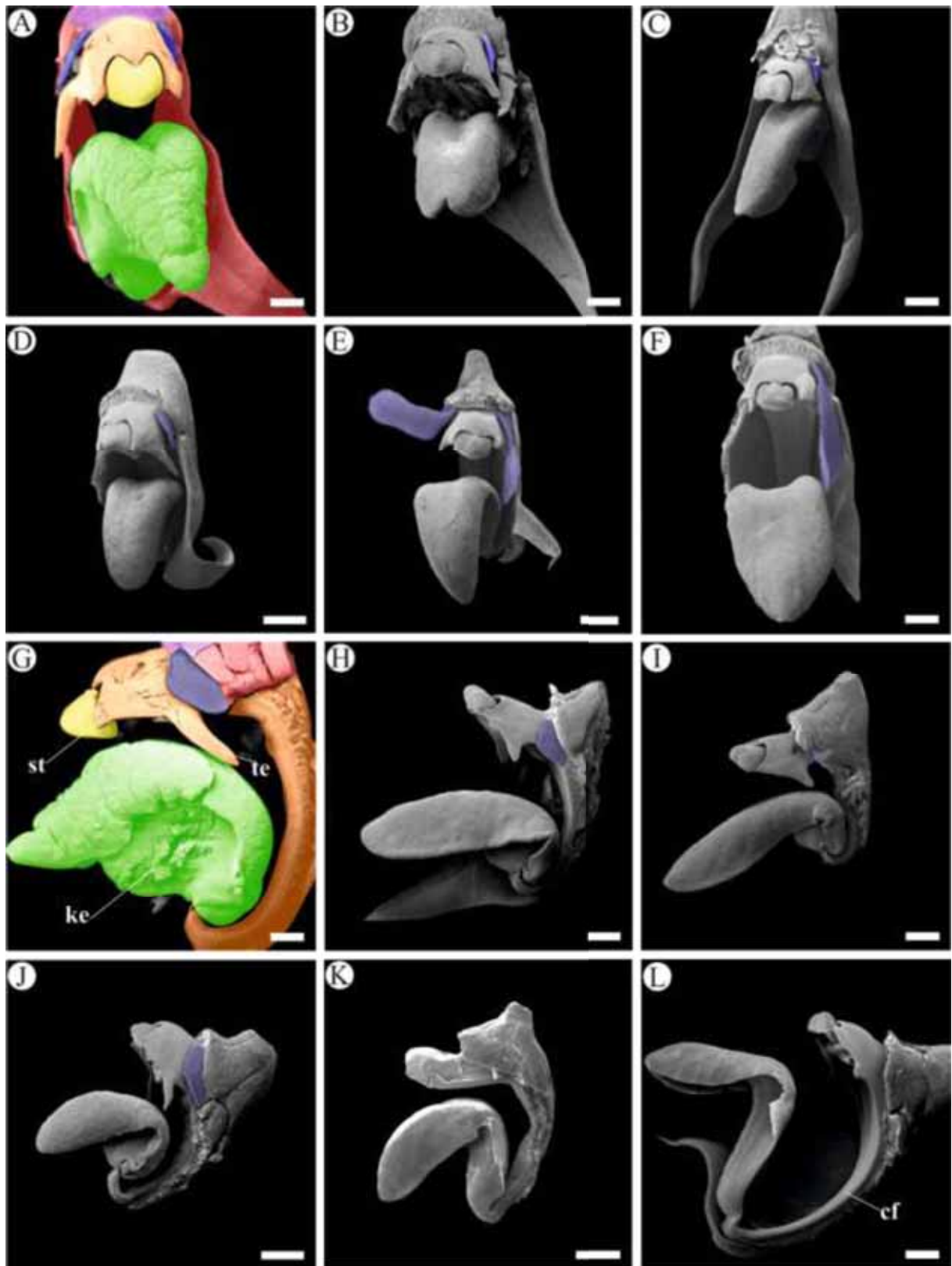


Figure 2. Micromorphology and anatomy of the dorsal sepal of *Bulbophyllum* sect. *Napelli*. Adaxial surface in A-D, K; abaxial surface in G-H, J; transverse sections in E-F, I, L-N. A, G, I, N) *B. malachadenia*; B, F, H, K) *B. atropurpureum*; C, E) *B. campos-portoi*; D, M) *B. regnellii*; J, L, O) *B. granulosum*. ri: idioblast with raphides, ih: idioblast with helical wall thickenings. The circles indicate the vascular bundles. Scale bars = 40 μm in A-D, G-I, K; 200 μm in E, F, J; 50 μm in L; 20 μm in M; 100 μm in N.

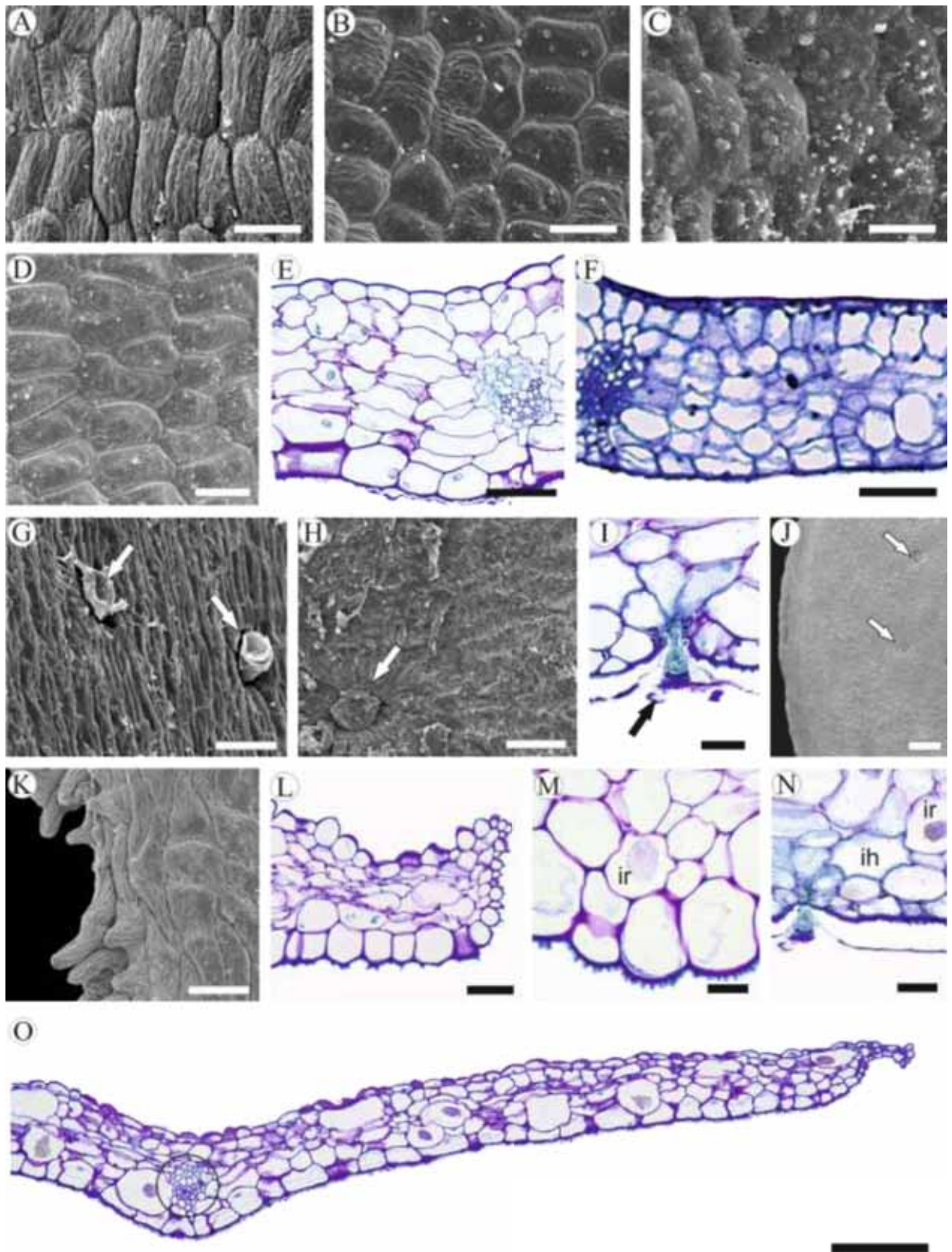


Figure 3. Anatomy and micromorphology of the sepals of *Bulbophyllum* sect. *Napelli*. Dorsal sepal in A-C; lateral sepal in D-H. Adaxial surface in D-E; transverse sections in A-C, F-H. A, E, H) *B. campos-portoi*; B) *B. napelli*; C, F) *B. malachadenia*; D, G) *B. granulosum*. ri: idioblast with raphids, ih: idioblast with helical wall thickenings. The circles indicate the vascular bundles. Scale bars = 500 μm in A-C, H; 40 μm in D, E; 200 μm in F-G.

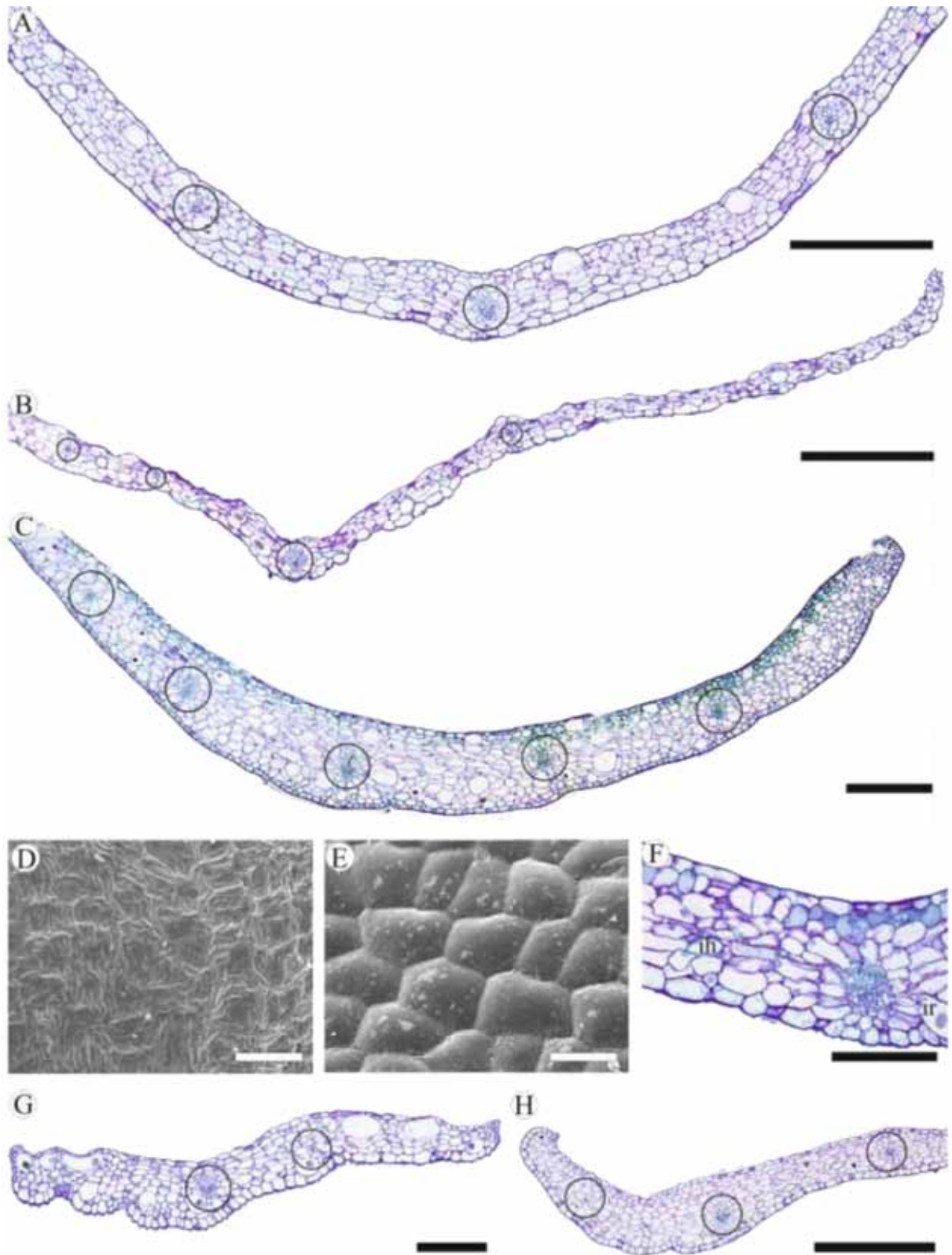


Figure 4. Anatomy and micromorphology of the lateral sepal, petal and labellum of *Bulbophyllum* sect. *Napelli*. Lateral sepal in A; petal in B-G; labellum in H-J. Transverse sections in A, E, G, I; adaxial surface in B, C, F, E, H, J. A, B) *B. malachadenia*; C, F-I) *B. granulosum*; D) *B. regnellii*; E) *B. campos-portoi*; J) *B. atropurpureum*; C: column, pe: petal, si: stigma. The circles indicate the vascular bundles. Scale bars = 500 μm in A, D; 40 μm in B, C, F, H, J; 100 μm in E, K; 200 μm in G.

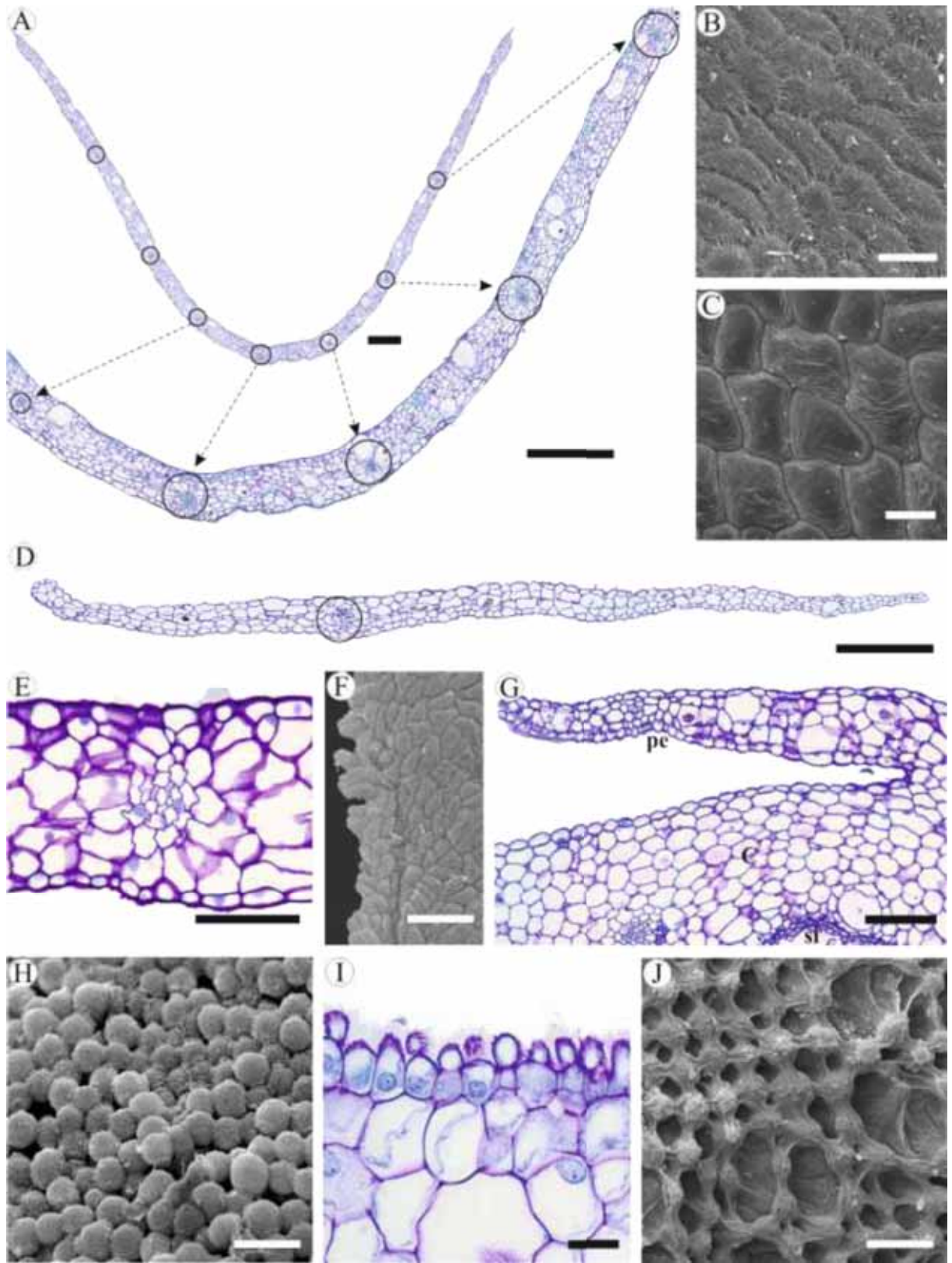


Figure 5. Anatomy and micromorphology of the labellum of *Bulbophyllum* sect. *Napelli*. Median third in A-I; proximal third in J, K. Transverse sections in A, C, G-K; adaxial surface in B; abaxial surface in D-F. A) *B. campos-portoi*; B, C, F, H, K) *B. malachadenia*; D, G) *B. atropurpureum*; E, I) *B. napelli*; J) *B. regnellii*. The circles indicate the vascular bundles. Scale bars = 100 μm in A, E; 40 μm in B-D, F; 500 μm in G, I-K; 250 μm in H.

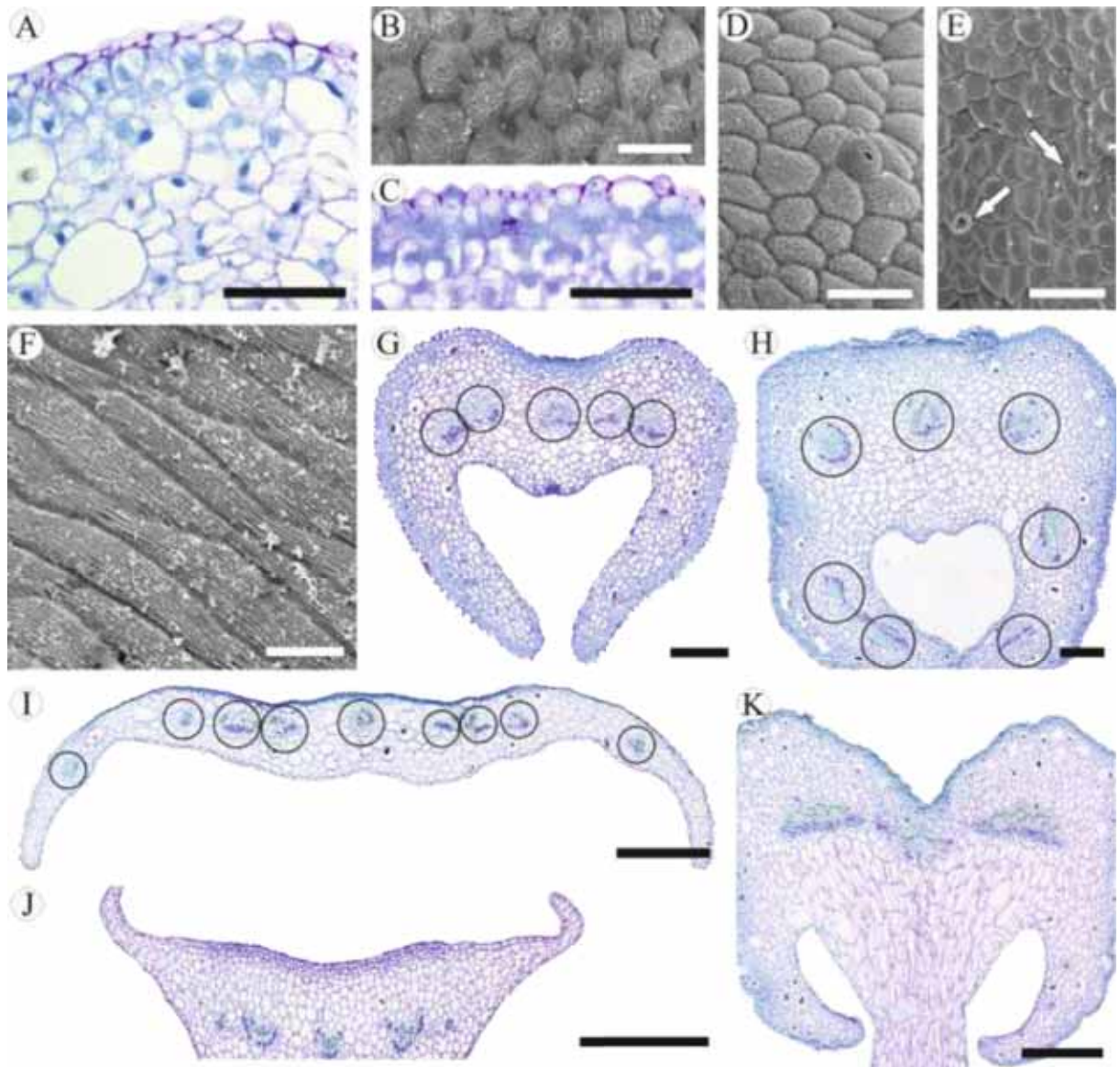


Figure 6. Micromorphology and anatomy of the labellum and column of *Bulbophyllum* sect. *Napelli*. Adaxial surface in A, B, H, I, K, L; abaxial surface in D, E; transverse sections in C, F, G; longitudinal section in J. A, I) *B. malachadenia*; B, D, J) *B. napelli*; C, E) *B. campos-portoi*; F, L) *B. atropurpureum*; G) *B. granulosum*; H, K) *B. regnellii*. an: anther, cf: column foot, ih: idioblast with helical wall thickenings, ov: ovary, sd: dorsal sepal, si: stigma, vi: viscidium. Scale bars = 40 μm in A, B, H, I, K, L; 100 μm in C-F; 200 μm in G; 500 μm in J.

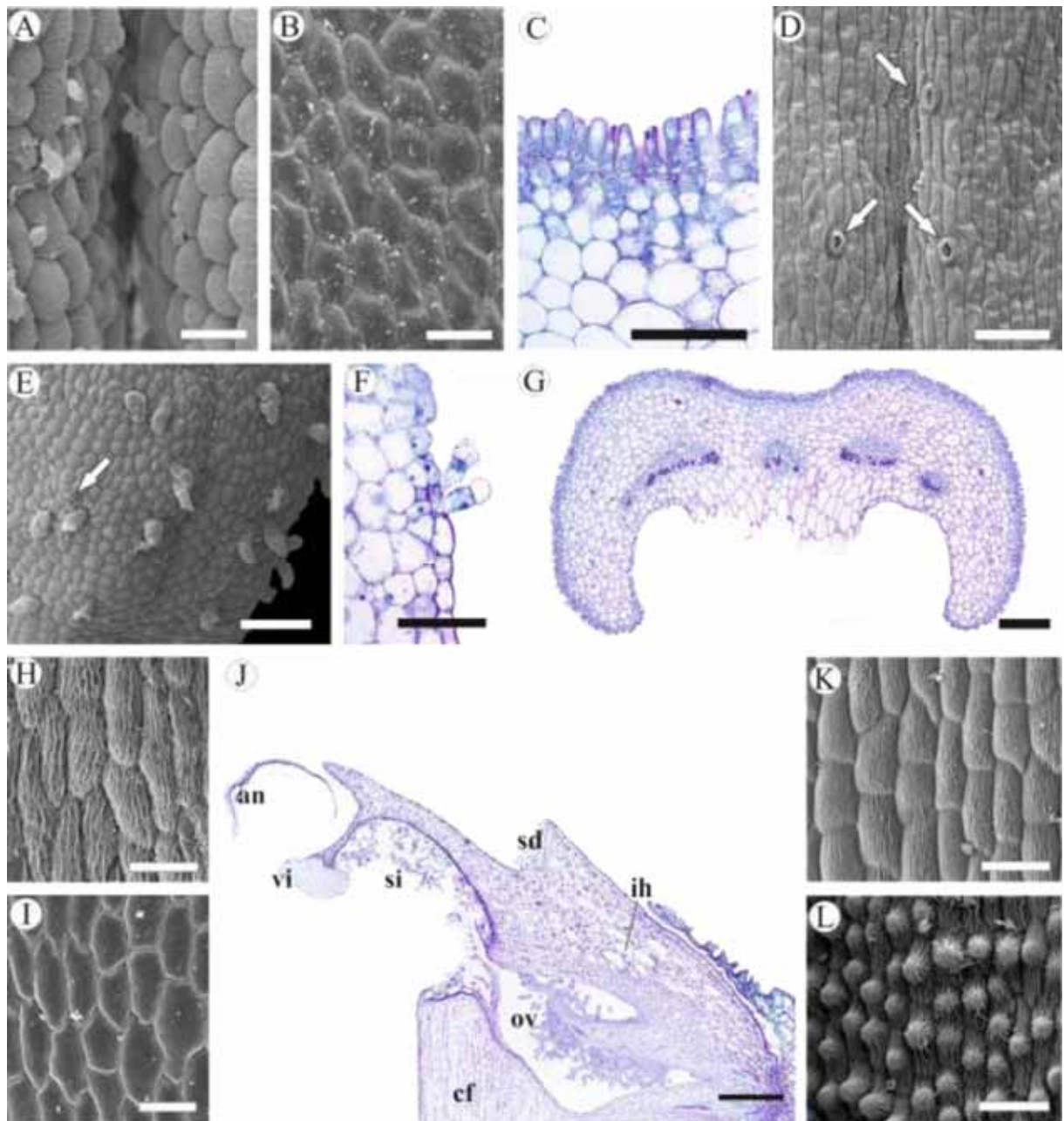
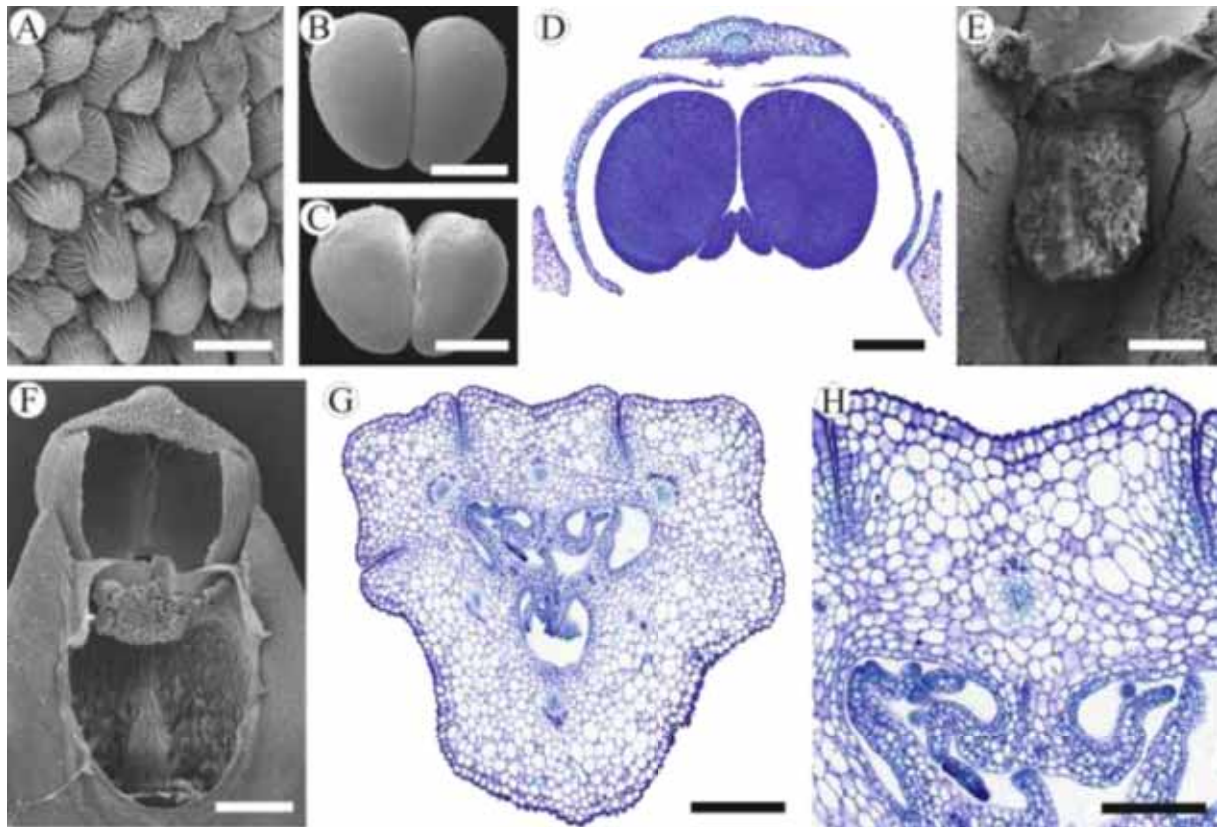


Figure 7. Micromorphology of anther and stigma and anatomy of pollinium and ovary of column of *Bulbophyllum* sect. *Napelli*. Dorsal view in A, B; Ventral view in C, E, F; transverse sections in D, G, H. A, G, H) *B. granulosum*; B, D) *B. atropurpureum*; C, F) *B. campos-portoi*; E) *B. malachadenia*. Scale Bars: 40 μm in A; 400 μm in B, C; 500 μm in D-G; 200 μm in H.



CAPÍTULO III

**Floral micromorphological and anatomical features in Neotropical
Bulbophyllum sect. *Micranthae* (Orchidaceae: Bulbophyllinae) and their
systematic value**

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Alessandra Ike Coan**

(A ser submetido ao periódico *Botanical Journal of the Linnean Society*)

ABSTRACT

Bulbophyllum sect. *Micranthae* comprises 12 species distributed in central South America showing problems in specific differentiation due to high similarity between species. Floral anatomical and micromorphological features of eight species belonging to this section were analysed using conventional techniques to verify: which are common to the representatives of *B.* sect. *Micranthae*; which distinguish between these species; and which may be related to their habitat and pollination. All species show homogeneity in 55 characteristics, the most relevant being: glabrous sepals with sunken glandular trichomes; petals with a single vascular bundle; labellum with central differentiated areas (sulcus and keel); anther with striate and papillose epidermis; pollinarium with four pollinia; ovary with the outer epidermal cells square and inner elliptic and with convex outer periclinal walls; mesophyll with raphide idioblasts in all floral parts. Another 55 characteristics were variable and allowed specific floral characterization, and also the differentiation between *B. epiphytum* and *B. rupicolum*, species similar in morphology and phylogenetically related. In addition, we discuss the positioning of *B. mentosum*: although its inclusion as sister group to the remaining of the species is supported by molecular data, the floral anatomical and micromorphological data presented here do not support this view.

KEY WORDS: *Bulbophyllum adiamantinum*, *B. chloroglossum*, *B. epiphytum*, *B. insectiferum*, *B. mentosum*, *B. micranthum*, *B. mucronifolium*, *B. rupicolum*, nectary, osmophore.

INTRODUCTION

In spite of *Bulbophyllum* Thouars representativeness, the largest genus of Orchidaceae (Frodin, 2004) with 1877 species (WCSP, 2014), little is still known on their floral morphology and anatomy. The first report dealt with gynostemium development and morphology of the Asian *B. ecornutum* (Rasmussen, 1985). Teixeira, Borba & Semir (2004) reported the labellum anatomy of one Asian and six Brazilian species of *Bulbophyllum* and its implication for pollination biology. Those authors suggest a link between the need of air currents to effective pollination and nectar presence in some species and also that labellum structure is homogeneous and highly conservative for the genus.

More recently, Nunes *et al.* (2014) studied the floral micromorphology and anatomy of the Neotropical *Bulbophyllum* sect. *Didactyle*, investigating features that could be used to delimitate and distinguish between species. Besides many useful characteristics, they found out a greater diversity on labellum micromorphology than previously anticipated, such as epidermal cell shape and secretory sulcus size.

In the Neotropics, six sections of *Bulbophyllum* were recognised based on morphological and molecular data (Smidt *et al.*, 2011): *B.* sect. *Xiphizusa* (Rchb.f.) Cogn., *B.* sect. *Didactyle* (Lindl.) Cogn., *B.* sect. *Napelli* Rchb.f., *B.* sect. *Furvescens* E.C. Smidt, Borba & Van den Berg, *B.* sect. *Bulbophyllaria* (Rchb.f.) Griseb., and *B.* sect. *Micranthae* Barb.Rodr. The latter comprises 12 species distributed in the central South America, mainly Brazil, most of them known for its occurrence in Cerrado Vegetation and “Campos Rupestres” (rocky outcrops), rupicolous habit and high endemism (Smidt *et al.*, 2011). The molecular analysis of Smidt *et al.* (2011), combining two plastid DNA regions and one nuclear, revealed three clades in *B.* sect. *Micranthae*: the first comprises *B. mentosum* Barb.Rodr., the sister group to the remaining species; the second one is a highly supported and basalmost clade composed by *B. adiamantinum* Brade and *B. insectiferum* Barb.Rodr.; and a third clade representing the section core (*B. chloroglossum* Rchb.f. & Warm., *B. epiphytum* Barb.Rodr., *B. macroceras* Barb.Rodr., *B. micranthum* Barb.Rodr., *B. mucronifolium* Rchb.f. & Warm., *B. pitengoense* Campacci, *B. rupicolum* Barb.Rodr., *B. tricolor* L.B.Sm. & S.K.Harris, and *B. uhlgabrielianum* Chiron & V.P.Castro). This latter clade grouped species of difficult distinction that possess white flowers and yellow labella (Smidt *et al.*, 2011).

Smidt, Gallo & Scatena (2013) studied leaf anatomical characters and compared them to a molecular dataset to improve the understanding of the relationships within *Bulbophyllum* sect. *Micranthae*. Although their data gave a better insight on the relationship between species and revealed some apomorphies, much remains to be solved and more data are needed to better distinguish species.

Therefore, the aims of the present study were to describe the floral micromorphology and anatomy of *Bulbophyllum* sect. *Micranthae*, answering the following questions: a) which micromorphological and anatomical floral features are common to the representatives of *B.* sect. *Micranthae*? b) which micromorphological and anatomical floral features distinguish between the species of this section? c) are there characters related to the habit, habitat and pollination of the representatives of this section?

MATERIAL AND METHODS

Inflorescences of seven species representing *Bulbophyllum* sect. *Micranthae* were collected on natural populations and were analysed by light microscopy (LM) and scanning electron microscopy (SEM) for character assessment (Table 1). At least four flowers from different specimens were analysed for each species, whenever possible, sourced from different localities (Table 1).

One additional species (*B. adiamantinum*) was also analysed, but few samples were available, resulting in poor anatomical results. We chose to keep those results because *B. adiamantinum* is a micro endemic species that was recovered as sister group of *B. insectiferum* in all available analyses, both molecular and morphological (Smidt *et al.*, 2011; Smidt *et al.*, 2013).

Flowers were fixed either in FAA 50 (3.7% v/v formaldehyde, 50% ethanol, 5% acetic acid; Johansen, 1940) or 2.5% glutaraldehyde and 2% formaldehyde in phosphate buffer 0.1 M, pH 7.2 (Karnovsky, 1965).

The material for LM analysis were embedded in historesin Leica (Leica Microsystems GmbH, Heidelberg, Germany) or Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim, Germany), according to the instructions of the suppliers and sectioned in a rotary microtome RM 2245 (Leica Microsystems GmbH, Wetzlar, Germany) from 5 to 7 µm with disposable blades. The sections were stained with 0.05% Toluidine Blue O in 0.1 M sodium phosphate buffer pH 6.8 (O'Brien, Feder & McCully, 1965) for general analysis.

The following histochemical tests were carried out on resin-embedded sections: Coomassie Brilliant Blue for proteins (Southworth, 1973), Calcofluor White MR2 for cellulose (Hughes & McCully, 1975), and Periodic Acid-Schiff reaction for total polysaccharides (Feder & O'Brien, 1968). Autofluorescence was observed on a DMLB microscope (Leica Microsystems GmbH, Wetzlar, Germany) using filter cubes D, N2.1 and I3.

Tests with iodine/potassium iodide solution (IKI) for starch (Johansen, 1940), Sudan III for lipids (O'Brien & McCully, 1981), Neutral Red under UV excitation for total lipids (Kirk, 1970), and Ruthenium Red for pectic acids/mucilage (Johansen, 1940) were performed with fresh material, hand sectioned with razor blades.

Photomicrographies were obtained through a Leica DMLB microscope coupled with a digital camera and using the software LAS (Leica Application Suite V3.3.0).

For SEM analysis, the samples were transferred from 70% ethanol to formaldehyde-dimethylacetal for 16 to 24h (Gerstberger & Leins, 1978), then dehydrated through a critical

point drier (CPD 030 - Bal-Tec AG, Balzers, Liechtenstein), and sputter coated with gold (SCD 050 - Bal-Tec AG, Balzers, Liechtenstein). SEM observations were made in a Zeiss DSM 950 (Carl Zeiss, Jena, Germany) and images were acquired through Digital Image Processing Software 2.2 (DIPS-Leipzig, Germany). The exceptions were *B. chloroglossum*, *B. mucronifolium* and *B. insectiferum* whose better results were obtained through slow ethanol dehydration (the remaining of the processing was similar to that previously described). Observations of *B. mucronifolium* and *B. insectiferum* were made on a TM3000 (Hitachi, Tokyo, Japan) SEM and the images were acquired through its software.

RESULTS

The flowers of *Bulbophyllum* sect. *Micranthae* are pedicellate (Fig. 1A), with free sepals that are usually erect (Fig. 1A–G), except the lateral ones of *B. micranthum* which are erect-patent (Fig. 1H). The labellum is fleshy (Fig. 1B–O), not differentiated in hypochile and epichile, usually trilobed with dentiform lateral lobes (Fig. 1K, M–O) - except for *B. mentosum* (Fig. 1I), *B. insectiferum* (Fig. 1J), and *B. mucronifolium* (Fig. 1L) whose lobes are inconspicuous or absent. The column has short stelia (Fig. 1D–H, K–O) - except for *B. mentosum* (Fig. 1B, I) and *B. insectiferum* (Fig. 1C, J) where they are longer - and inconspicuous teeth (Fig. 1I–O); the column foot is usually longer than the column itself (Fig. 1I–O).

The dorsal sepal of the species studied, on the adaxial surface, usually do not show a conspicuous ornamentation (Fig. 2A), but it is reticulate in *B. adiamantinum* and *B. insectiferum* (Fig. 2B) or granulose in *B. micranthum* (Fig. 2C). In frontal view, the epidermal cell shape vary between transversely elongate (Fig. 2A), longitudinally elongate (Fig. 2B), or irregular-shaped (Fig. 2C, D). In transverse section, the outer periclinal wall is convex and thin-walled (Fig. 2E), except for *B. epiphytum*; *B. insectiferum* and *B. mentosum* which show thicker outer periclinal walls (Fig. 2F–H). In *B. mentosum* (Fig. 2A, F, G) and *B. epiphytum*, there are also some papillose cells. On the abaxial surface, the ornamentation is similar to that of the adaxial surface or is striate (Fig. 2I) and the epidermal cells are similar to those of the adaxial surface; the exception is *B. mentosum* in which these cells are more rectangular and epicuticular wax is present (Fig. 2J). In transverse sections, the outer periclinal wall is convex and thicker than those of the adaxial surface (Fig. 2E–G, I). All species have sunken multicellular glandular trichomes that react positively to an *in vivo* test for osmophore

localization (Fig. 2K, L); stomata were also observed on the abaxial surface of the dorsal sepal (Fig. 2K), except for *B. mentosum*, *B. rupicolum* and *B. micranthum*.

The margin of the dorsal sepal is smooth (Fig. 2J) and consists of both epidermal and mesophyll cells (Fig. 2E, G). The mesophyll is homogeneous (Fig. 2E) (with more cell layers than the other species in *B. mentosum* - Fig. 2F, G), with regular parenchyma and two types of idioblasts (Fig. 2E–G): one with helical wall thickenings (Fig. 2E, F) (absent in *B. insectiferum*) and the other with raphides (Fig. 2G). In *B. epiphytum*, in addition to the two aforementioned idioblast types, mixed idioblasts (with both helical wall thickenings and raphides) were occasionally observed (Fig. 2M). The helical wall thickenings seem to be lignified, comparing the results of Calcofluor White MR2 test (Fig. 2N) to the intrinsic fluorescence of them (Fig. 2O). At the middle third of the dorsal sepal, there is a single collateral vascular bundle (Fig. 2E), except for *B. mentosum* which has three (Fig. 2G).

The lateral sepals show similar characteristics to the dorsal one, although they may vary between species (refer to Table 3 for differences). At the middle third of each lateral sepal, there are three collateral vascular bundles (Fig. 2P), except for *B. rupicolum* which has only one (Fig. 2Q).

The petals are clawed only in *B. adiamantinum* (Fig. 3A) and *B. insectiferum*. The adaxial surface shows an ornamentation that vary between smooth (Fig. 3B), reticulate (Fig. 3C) or striate (Fig. 3D), while the epidermal cell shape vary from longitudinally elongate (Fig. 3B, C) to irregular-shaped (Fig. 3D, E) in frontal view. The outer periclinal walls of those cells are usually convex (Fig. 3C, D, F), but in *B. mentosum* they are papillose (Fig. 3B, G) and in *B. epiphytum* there are both convex and papillose cells (Fig. 3E, H). The abaxial surface is more or less similar to the adaxial one, with some variation in ornamentation and epidermal cells shape (see Table 3 for differences). The margin of the petals is smooth (Fig. 3I), except for *B. mentosum* that shows long unicellular trichomes (Fig. 3J); in transverse sections, the margin is composed both by epidermal and mesophyll cells (Fig. 3F–H), except for *B. adiamantinum* and *B. insectiferum* (Fig. 3K) where it is composed by epidermal cells only. The mesophyll is homogeneous with both idioblasts with raphides and with helical wall thickenings (Fig. 3F–H); the latter are absent in *B. insectiferum* (Fig. 3K). There is a single collateral bundle in all species (Fig. 3F–H, K).

The labellum is shorter in *B. mentosum* (Fig. 4A), *B. adiamantinum* (Fig. 4B) and *B. insectiferum* (Fig. 4C) when compared to the remaining species (Fig. 4D–H). Five species show two lateral lobes at the proximal portion of the labellum (Fig. 4B, D, F–H).

On its distal portion, the adaxial surface of the labellum either does not show an obvious ornamentation (Fig. 4I) or it is striate (Fig. 4J). The epidermal cells are papillose (Fig. 4I) or scale-like (Fig. 4J) in frontal view. At the abaxial surface of this portion of the labellum, the ornamentation is similar to that of the adaxial surface and epidermal cells are also papillose (Fig. 4K), varying in the prominence of their outer periclinal wall (Fig. 5A–C). In *B. rupicolum*, however, the outer periclinal wall is convex (Fig. 5D). The margin is clothed with similar cells to those of the adaxial surface, except for *B. mentosum*, that have both papillae and long unicellular trichomes (Fig. 4A), and *B. mucronifolium*, that have both papillae and short unicellular trichomes (Fig. 4E). The mesophyll is homogeneous, with regular parenchyma and idioblasts with raphides (Figs 4K, 5D–F); idioblasts with helical wall thickenings are present only in *B. chloroglossum* and *B. micranthum*. The vascular bundles are collateral and vary between two (Figs 4K, 5D) or three (Fig. 5E); in *B. mentosum* the vascular bundles do not reach the labellum apex (Fig. 5F). Stomata were observed only in *B. mucronifolium* (Fig. 5G) at this portion.

The adaxial surface of the proximal third of the labellum shows similar epidermal cells and surfaces to those of the distal portion. However, this region shows, along the labellum length, a differentiated central region (Figs 4A–H, 5H), that may be below the level of the remaining cells (usually shallow; but deep in *B. adiamantinum* - Fig. 4B) or more or less elevated (Fig. 4A). The epidermal cells of the sulcus are scale-like papillae (Fig. 5I) – except for *B. rupicolum* (Fig. 5J) that have convex epidermal cells – and usually striate (Fig. 5H). The margin is clothed with similar cells to those of the adaxial surface, except for *B. mentosum*, that have both papillae and long unicellular trichomes (Fig. 4A), and *B. mucronifolium*, that have both papillae and short unicellular trichomes (Fig. 4E). The abaxial surface of the proximal portion of the labellum has similar cells to those of the distal portion (Fig. 5K–M). A differentiated keel occurs in the middle-line, where cells may be more papillose and some scattered stomata are observed (Fig. 5K, L), except for *B. adiamantinum* where stomata are lacking (Fig. 5M). The mesophyll and vascular bundles are similar to that described to the remaining of the labellum, but *B. insectiferum* shows parenchymatic cells more plicate surrounding the vascular bundles (Fig. 6A); the bundles vary between three (Figs 5J, 6B–D), five (Fig. 6A) or eleven (Fig. 6E, F).

The lateral lobes of the labellum are usually erect and dentiform (Fig. 4D, F–H), but are patent and ear-shaped in *B. adiamantinum* (Fig. 4B). The adaxial and abaxial epidermal cells of the lateral lobes are longitudinally elongate (Fig. 6G). The margin of the lobes of *B. adiamantinum* show papillose cells (Fig. 6H).

In the entire labellum, *B. rupicolum* shows a notably thick cuticle when compared to the other species (Fig. 5D, J), and the ornamentation is due to cuticle striate pattern, and not caused by the outer periclinal cell wall striation (Fig. 5D - detail).

The gynostemium surface either lacks conspicuous ornamentation (Fig. 6I) or shows a striate pattern (Fig. 6J); the epidermal cells, in frontal view, are longitudinally elongate and convex (Fig. 6I, J), but slightly papillose in *B. insectiferum* (Fig. 7A). The column foot usually shows striate ornamentation on its surface (Fig. 7B, C), but in *B. mentosum* (Fig. 7D) and *B. adiamantinum* it is smooth. In *B. insectiferum*, the epidermal cells of the column foot are papillae or unicellular trichomes (Fig. 7B, C). The mesophyll of the column is homogeneous, with regular parenchyma containing idioblasts with raphides and helical wall thickenings (Fig. 7E–G).

The anther is papillose and its irregularly shaped epidermal cells have striate ornamentation (Fig. 7H). The pollinarium is composed of four pollinia with smooth tetrads (the central two pollinia being smaller than the outer pollinia; Fig. 7I, J) and a viscidium formed by the disorganization of rostellar cells (Fig. 7E).

The stigma cavity is longitudinally elongate in *B. chloroglossum* and *B. epiphytum* (Fig. 7K) and wide on the remaining species (Fig. 7L). The ovary is a complex, six-lobed structure, the lobes of which are arranged in two alternating whorls of three (Fig. 7M). The three outer lobes occur along the same radius as the sepals, and the three inner lobes occur along the same radius as the petals and carpels. In transverse section, the outer epidermal cells of the ovary are square to rectangular in shape, with convex and ornamented outer periclinal walls (Fig. 7N). The inner epidermal cells are elliptic with convex outer walls (Fig. 7N). The ovary mesophyll is homogeneous and formed by regular parenchyma (Fig. 7M, N) containing idioblasts with raphides (except for *B. chloroglossum*) and others having helical wall thickenings (except for *B. mentosum*, *B. insectiferum* and *B. micranthum*). Each lobe of the ovary contains a single collateral vascular bundle (Fig. 7M).

Histochemical tests were performed on all species, and the results are summarized in Table 2. The characteristics that allow distinction between the species are summarized in Table 3.

DISCUSSION

In the present study, ca. 110 floral micromorphological and anatomical characteristics were analysed for eight out of twelve species of *B. sect. Micranthae* and from those 55 were

constant for all species. From the micromorphological and anatomical perspective, the flowers of *Bulbophyllum* sect. *Micranthae* can be characterised as follows: sepals with glabrous margin, covered with an epidermis with convex outer periclinal walls and showing sunken multicellular glandular trichomes on the abaxial surface; petals with a single collateral vascular bundle; labellum with a central differentiated area on both adaxial (sulcus) and abaxial (keel) surfaces of the proximal portion; gynostemium with longitudinally elongate epidermal cells; column foot with longitudinally elongate epidermal cells; anther without a distinctive beak, but with striate, papillose epidermal cells; pollinarium composed by four pollinia with smooth tetrads; ovary with the outer epidermal cells square and the inner elliptic, besides convex outer periclinal walls; homogeneous mesophyll with idioblasts with raphides in all floral parts.

All species studied here showed sunken glandular trichomes on the abaxial surfaces of the sepals. Such trichomes were also found in species belonging to *B.* sect. *Didactyle* (Nunes *et al.*, 2014) and *B.* sect. *Napelli* (Nunes *et al.*, submitted – Capítulo II), and in the former, Nunes *et al.* (2014) suggested a possible osmophore function. Here, we found some indicatives of this function (positive result for neutral red *in vivo* test, prominent nucleus in the basal cell), although the histochemical tests were not conclusive. Verola (2002), when studying the reproductive biology of some *Bulbophyllum* species, reported sweet and citric odours for three species of this section (*B. adiamantinum*, *B. insectiferum* and *B. epiphytum*), but no indication of the origin of the odour could be found. Besides the sepal sunken glandular trichomes, the sulcus and some papillae of the adaxial surface of the labellum also gave positive reaction to the neutral red test, meaning that they are also metabolic active.

Based on histochemical tests that revealed high metabolic activity (Neutral red, Coomassie Blue) and the large size of the nucleus of the epidermal cells of the labellum sulcus, most species studied here show clear secretory characteristics. Considering these findings and that no other secretory sites were found in other floral parts, together with the report of nectar presence for the three species studied by Verola (2002), the labellum sulcus are most likely the nectary. Teixeira *et al.*, (2004), on the other hand, did not find histological evidence for a nectary on the labellum of *B. epiphytum*, in spite of having observed nectar. Based on that, those authors suggested that the nectar might be produced in other floral organs, hypothesis that is not corroborated by our data.

Idioblasts with raphides were found in all organs of nearly all species studied here. Such idioblasts are largely known for Orchidaceae (Stern *et al.*, 1993; Prychid & Rudall, 1999; Kocyan & Endress, 2001; Stern & Judd, 2001; Ascensão *et al.*, 2005), and have also

been reported for the labellum of species of *Bulbophyllum* (Teixeira *et al.*, 2004). Their primary function is mechanical and chemical protection against herbivory (Hartmann, 2008; Konno, Inoue & Nakamura, 2014), although Nunes *et al.* (submitted – Capítulo II) have also suggested some participation in pollinator attraction in species belonging to *B. sect. Napelli*. A second type of idioblasts of common occurrence for Orchidaceae, including *Bulbophyllum* flowers, was also recorded here (Olatunji & Nengim, 1980; Pridgeon, 1981, 1982; Nunes *et al.*, 2014). These idioblasts possess helical wall thickenings and are traditionally associated with epiphytic Orchidaceae and hydric stress (Burr & Barthlott, 1991; Olatunji & Nengim, 1980) and although the species belonging to *Bulbophyllum* sect. *Micranthae* are more commonly rupicolous, they occur in open dry areas. Differently from *B. sect. Didactyle*, the species studied here showed some secondary deposition of some type lignin as shown by histochemical tests and autofluorescence. And it is interesting to note that there were differences on the fluorescence spectra emitted by *B. mentosum* and the other species studied here (personal observation).

It is noteworthy that, considering the current circumscription of *B. sect. Micranthae*, so many floral characters are variable and thus useful to better distinguish between species. Compared to the other Neotropical sections of *Bulbophyllum* to which similar data are available, *B. sect. Micranthae* showed much more variation (55 characteristics with variable states), once *B. sect. Didactyle* (Nunes *et al.*, 2014) and *B. sect. Napelli* (Nunes *et al.*, submitted – Capítulo II) showed less than 30.

The species that showed the highest number of exclusive characteristics was *B. mentosum* (14), the basalmost in the section (*sensu* Smidt *et al.*, 2011). Amongst these characteristics, the noteworthy are: three vascular bundles in the middle third of the dorsal sepal; papillose epidermal cells on the petals; unicellular trichomes on the margin of the petals; smooth epidermal cells; and margin with unicellular trichomes in the labellum. Smidt *et al.* (2011), when building their phylogeny, found incongruence between molecular datasets (plastid DNA data suggests the insertion of this species in *B. sect. Xiphizusa*, while ribosomal nuclear spacer suggests its insertion in *B. sect. Micranthae*), so that *B. mentosum* had to be excluded from the combined analysis. Besides the higher differentiation of this species found in the present study, *B. mentosum* also show other morphological characters considered unique by Smidt *et al.* (2011): ribbon-like scape (cylindrical in all the other species), some vegetative characters similar to *B. sect. Didactyle* and others, such as fleshy leaves, similar to *B. sect. Micranthae*. On the other hand, floral morphological aspects, like spirally flowers arrangement, short pedicel and entire lip, without lateral lobes (only the margin erect,

flattened and ciliated), are similar to those of *B. sect. Furvescens*, as noted by Smidt *et al.* (2011). Interestingly, on Nunes *et al.* (in prep. – Capítulo VI) a morphological based phylogeny shows *B. mentosum* as sister to a group including (*B. sect Furvescens* + *B. sect. Bulbophyllaria* (*B. sect. Napelli* + *B. sect. Didactyle*)). Currently, there are no published information on the floral anatomy and micromorphology of *B. sect. Furvescens* and *B. sect. Bulbophyllaria* but previous results (Nunes *et al.*, in prep. – Capítulo V) show some remarkable similarities between *B. mentosum* and species belonging those sections.

Still regarding *B. mentosum*, it should be noted that no information on its pollination biology is available and, since pollinator specialization in Angiosperms (van der Niet & Johnson, 2012), especially in Orchidaceae, is a major factor in species diversification (Kay & Sargent, 2009; Schiestl & Schüter, 2009), it could explain the differences found between this species and the remaining species of *B. sect. Micranthae*. Another factor that cannot yet be ruled out as responsible for *B. mentosum* differentiation is the occupation of a more xeric habitat. According to Smidt (2007), this is a rupicolous species that occurs in “campos rupestres” and in interfaces between them and Caatinga, probably being endemic to Chapada Diamantina (Bahia State, Brazil) and the only *Bulbophyllum* species occurring in the Caatinga.

A pair of species that has called attention on the available analyses, by forming a well-supported clade and showing some genetic identity (Azevedo *et al.*, 2007; Smidt *et al.*, 2011, 2013), is *B. adiamantinum* and *B. insectiferum*. Our data offer additional support to those previous findings, even though we do not have the complete anatomical information on *B. adiamantinum*, both species share at least 32 characteristics (from the 55 variable between all species), six of them being exclusive to this pair of species (reticulate ornamentation on the dorsal sepal; reticulate ornamentation on the lateral sepals; reticulate ornamentation on the petals; clawed insertion of the petals; margin of the petal formed by epidermal cells only; and unicellular trichomes on the column foot). It is interesting to note that from those 32 shared characteristics, 12 are also shared with *B. mentosum*, although only two of them (smooth ornamentation on the adaxial surface of the labellum and elongate stelidia) are exclusive to those three species.

The *core* of *B. sect. Micranthae* comprises nine species of difficult distinction (Smidt *et al.*, 2011) and we analysed five of them, which were homogeneous regarding 19 characteristics, three of them being exclusive to this group (striate ornamentation on the adaxial surface of the labellum; three vascular bundles on the proximal portion of the labellum; and short stelidia). From these 19 homogeneous characteristics, five were shared

with *B. adiamantinum* and *B. insectiferum* (one vascular bundle on the dorsal sepal; flat margin on the petals; striate ornamentation on the abaxial surface of the distal portion of the labellum; and striate ornamentation on both surfaces of the proximal portion of the labellum), while seven were shared with *B. mentosum*. In addition, 34 characteristics were variable between the five studied species belonging to *B. sect. Micranthae core*, thus allowing a better distinction between them.

Embedded within the section *core* is a pair of species that deserve attention: *B. epiphytum* and *B. rupicolum*. They are closely related and distinguished solely by their habit, shape of pseudobulb, flower size and some labellum characteristics (Azevedo *et al.*, 2007; Smidt, 2007; Smidt *et al.*, 2011). We found that those species differ on 19 states (including some of more gross morphology as stigma shape) of the 55 variable characters, and even though they seem to be phylogenetically close and introgression has been suggested (Azevedo *et al.*, 2007), we were able to discriminate between them. It is interesting to note that *B. epiphytum* showed a nectary on the labellum (this study; Verola, 2002), while *B. rupicolum* do not seem to secrete nectar, based on anatomical and histochemical evidence. Since those two species are more or less difficult to discriminate, the former offers nectar and shows the widest distribution within this section (Barros *et al.*, 2014) while the latter does not offer nectar and show a more restrict distribution (Barros *et al.*, 2014) and their distribution can overlap (Smidt, 2007), it seems tempting to speculate on the possibility of mimicry.

Batesian mimicry in plants refers to non-rewarding species with flowers that imitate those of rewarding species (Johnson, 1994; Gigord *et al.*, 2002; Johnson, Alexandersson & Linder, 2003). On the other hand, keeping in mind that mimicry can be defined as ‘adaptive resemblance’ (Starret, 1993) and that characteristics present merely by common descent cannot be described as adaptations to current ecological conditions (Gould & Vrba, 1982; Coddington, 1988), mimicry does not seem to occur with *B. epiphytum* and *B. rupicolum*. It seems more likely that stabilizing selection may maintain these closely related species with similar floral morphology (Gould & Vrba, 1982; Coddington, 1988). Since no data is currently available on the pollination biology of *B. rupicolum* and population genetic information is scarce, such kind of studies are essential to understand the relationship and processes underlying these two species.

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Table 1. List of species of *Bulbophyllum* sect. *Micranthae* studied and collection data.

Species	Collector data	Collection site
<i>B. adiamantinum</i> Brade	E. C. Smidt 802	Joaquim Felício – MG
<i>B. chloroglossum</i> Rchb.f.	W. S. Mancinelli 1129	Sengés – PR
<i>B. epiphytum</i> Barb.Rodr.	E. C. Smidt et al. s.n.	Caldas – MG
<i>B. insectiferum</i> Barb.Rodr.	T. M. Rodrigues s.n.	Carajás – PA
<i>B. mentosum</i> Barb.Rodr.	A. Massenssini Jr s.n.	Palmeiras – BA
<i>B. micranthum</i> Barb.Rodr.	W. S. Mancinelli 1172	Tibagi – PR
<i>B. mucronifolium</i> Rchb.f. & Warm.	A. I. Coan et al. 155	São Gonçalo do Rio das Pedras – MG
<i>B. rupicolum</i> Barb.Rodr.	E. C. Smidt 741, 766	Caldas – MG

Table 2. List of histochemical tests performed on species of *Bulbophyllum* sect. *Micranthae* with common results and reaction sites.

Reagent	Reaction	Location
Acidified phloroglucinol	Lignin stains red	Secondary walls of xylem elements
Calcofluor White MR2	Cellulosic cell walls fluoresce bright blue	Idioblasts with helical wall thickenings; mesophyll cell walls
Coomassie Brilliant Blue	Proteins stains blue	Cytoplasmic proteins
Intrinsic fluorescence under UV light	Suberin and lignin fluoresce blue or green Cutin fluoresce blue or white	Secondary walls of xylem elements; idioblasts with helical wall thickenings; cuticle
IKI	Starch stains blue-black	Labellum mesophyll starch
Neutral Red <i>in vivo</i>	Lipids/volatile compounds stain red	Sepal glandular trichomes; labellum scale-like papillae (sulcus)
Neutral Red under UV light	Lipids fluoresce greenish blue	Labellum scale-like papillae; adaxial surface of the labellum
PAS	Polysaccharides stain purplish red	Cell walls; labellum mesophyll starch
Ruthenium Red	Pectic acids stain pink to red.	Sepal glandular trichomes
Sudan III	Lipids stain orange to red	Labellum small drops in the mesophyll cells

Table 3. List of variable characters between species of *Bulbophyllum* sect. *Micranthae*. States of a characteristic that are exclusive to a single species are marked in bold. *B. men.*: *B. mentosum*; *B. adi.*: *B. adiamantinum*; *B. ins.*: *B. insectiferum*; *B. chl.*: *B. chloroglossum*; *B. muc.*: *B. mucronifolium*; *B. epi.*: *B. epiphytum*; *B. rup.*: *B. rupicolum*; *B. mic.*: *B. micranthum*; pw: periclinal walls.

Characteristic	<i>B. men.</i>	<i>B. adi.</i>	<i>B. ins.</i>	<i>B. chl.</i>	<i>B. muc.</i>	<i>B. epi.</i>	<i>B. rup.</i>	<i>B. mic.</i>
Adaxial epidermis	Surface ornamentation on pw	reticulate	reticulate	smooth	smooth	smooth	smooth	granulose
	Cell shape in frontal view	transversely elongate	longitudinally elongate	irregular	irregular	irregular	irregular	irregular
	Outer periclinal wall shape	convex and papillose	convex	convex	convex	convex and papillose	convex	convex
	Surface ornamentation on pw	reticulate	striate	granulose	striate	striate	granulose	granulose
Abaxial epidermis	Cell shape in frontal view	rectangular	irregular	longitudinally elongate	irregular	irregular	irregular	irregular
	Stomata	absent	present	present	present	present	absent	absent
Mesophyll	Idioblasts with helical thickenings	present	?	present	present	present	present	present
	Vascular bundles	3	1	1	1	1	1	1
	Surface ornamentation on pw	smooth	reticulate	striate	striate	smooth and striate	smooth	granulose
Lateral sepal	Cell shape in	transversely	irregular	longitudinally	longitudinally	irregular	irregular	irregular

	frontal view	elongate				
	Surface					
Abaxial epidermis	ornamentation on pw	striate	reticulate	striate	granulose	granulose
	Cell shape in frontal view	irregular	irregular	longitudinally elongated	irregular	irregular
	Stomata	absent	present	present	absent	absent
	Idioblasts with					
Mesophyll	helical	present	?	absent	present	present
	thickenings					
	Vascular bundles	3	?	3	3	1 3
Insertion		sessile	clawed	clawed	sessile	sessile
	Surface					
Adaxial epidermis	ornamentation on pw	smooth	reticulate	reticulate	smooth	striate
	Cell shape in frontal view	longitudinally elongated	longitudinally elongated	longitudinally elongated	irregular	longitudinally elongated
	Outer					
	periclinal wall shape	papillose	convex	convex	convex	convex and papillose convex
Margin	Cell type	unicellular trichome				
	Surface					
Abaxial epidermis	ornamentation on pw	smooth	smooth	reticulate	slightly striate	striate
	Cell shape in frontal view	longitudinally elongated	longitudinally elongated	longitudinally elongated	irregular	longitudinally elongated
	Outer					
	periclinal wall	papillose	convex	convex	convex	convex and papillose convex
Petal						

shape									
Mesophyll	Margin	in	epidermis						
	transverse		plus	epidermis	epidermis	epidermis	epidermis	epidermis	epidermis
	section		mesophyll		plus	plus	plus	plus	plus
	Idioblasts with				mesophyll	mesophyll	mesophyll	mesophyll	mesophyll
Adaxial epidermis	helical		present	?	absent	present	present	present	present
	thickenings								
	Surface								
	ornamentation		smooth	smooth	smooth	striate	striate	striate	striate
Abaxial epidermis	on pw								
	Outer								
	periclinal		papillose	papillose	papillose	scale-like papillae	scale-like papillae	convex	papillose
	shape								
Margin	Cell Type		papillae plus trichomes	smooth	smooth	smooth	smooth	smooth	smooth
Abaxial epidermis	Surface								
	ornamentation		smooth	striate	striate	striate	striate	striate	striate
	on pw								
	Outer								
Distal portion	periclinal		papillose	papillose	papillose	papillose	papillose	convex	papillose
	wall								
	shape								
	Stomata		absent	absent	absent	absent	present	absent	absent
Meso-phyll	Idioblasts with								
	helical		absent	absent	absent	absent	absent	absent	present
	thickenings								
	Vascular Bundles		0	3	2	2	3	2	2
Proximal portion	Surface								
	ornamentation		smooth	striate	striate	striate	striate	striate	striate
Adaxial epidermis	on pw								

Cell shape in frontal view		isodiametric	isodiametric	isodiametric	isodiametric	longitudinally elongate	longitudinally elongate	isodiametric	isodiametric
Margin	Outer periclinal wall shape	scale-like papillae	scale-like papillae	scale-like papillae	scale-like papillae	scale-like papillae	scale-like papillae	convex	scale-like papillae
	Cell type	papillae plus trichomes	smooth	smooth	smooth	papillae plus trichomes	smooth	smooth	smooth
Surface									
Abaxial epidermis	ornamentation on pw	smooth	striate	striate	striate	striate	striate	striate	striate
	Outer periclinal wall shape	convex papillose	/	papillose	convex	papillose	papillose	convex	convex
	Keel cell shape in frontal view	isodiametric	irregular	isodiametric	longitudinally elongated	isodiametric	longitudinally elongate	longitudinally elongated	longitudinally elongate
	Keel cells outer periclinal wall shape	convex	papillose	papillose	convex	papillose	papillose	papillose	papillose
Keel stomata		present	absent	present	present	present	present	present	present
Meso- phyll	Vascular bundles	11	11	5	3	3	3	3	3
Lateral Lobes	Presence	absent	present	absent	present	absent	present	present	present
	Shape	-	patent and ear-shaped	-	erect and dentiform	-	erect and dentiform	erect and dentiform	erect and dentiform
Surface									
Epider- mis	ornamentation on pw	smooth	?	smooth	slightly striate	striate	smooth	smooth	smooth
	External periclinal wall shape	convex	convex	slightly papillose	convex	convex	convex	convex	convex

Column
Gynostemium

Stelidia	Size	elongated	elongated	elongated	short	short	short	short
Stigma	Shape	wide	wide	wide	longitudinally elongated	wide	longitudinally elongated	wide
Foot	Surface ornamentation on pw	smooth	smooth	striate	?	striate	striate	slightly striate
	Epidermis External periclinal wall shape	convex	unicellular trichome?	unicellular trichome	convex	convex	convex	convex
	Meso-phyll	present	present	present	absent	present	present	present
Ovary	Idioblasts with raphides	absent	absent	absent	present	present	present	absent
	Idioblasts with helical thickenings	absent	present	absent	present	present	present	absent

FIGURES

Figure 1. General floral morphology of the species studied of *Bulbophyllum* sect. *Micranthae* in frontal (A–H) and lateral views (I–O). A, E, L) *B. mucronifolium*, B, I) *B. mentosum*; C, J) *B. insectiferum*; D, K) *B. chloroglossum*; F, M) *B. epiphytum*; G, N) *B. rupicolum*; H, O) *B. micranthum*. cf: column foot, ds: dorsal sepal, ke: keel, ll: lateral lobe, ls: lateral sepal, p: petal, st: stelidia, te: teeth. Scale bars = 2mm in A; 1 mm in B–O.

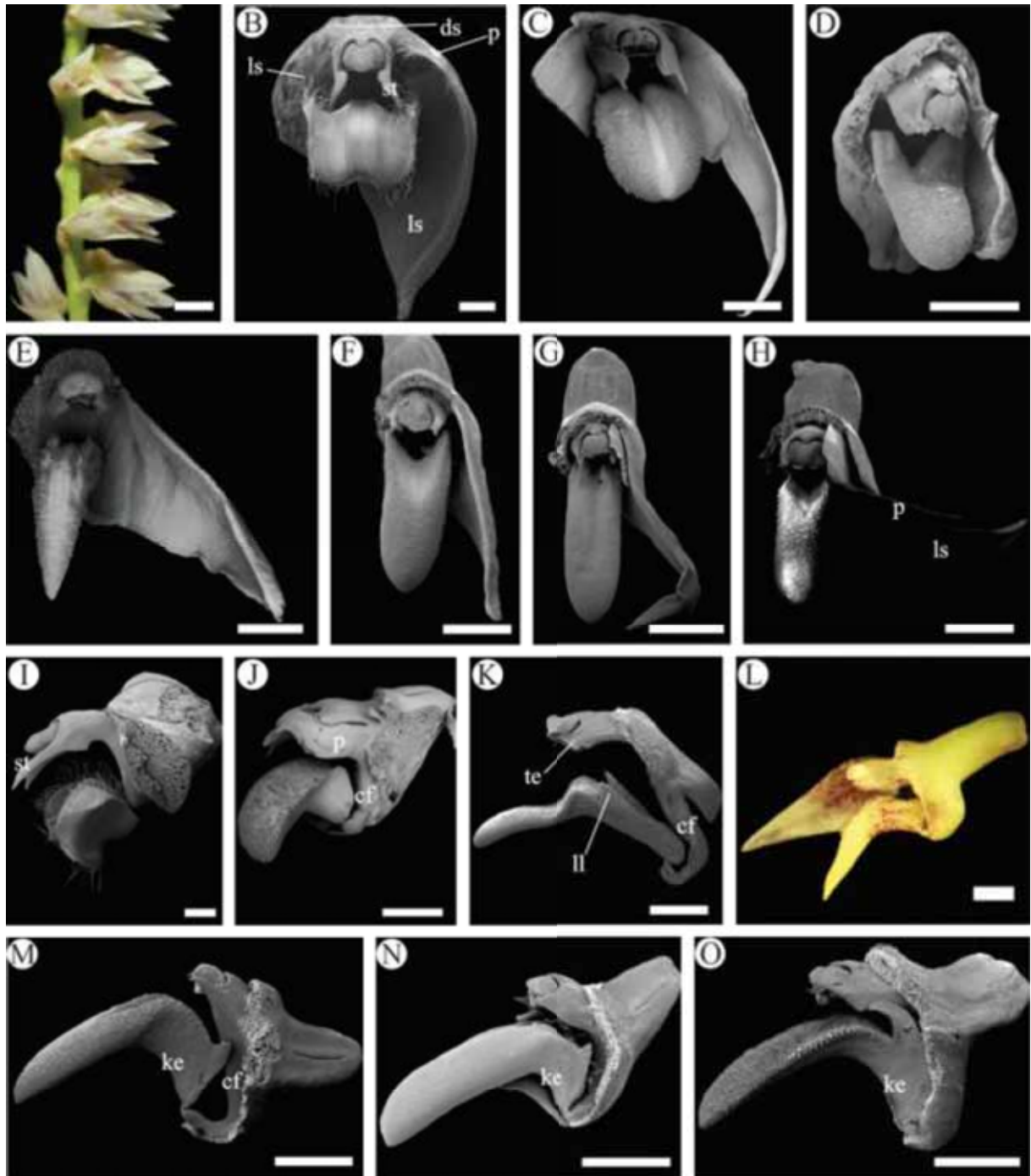


Figure 2. Micromorphology and anatomy of the sepals of *Bulbophyllum* sect. *Micranthae*. Adaxial surface in A–D; transverse sections in E–I, L–Q, and abaxial surface in J, K; Calcofluor White MR2 reaction in N; intrinsic fluorescence under UV in O. A, F, G, J, L) *B. mentosum*; B, K) *B. insectiferum*; C) *B. micranthum*; D) *B. mucronifolium*; E) *B. chloroglossum*; H, I, M–P) *B. epiphytum*; Q) *B. rupicolum*. black arrow: sunken trichome, white arrow: stomata, red rectangle outline: cell shape in frontal view, red circle outline: vascular bundle; ih: idioblast with helical wall thickenings, ri: idioblast with raphides. Scale bars = 100 μm in A, E–G, J, P, Q; 25 μm in B, L; 40 μm in C; 50 μm in D, K, O; 10 μm in H, I, M; 20 μm in N.

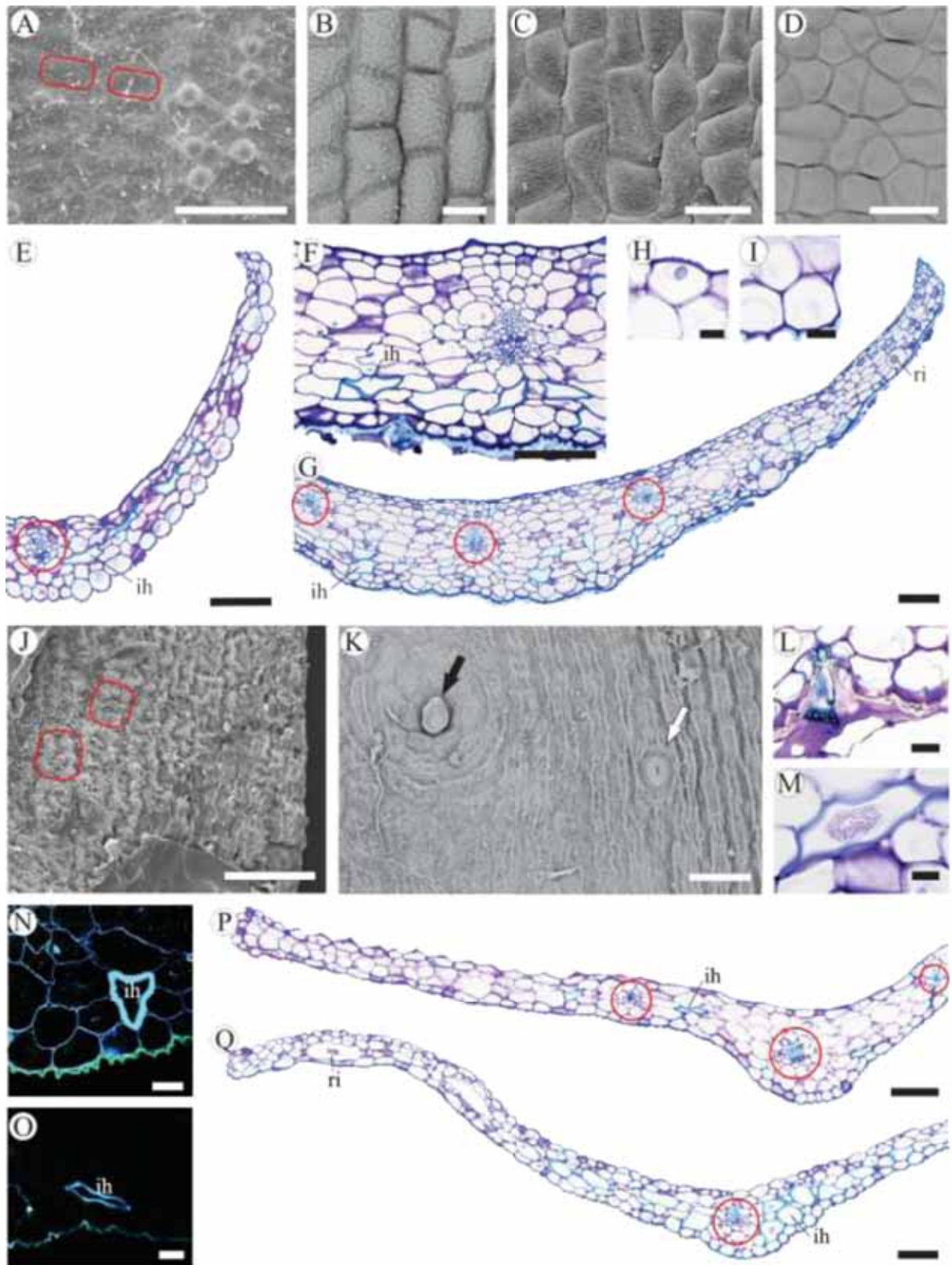


Figure 3. Micromorphology and anatomy of petals of *Bulbophyllum* sect. *Micranthae*. Abaxial surface in A; adaxial surface in B–E, I, J; transverse sections in F–H, K. A, C, K) *B. insectiferum*; B, G, J) *B. mentosum*; D) *B. mucronifolium*; E, H) *B. epiphytum*; F) *B. micranthum*; I) *B. adiamantinum*. ih: idioblast with helical wall thickenings, ri: idioblast with raphides. Scale bars = 250µm in A; 40µm in B, D, E; 50µm in C; 100µm in F–H; 125µm in I; 200µm in J; 80µm in K.

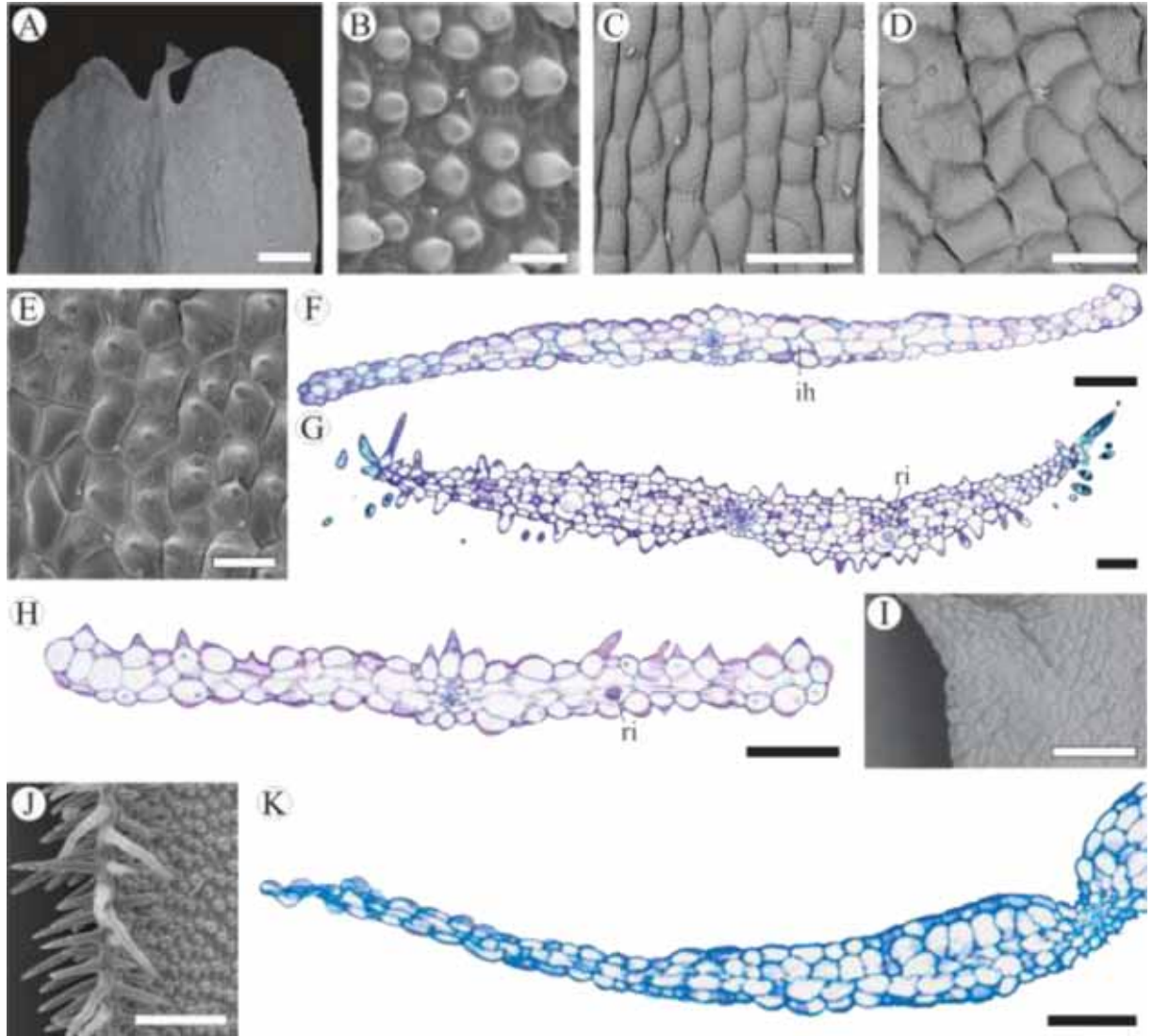


Figure 4. Micromorphology and anatomy of the labellum of *Bulbophyllum* sect. *Micranthae*. Adaxial surface in A–J; transverse section in K. A) *B. mentosum*; B) *B. adiamantinum*; C, I) *B. insectiferum*; D, K) *B. chloroglossum*; E) *B. mucronifolium*; F) *B. rupicolum*; G, J) *B. epiphytum*; H) *B. micranthum*. ri: idioblast with raphides. Scale bars = 500µm in A–H; 40µm in I, J; 100µm in K.

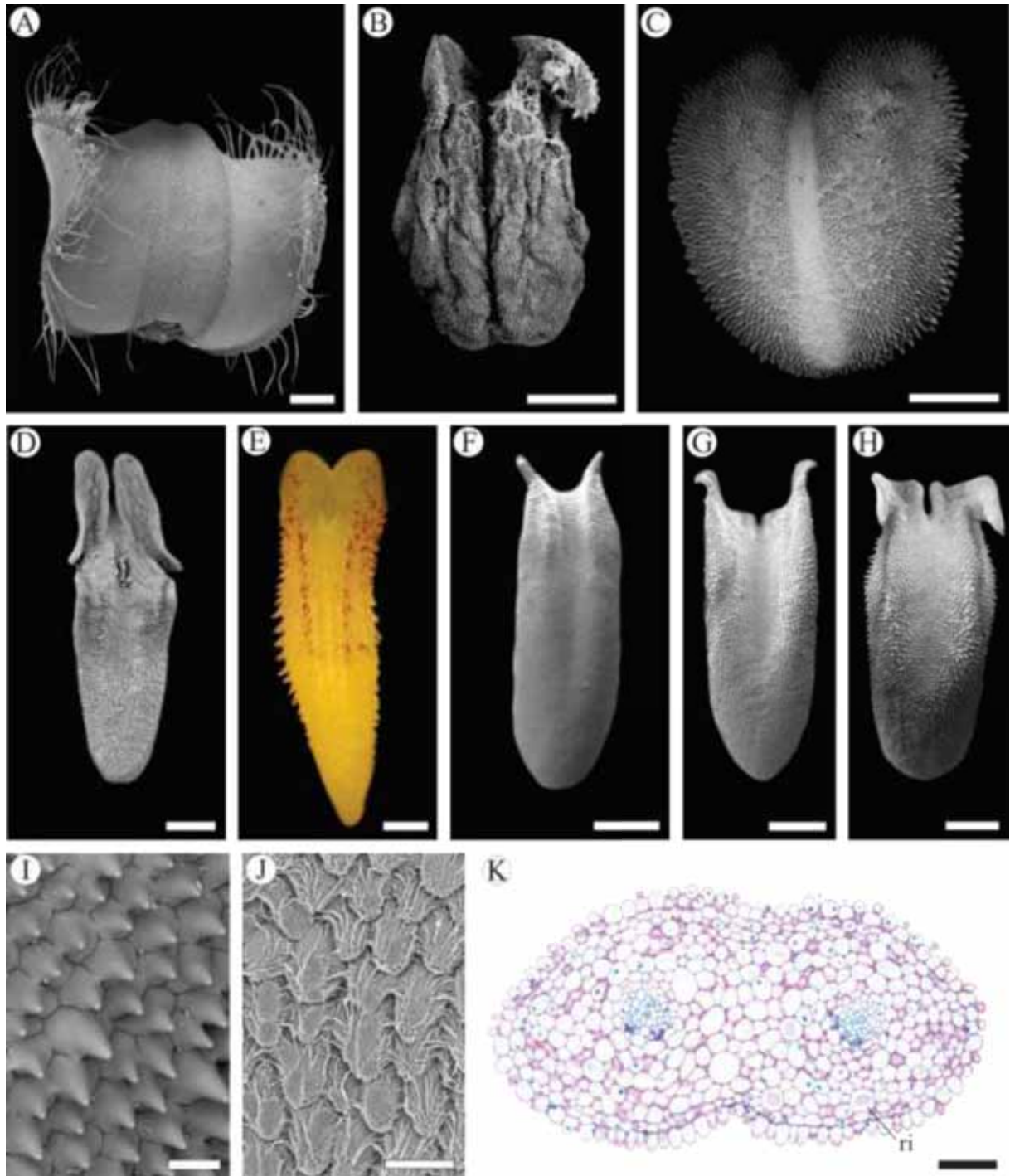


Figure 5. Micromorphology and anatomy of the labellum of *Bulbophyllum* sect. *Micranthae*. Abaxial surface in A–C, G, K–M; transverse sections in D–F, J, adaxial surface in H; longitudinal section in I. A, F, I) *B. mentosum*; B) *B. chloroglossum*; C, M) *B. adiamantinum*; D, J) *B. rupicolum*; E, H, L) *B. epiphytum*; G) *B. mucronifolium*; K) *B. micranthum*. white arrow: stomata; ri: idioblast with raphides. Scale bars = 100µm in A, D, E, H, J, K; 40µm in B, C; 10µm in D (detail); 200µm in F, I, L; 50µm in G, M.

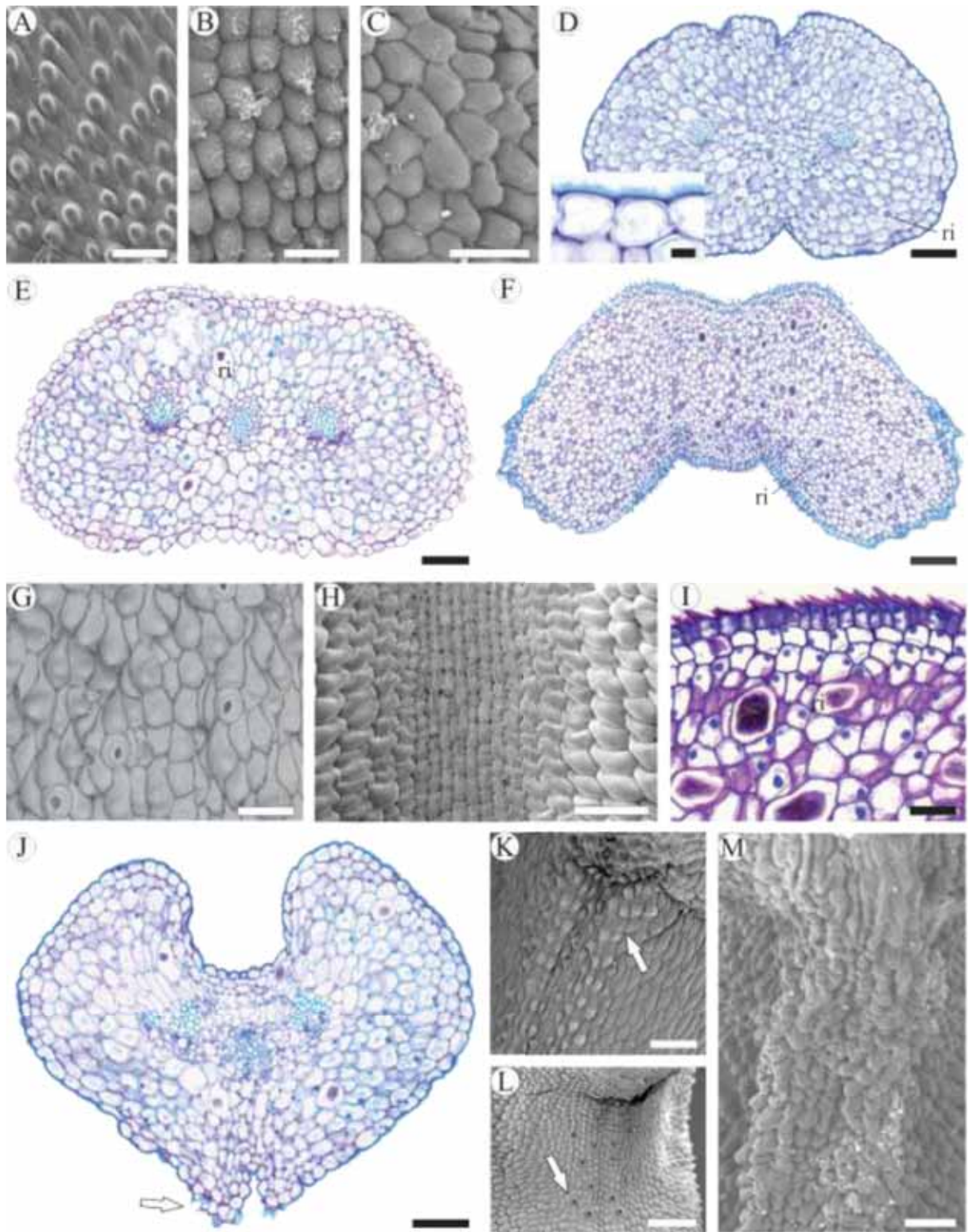


Figure 6. Micromorphology and anatomy of the labellum and gynostemium of *Bulbophyllum* sect. *Micranthae*. Transverse sections in A–F; adaxial surface in G, H; ventral surface in I, J. A) *B. insectiferum*; B, G) *B. micranthum*; C) *B. epiphytum*; D) *B. chloroglossum*; E, I) *B. mentosum*; F, H) *B. adiamantinum*; J) *B. mucronifolium*. ih: idioblast with helical wall thickenings, ri: idioblast with raphides. Scale bars = 80µm in A; 100µm in B–D, F, G; 200µm in E; 90µm in H; 40µm in I, J.

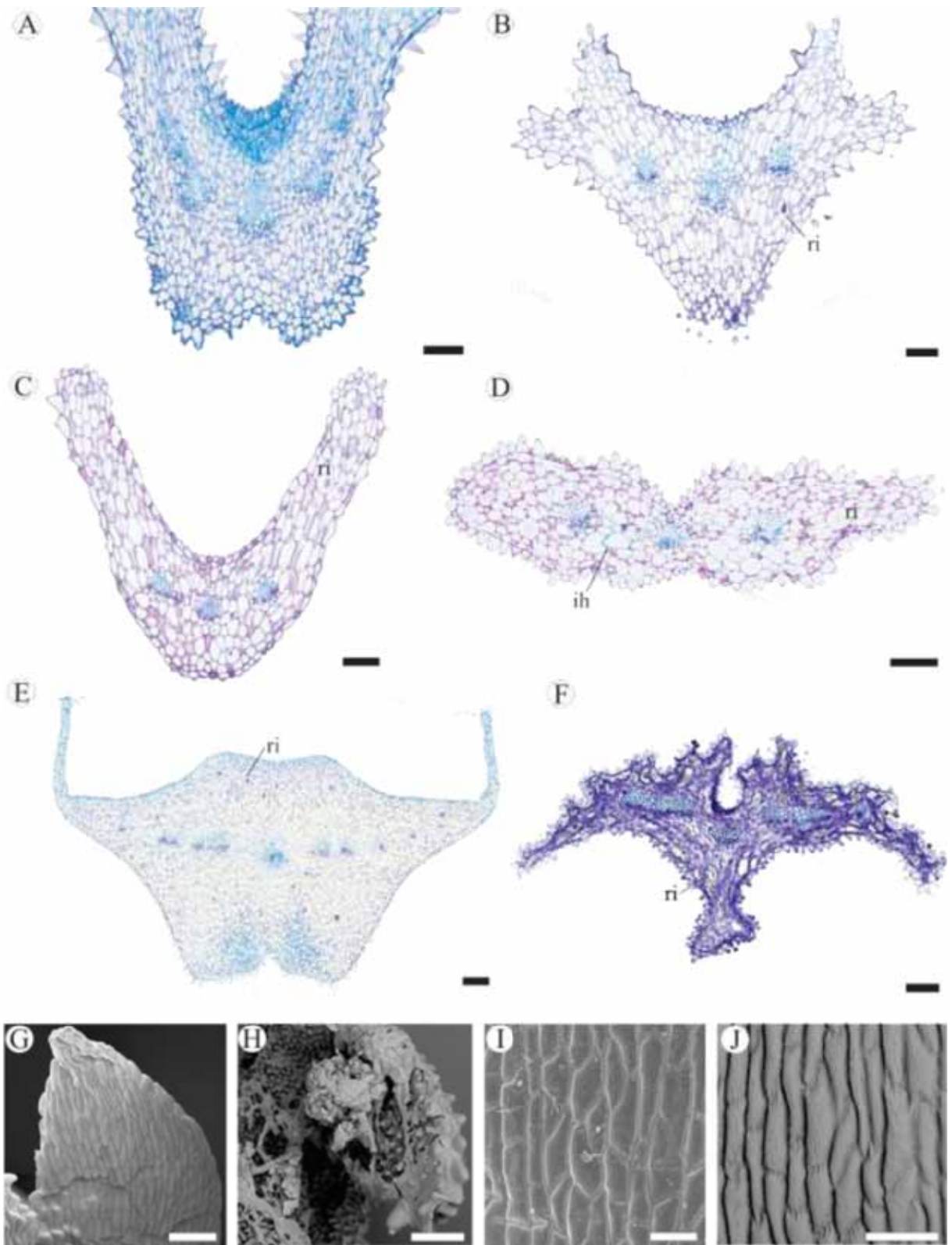
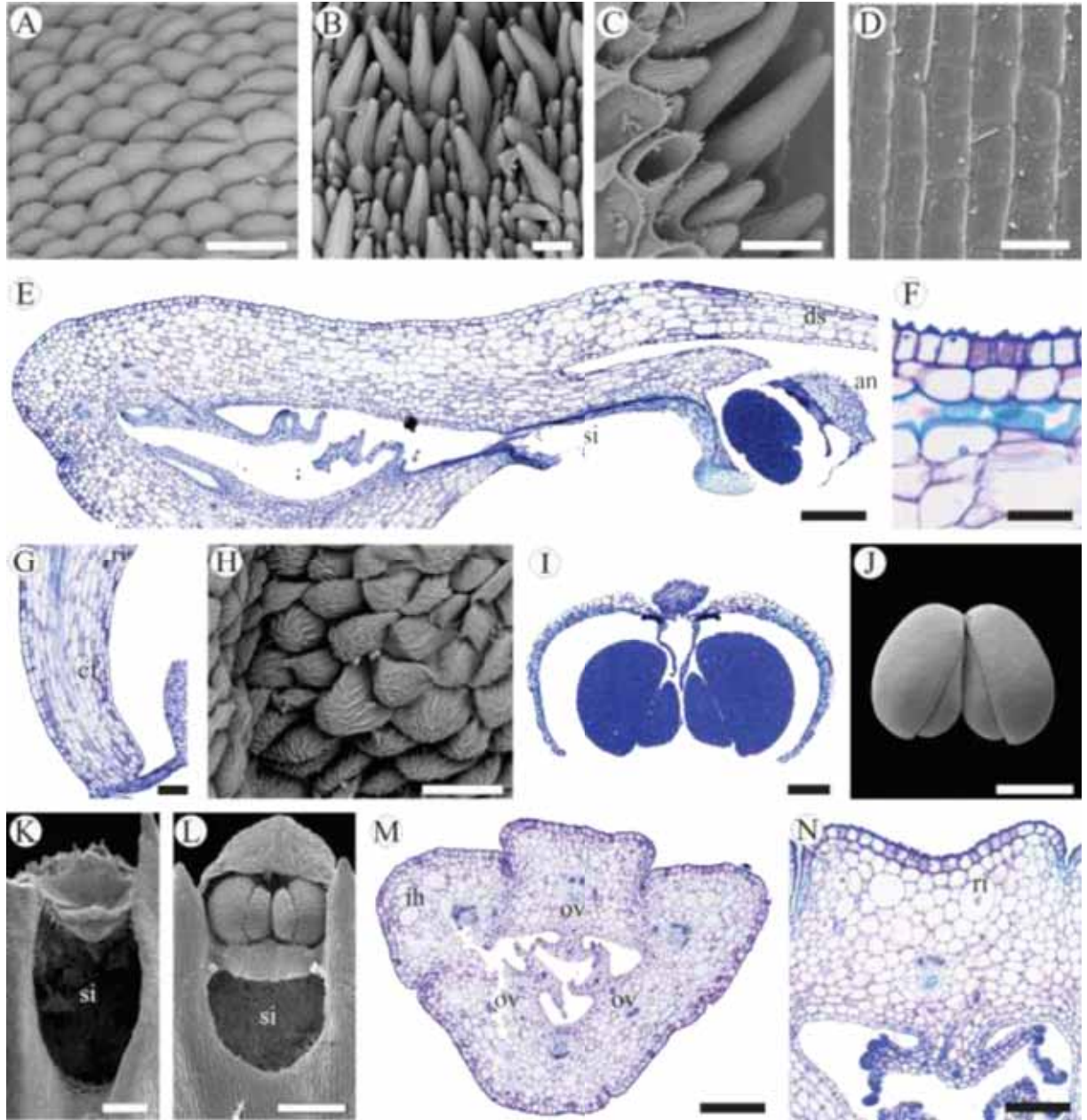


Figure 7. Micromorphology and anatomy of the column of *Bulbophyllum* sect. *Micranthae*. Dorsal surface of the gynostemium in A; ventral surface of the column foot in B, D; lateral view of the column foot in C; longitudinal section of the column in E–G; frontal view of the anther in H; transverse section of the anther in I; dorsal view of the pollinarium in J; frontal view of the stigma in K, L; transverse sections of the ovary in M, N. A) *B. mucronifolium*; B, C) *B. insectiferum*; D, I, J) *B. mentosum*; E) *B. micranthum*; F, K, N) *B. epiphytum*; G, L) *B. rupicolum*; H) *B. adiamantinum*, M) *B. chloroglossum*. an: anther, cf: column foot, ds: dorsal sepal, ih: idioblast with helical wall thickenings, ov: ovary, ri: idioblast with raphides, si: stigma. Scale bars = 40µm in A, B, D, H; 50µm in C, F; 200µm in E, I, K–M; 100µm in G, N; 400µm in J.



CAPÍTULO IV

Floral micromorphology and anatomy in *Bulbophyllum* sect. *Xiphizusa* (Orchidaceae: Asparagales) and their systematic significance

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(A ser submetido ao periódico *Botany*)

Abstract

Bulbophyllum sect. *Xiphizusa* (Rchb.f.) Cogn., comprises 23 species, mostly micro-endemic and epiphytic in the Atlantic Forest, gallery forests and Campos Rupestres throughout South America. In order to assess the floral micromorphological and anatomical features of *B.* sect. *Xiphizusa*, six species were analysed through light and scanning electron microscopies to characterize the section and verify the congruence with the systematic and pollination biology data available. The species were homogeneous regarding 54 characteristics and the section can be characterised as follows: sepals without conspicuous wall ornamentations on the adaxial surface, with sunken glandular trichomes; lateral sepals fused in a synsepal by intertwining of marginal trichomes; petals with indumented margins; trilobed lip without thickened idioblasts, hypochile with a callus covered by smooth scale-like papillae on the adaxial surface; anther with papillose cells and striated outer walls, and four pollinia. Fifty-one characteristics showed variation and were useful to differentiate between species. Four species showed secretory tissues in the sepals and/or labellum, which differ from other Neotropical species of the genus, whose secretory tissues are exclusively labellar. *Bulbophyllum ciluliae* Bianch. & J.A.N. Bat. was the most distinctive species, in agreement with their basalmost status according to a previous molecular phylogenetic study.

Key words: *Bulbophyllum*, fly pollination, idioblasts with helical wall thickenings, nectary, osmophore.

Introduction

Bulbophyllum Thouars is a pantropical genus that occurs in Australasia and American tropics, but its greatest diversity is found in the Asian South-East (Vermeulen 1987, 1993; Dressler 1993; Siegerist 2001). In the Neotropics, 60 species are currently recognized (Smidt et al. 2011; Gravendeel et al. 2014), and its greatest diversity is found in the contact areas between Cerrado vegetation and Atlantic Rain Forest in Brazil South-East (Smidt et al. 2007).

From the present six sections recognized among the Neotropical *Bulbophyllum* (Smidt et al. 2011), *B.* sect. *Xiphizusa* (Rchb.f.) Cogn. is the most diverse (Smidt et al. 2011). This section includes 23 species distributed from Mexico to the southern Brazilian state of Paraná, and with East limit in Bahia State (Brazil) (Smidt et al. 2011). Most species of this section are micro-endemic and epiphytic in the Atlantic rainforest, Cerrado vegetation or

gallery forests, but some are rupicolous in “campos rupestres” (rocky outcrops) areas; the greatest species diversity is found in the mountains in the interior of Brazil between the states of São Paulo and Bahia (Smidt et al. 2011).

Molecular phylogenetic studies performed by Smidt et al. (2011) analysed nine species currently included within *B. sect. Xiphizusa* and found *Bulbophyllum ciluliae* Bianch. & J.A.N. as sister to a clade that contained the remaining species with high support; the relationship within this clade, however, was poorly resolved and polytomies were present. The remarkable floral characteristics for this section are: pedicellate flowers distichously arranged in the inflorescence; lateral sepals connate forming a synsepal; a trilobed labellum whose lateral lobes are erect, with a pronounced callus between the lobes; column with stelidia, teeth and a short foot (Smidt et al. 2011). Nevertheless, the synsepal is absent in the basalmost species of *B. sect. Xiphizusa*, *B. ciluliae* (Smidt et al. 2011).

Verola (2002) studied two species of *B. sect. Xiphizusa* regarding their reproductive biology: *B. plumosum* Cogn. and *B. bidentatum* Cogn, although the second species was not pollinated during the study. That author did not observe nectar or floral odour for those species, although species belonging to other sections were reported to present those characteristics (Verola 2002; Teixeira et al. 2004; Nunes et al. 2014). *Bulbophyllum plumosum* is pollinated by a Chloropidae fly, in a mechanism dependent upon the wind similar to that described to *B. sect. Didactyle* (Borba and Semir 1998), sister group of *B. sect. Xiphizusa*.

No anatomical investigation was performed in flowers of species belonging to *B. sect. Xiphizusa* so far, and previous studies concerning neotropical species of this genus found high micromorphological and anatomical differentiation between species of a section (Nunes et al. 2014 – Capítulo I). In addition, nectaries and osmophores were reported (Teixeira et al. 2004; Nunes et al. 2014 – Capítulo I).

Therefore, the present work aims to answer the following questions: a) which floral anatomical and micromorphological features characterize species belonging to *B. sect. Xiphizusa*; b) are there floral anatomical and micromorphological characteristics that distinguish between species; c) are nectaries and osmophores absent from species belonging to *B. sect. Xiphizusa*?

Materials and methods

The material used in this study was previously and fixed in FAA 50 (3.7% v/v

formaldehyde, 50% ethanol, 5% acetic acid; Johansen 1940) and stored in 70% ethanol. The collection data are listed in Table 1.

To the assessment of micromorphological and anatomical characteristics, usual techniques of light microscopy (LM) and scanning electron microscopy (SEM) were used. The samples for LM analysis were embedded in Leica historesin, according to the supplier instructions and sectioned at 5–8 μm with disposable blades in a rotary microtome. Sections were stained with 0.05% toluidine blue O in 0.1 M sodium phosphate buffer, pH 6.8 (O'Brien et al. 1965), for general analysis. The following histochemical tests were performed on resin-embedded sections: Coomassie Brilliant Blue for proteins (Southworth 1973), Calcofluor White MR2 for cellulose (Hughes and McCully 1975), Ruthenium Red for pectic acids/mucilage (Johansen 1940), and the periodic acid-Schiff reaction (PAS) for total insoluble polysaccharides (Feder and O'Brien 1968). The appropriate controls were run simultaneously whenever applicable. Photomicrography was achieved using a Leica DMLB microscope that was coupled with a digital camera using the LAS (Leica Application Suite V3.3.0) software.

For SEM analysis, the samples were transferred from 70% ethanol to formaldehyde-dimethyl acetal for dehydration for 16 to 24 h (Gerstberger and Leins, 1978), critical-point-dried (CPD 030 - Bal-Tec AG, Balzers, Liechtenstein), and sputter-coated with gold (SCD 050 - Bal-Tec AG, Balzers, Liechtenstein). The observations were made using a Zeiss DSM 950 SEM (Carl Zeiss, Jena, Germany), and images were obtained using Digital Image Processing Software 2.2 (DIPS-Leipzig, Germany). Samples from *B. melloi* Pabst were dehydrated through a propanone series (critical-point drying and sputter-coating were similar to the aforementioned) and observed in a TM 3000 SEM (Hitachi, Tokyo, Japan) using its software.

Results

The flowers of the studied species belonging to *Bulbophyllum* sect. *Xiphizusa*, are characterised by a synsepal formed by the lateral sepals (Fig. 1A–D) except for *B. ciluliae* (Fig. 1E); reduced petals (Fig. 1E, F); trilobed labellum in which the median lobe is differentiated in hypochile (proximal part usually fleshy) and epichile (distal part usually membranaceous) (Fig. 1A–J), while the lateral lobes are erect inconspicuous; gynostemium with cylindrical stelia, which are longer than the anther (Fig. 1A–J); and elongate ovary (Fig. 1A–J).

The adaxial surface of the dorsal sepal lacks conspicuous ornamentation and, in frontal view, their epidermal cells are irregular shaped (Fig. 2A), longitudinally elongate (Fig. 2B) or more or less isodiametric (Fig. 2C). In transverse section, the outer periclinal wall is convex (Fig. 2D) or papillose (Fig. 2C, E). On the abaxial surface, the ornamentation is granulose (Fig. 2F) and the epidermal cells, in frontal view, are irregular shaped (Fig. 2F); in transverse section, the outer periclinal wall is either convex (Fig. 2D) or straight (Fig. 2E). At this surface, all species possess sunken multicellular glandular trichomes (Fig. 2G), but stomata are present only in *B. ciluliae* (Fig. 2F) and *B. plumosum*. The margin is either papillose (Fig. 2H) or has unicellular trichomes (Fig. 2I), and is formed by both epidermal and mesophyll cells (Fig. 2D, E). The mesophyll is usually homogeneous, with regular parenchyma, and shows both idioblasts with raphides and with helical wall thickenings (Fig. 2E, G). However, *B. melloi* and *B. plumosum* (Fig. 2E, J) have two to three layers of a secretory subepidermal epithelium. There may be one (Fig. 2D), two, three (Fig. 2K) or five (Fig. 2E) collateral vascular bundles.

The lateral sepals usually lack conspicuous ornamentation on their adaxial surface (Fig. 2L). The epidermal cells, in frontal view, are irregular shaped (Fig. 2L), and their outer periclinal walls, in transverse section, are either convex (Fig. 3A) or papillose (Fig. 3B, C). On the abaxial surface, the ornamentation is irregular (Fig. 3D) or slightly striate (Fig. 3E). The epidermal cell of this surface, in frontal view, are either irregular shaped (Fig. 3D) or longitudinally elongate (Fig. 3E); the outer periclinal walls are convex in transverse sections (Fig. 3A, B). All species possess sunken multicellular glandular trichomes (Fig. 3F) and stomata (Fig. 3D, E); the latter are absent in *B. manarae* only. The margin is papillose (Fig. 3G), except for *B. plumosum* which has also unicellular trichomes (Fig. 3H). The synsepal is formed by the intertwining of papillae and/or trichomes of the inner margins (Fig. 3G, H). The mesophyll is either homogeneous (Fig. 3A) or heterogeneous (Fig. 3B). All species show three collateral vascular bundles (Fig. 3A, B), except *B. ciluliae* which shows one.

The petals lack a conspicuous ornamentation (Fig. 3I), except for *B. bidentatum* where it is striate (Fig. 3J). In frontal view, the epidermal cells are either irregular shaped (Fig. 3I) or longitudinally elongate (Fig. 3J); their outer periclinal walls are convex in transverse sections (Fig. 3K), but papillose cells may also be present at the margin (Fig. 3K, L). On the abaxial surface, ornamentation, cell shape in frontal view and outer periclinal wall in transverse section are similar to those of the adaxial surface. The margin has papillose cells (Fig. 3M), many long trichomes (Fig. 3N) or papillose cells with a few long trichomes (Fig. 3O). The mesophyll is homogeneous, with regular parenchyma and shows only

idioblasts with raphides (Fig. 3K, L). A single collateral vascular bundle is present (Fig. 3K, L).

The labellum epichile has a striate adaxial surface (Fig. 4A), except for *B. ciluliae*, where it is smooth (Fig. 4B). The epidermal cells, in frontal view, are more or less isodiametric (Fig. 4A), but in *B. plumosum* they are longitudinally elongate (Fig. 4C). In transverse section, the outer periclinal walls of those cells are either papillose (Fig. 4D) or convex (Fig. 4E). On the abaxial surface, ornamentation, cell shape of epidermal cells in frontal view and outer periclinal wall in transverse section are similar to those of the adaxial surface; however, *B. ciluliae* has papillose cells (Fig. 4D). The margin is smooth (Fig. 1B, D–H), except for *B. gladiatum* (Figs 1C, J, 4F) and *B. plumosum* (Fig. 1A, I) which shows long trichomes on the epichile margin. The mesophyll is homogeneous and formed by regular parenchyma in most species (Fig. 4D); however, in *B. bidentatum* the parenchyma is secretory (Fig. 4E) and in *B. melloi* (Fig. 4G) and *B. plumosum* there are more intercellular spaces. Idioblasts with raphides are also present (Fig. 4D, E). The collateral vascular bundles vary between two (Fig. 4D) or three (Fig. 4E, G).

The hypochile has a callus on its adaxial surface that does not show conspicuous ornamentation, and whose cells are scale-like papillae (Fig. 4H). On the proximal portion of the hypochile, four species (*B. ciluliae*, *B. manarae* – Fig. 1B, *B. bidentatum* – Fig. D, and *B. melloi*) show a discrete sulcus; in *B. manarae* and *B. melloi* (Fig. 4I) this sulcus shows secretory characteristics (cells with large prominent nucleus, high cytoplasmic content and cytoplasmic proteins). In *B. plumosum*, there are orifices aligned along the callus midline (Figs 4J, 5A, B). On the abaxial surface, the hypochile either lacks conspicuous ornamentation (Fig. 5C) or is striate (Fig. 5D); the epidermal cells, in frontal view, are longitudinally elongate (Fig. 5C, D), and in transverse section their outer periclinal walls are papillose (Figs 4I, 5A, E). The mesophyll is homogeneous and formed by regular parenchyma that shows idioblasts with raphides (Fig. 5A, B). *Bulbophyllum bidentatum* shows a secretory epithelium below the epidermis, at the hypochile periphery (Fig. 5E). The vascular bundles are collateral and vary between three (Fig. 5A), five (Fig. 5E) or seven (Fig. 4I).

At the proximal portion of the labellum, there are two lateral lobes (Fig. 4B, E), whose surfaces lack conspicuous ornamentation; the epidermal cells are more or less isodiametric and show convex to papillose outer periclinal walls (Fig. 5F, G). On the margin, there are long trichomes (Fig. 5F) or papillae (Fig. 5G).

The gynostemium surface either lacks conspicuous ornamentation (Fig. 5H) or is

slightly striate (Fig. 5I). Their epidermal cells, in frontal view, are longitudinally elongate (Fig. 5H, I) and their outer periclinal walls are convex (Fig. 5J). The mesophyll is homogeneous and formed by regular parenchyma, with both idioblasts with raphides and with helical wall thickenings (Fig. 5J, K). The column foot usually shows striate ornamentation (Fig. 6A), but it is sometimes inconspicuous (Fig. 6B). Their epidermal cells, in frontal view, are longitudinally elongate and their outer periclinal walls are papillose (Fig. 6A, B); the mesophyll is similar to that of the gynostemium (Fig. 8J, K).

The anther has striate epidermal cells (Fig. 6C), whose outer periclinal walls are papillose (Fig. 6C). The pollinarium is composed by four pollinia, whose the central are smaller (Fig. 6D, E), and a viscidium formed by the disorganization of rostellar cells (Fig. 5J, K). The stigmatic cavity is more or less wide (Fig. 6F) and has long, narrow cells embedded, but loosely arranged, in a mucilaginous matrix (Fig. 5J, K).

The ovary is six-lobed and unilocular (Fig. 6G), with the outer epidermis formed by rectangular cells, in transverse section, and whose outer periclinal walls are convex and ornamented (Fig. 6H); the inner epidermis epidermis is formed by smaller elliptic to isodiametric cells with convex outer periclinal walls (Fig. 6I). The mesophyll is homogeneous, shows only idioblasts with raphides (Fig. 6G–I) and one collateral vascular bundle per lobe (Fig. 6G).

The results of the histochemical testes that were performed are summarised in the Table 2 and the variable characters between species are in Table 3.

Discussion

From the ca. 110 characteristics analysed here, the six species of *Bulbophyllum* sect. *Xiphizusa* studied showed homogeneity in 55, being characterised as follow: sepals without conspicuous ornamentation on the adaxial surface, but with sunken glandular trichomes on the abaxial surface; petals with a homogeneous mesophyll that lacks idioblasts with helical wall thickenings and possess a single collateral vascular bundle; labellum without idioblasts with helical wall thickenings, with the adaxial surface of the hypochile covered by scale-like papillae; gynostemium with elongated epidermal cells; anther with papillose epidermal cells that show striate ornamentation, and pollinarium composed by four pollinia and a viscidium derived from rostellar cells.

In our study, we found secretory tissue in four out of six species studied. They may occur in all sepals (*B. melloi* and *B. plumosum*), only in the lateral ones (*B. manarae*) or in the

labellum (*B. manarae*, *B. bidentatum* and *B. melloi*). However, we were not able to characterize them as osmophores or nectaries due to inability to characterise the nature of their products in the absence of fresh material. *Bulbophyllum plumosum* is the only species belonging to this section whose pollination was unveiled, but neither nectar nor floral odour was perceived by Verola (2002).

In the present study, we observed some cavities on the labellum of this species that cannot be confirmed as secretory, but the sepals show tissues that are clearly secretory, most likely working as osmophores instead of a nectary, since Verola (2002) did not observe any feeding behaviour from the pollinator in his study. The paucity of secretory tissues on the labellum of the species studied here is somewhat surprising, since in *B. sect. Didactyle* (sister group of *B. sect. Xiphizusa*) it seems that there was an increase in the size of the nectaries (Nunes et al. 2014) and both groups are believed to share the same wind-assisted pollination mechanism (Verola 2002).

Too little is known on the pollination biology of species belonging to *B. sect. Xiphizusa*, but *B. plumosum* (Verola 2002) is pollinated by a species belonging to a different fly family – Chloropidae – than species belonging to the other *Bulbophyllum* sections. Milichiidae flies are reported for *B. setigerum* (*B. sect. Furvescens* – Braga 1977), three species of *B. sect. Didactyle* (Borba and Semir 1998), and *B. insectiferum* (*B. sect. Micranthae* – Verola 2002); Sciaridae for *B. epiphytum* (*B. sect. Micranthae* – Verola 2002); and Tachinidae for *B. glutinosum* (*B. sect. Napelli* – Verola 2002). So that, it is possible that pollinator shifts may have mediated such change in the nectary presence. From these previous studies, some degree of specificity in *Bulbophyllum* pollination have been demonstrated (Borba and Semir 1998; Verola 2002), regardless of the general belief that flies are inefficient and unreliable pollinators because of their random and casual behaviour (van der Pijl and Dodson 1966; Faegri and van der Pijl 1979; Christensen 1994).

Bulbophyllum and the subtribe Pleurothallidinae are the two largest groups of myophilous orchids (van der Pijl and Dodson 1966; Dressler 1993; Christensen 1994) and some degree of floral convergence has been suggested between them (van der Pijl and Dodson 1966). In addition, some specificity in the pollination of Pleurothallidinae (Borba and Semir 2001; Melo et al. 2011), has also been demonstrated. *Acianthera fabiobarrosii* and *A. johannensis* are pollinated by the same species of Chloropidae flies (*Tricimba* sp.), release a unpleasant fish odour and do not produce nectar (Borba and Semir 2001), while *A. teres* and *A. ochreatea* are pollinated by species of *Megaselia* (Phoridae flies), offer nectar as reward and smell of rancid cheese; *A. adamantinensis* showed faeces odour, offer nectar as reward and

are pollinated by another genus of Chloropidae flies (*Hippellates*) (Borba and Semir 2001). Four other species of *Acianthera* showed nectaries in their lip and also osmophores on their sepal adaxial surfaces, especially on the lateral ones, irrespective of being pollinated by Phoridae or Chloropidae flies (Melo et al. 2010). Such striking variability showed by *Acianthera* may be also occurring in a greater degree in *Bulbophyllum* sect. *Xiphizusa*, with some species showing secretory tissues and others not (releasing or not odour, offering or not nectar as pollinator reward). The presence of fused lateral sepals that may have secretory tissues both in *Bulbophyllum* sect. *Xiphizusa* and *Acianthera*, is a very interesting feature that accounts for the hypothesis of convergence between the two groups and it would not be such a surprise if Phoridae flies were also found to be pollinators of other *B.* sect. *Xiphizusa* species.

Another interesting characteristic shown by five of the species studied here is the lack of idioblasts with helical wall thickenings. Such cell type usually was reported as idioblasts or forming transfusion tissue in leaves in several Gymnosperms and Angiosperms (Venning 1946; Foster 1956; Rodin 1958; Koller and Rost 1988), including Orchidaceae (Olatunji and Nengim 1980; Pridgeon 1981, 1982). Some authors regard these cells both as water reservoirs and mechanical structures which support the mesophyll during drought (Haberlandt 1924; Olatunji and Nengim 1980, Pridgeon 1982), while others consider only the latter function (Whitner et al. 1974). Epiphyte Orchidaceae would also bear such cells as an adaptation to drought conditions (Olatunji and Nengim 1980; Burr and Barthlott 1991), but they have also been observed at least in one rupicolous *Pleurothallis* species in Campos Rupestres (Scatena and Nunes 1996). Thus, the presence of idioblasts with helical wall thickenings would be expected in many neotropical *Bulbophyllum* species, as indeed verified in leaves of *B.* sect. *Micranthae* species (Smidt et al. 2013) and many floral parts of species belonging to *B.* sect. *Didactyle* (Nunes et al. 2014), *B.* sect. *Micranthae* (Nunes et al. in prep. – Capítulo III), *B.* sect. *Napelli* (Nunes et al. submitted – Capítulo II), *B.* sect. *Furvescens* and *B.* sect. *Bulbophyllaria* (Nunes et al. in prep. – Capítulo V). The paucity of these cells in the studied species of *B.* sect. *Xiphizusa* which are epiphytes or rupicolous in areas usually subject to some degree of drought is intriguing and deserves deeper investigation.

All species of *B.* sect. *Xiphizusa* studied here showed variation in 52 characteristics, most of them of the labellum and sepals. The number of variable characteristics with exclusive states to a single species was usually reduced: four in *B. manarae*, two in *B. bidentatum* and *B. gladiatum* and three in *B. melloi*.

The exceptions were *B. ciluliae* (11) and *B. plumosum* (eight). The higher number of

exclusive states of characteristics shown by these two species is interesting and might be explained, in *B. ciluliae*, by its positioning on the molecular-based phylogeny as the basalmost species within *B. sect. Xiphizusa* (Smidt et al. 2011). *Bulbophyllum ciluliae* was the most distinctive species studied here and according to Smidt et al. (2011) shows floral characteristics that mostly resemble *B. sect. Micranthae* (notably being the only species assigned to *B. sect. Xiphizusa* without a synsepal) while vegetative ones are typically *B. sect. Xiphizusa*. In fact, we agree with the view that floral characteristics of this species are more similar to those of *B. sect. Micranthae*, such as: unfused lateral sepals without secretory tissues, with one (dorsal sepal) vascular bundle; labellum not (clearly) differentiated in hypochile and epichile, with dentiform lateral lobes, and scale-like papillae on the adaxial surface. Once it is known that Orchidaceae shows all sorts of floral diversity due to the complex interactions with pollinators (Faegri and van der Pijl 1979; van der Cingel 2001), information on reproductive biology of *B. ciluliae* is completely lacking and as this species may occur sympatrically with species from *B. sect. Micranthae* (Smidt. et al. 2011), floral convergence cannot be excluded. The differentiation of *B. plumosum*, however, is more difficult to explain phylogenetically, once it is embedded within a polytomy that includes four more species (Smidt et al. 2011), of which three have been analysed in this study (*B. gladiatum*, *B. melloi* and *B. bidentatum*).

Bulbophyllum manarae is not embedded within this clade and shows a more uncertain phylogenetic position (Smidt et al. 2011), but shares with *B. gladiatum*, *B. melloi* and *B. bidentatum* some lack of differentiation, from the micromorphological and anatomical point of view. Considering our limited sampling of the section (six out of 23 species), deep systematic discussions regarding the section core would be rather speculative. However, considering the results from other neotropical sections of the genus (Nunes et al. 2014, submitted – Capítulo II), we argue that the 52 characteristics that showed variation between species may be useful for better characterise each species, and differentiate between them, even though is advisable to broad the sampling for this group.

In spite of representing a small fraction of the diversity of *Bulbophyllum*, the floral diversity regarding their anatomy and morphology found so far in the neotropical species of the groups (Teixeira et al. 2004; Nunes et al. 2014, submitted – Capítulo II), including *B. sect. Xiphizusa* is remarkable. The understanding of the process involved in species richness is one of the most challenging problems in Angiosperm evolution and, studying such big genera like *Bulbophyllum* may be a great opportunity to understand rapid speciation, adaptative radiation, character evolution, key-innovations and more (Berry et al. 2005). In spite of that, attempts to

study consistently such big genera are rare, once detailed knowledge on the group is essential and this is unlikely to happen in short-term projects (Samain et al. 2009).

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Table 1. List of species studied and collection data.

Species	Collection Data
<i>B. ciluliae</i> Bianch. & J.A.N.Bat.	HUEFS (E. C. Smidt et al., 805: Bahia, Rio de Contas, Brazil)
<i>B. manarae</i> Foldats	HUEFS (E. C. Smidt et al. 747: Bahia, Rio de Contas, Brazil) HUEFS (E. C. Smidt 794: Bahia, Rio de Contas, Brazil)
<i>B. bidentatum</i> Cogn.	HUEFS (E. C. Smidt et al. 787: Minas Gerais, Tiradentes, Brazil) HUEFS (E. C. Smidt 788: Minas Gerais, Tiradentes, Brazil)
<i>B. gladiatum</i> Lindl	HUEFS (A. L. V. Toscano-de-Brito 1905 Bahia, Rio de Contas, Brazil)
<i>B. melloi</i> Pabst	Spirit material (N. Mota & M. Marques 656: Minas Gerais, São Gonçalo do Rio Preto, Brazil) HUEFS (E. C. Smidt 744: Minas Gerais, Caldas, Brazil)
<i>B. plumosum</i> Cogn.	HUEFS (E. C. Smidt 726: Minas Gerais, Caldas, Brazil) HUEFS (E. C. Smidt 754: Minas Gerais, Caldas, Brazil) JB-SP s.n.
HUEFS: Herbário Universidade Estadual de Feira de Santana; JB-SP: Instituto de Botânica de São Paulo.	

Table 2. Histochemical tests performed on species of *B.* sect. *Xiphizusa* and reaction sites.

Histochemical test	Reaction site
Coomassie Brilliant Blue	Cytoplasmic proteins (epidermis and subjacent tissue -labellum sulcus)
Calcofluor White MR2	Idioblasts with helical wall thickenings
Ruthenium Red	Sepal glandular trichomes

Table 3. List of variable characteristics between species of *Bulbophyllum* sect. *Xiphizusa*. States of a characteristic that are exclusive to a single species are marked in bold. pw: periclinal walls.

Floral Part	Characteristics	<i>B. ciluliae</i>	<i>B. manarae</i>	<i>B. gladiatum</i>	<i>B. bidentatum</i>	<i>B. melloi</i>	<i>B. plumosum</i>
Ad	Cell shape	irregular	irregular	longitudinally	isodiametric	longitudinally	longitudinally
	frontal view			elongated		elongated	elongated
	Outer pw shape	convex	convex	convex	papillose	papillose	papillose
Ab	Cell shape	irregular	irregular	irregular	irregular	irregular/longitudinally	irregular/longitudinally
	Frontal view					elongate	elongate
	Outer pw shape	convex	convex	convex	convex	convex	straight
Dorsal	Stomata	present	absent	present	present	absent	present
Sepal	Margin	papillae	papillae	papillae	papillae	papillae	unicellular trichome/papillae
	Type	homogeneous	homogeneous	homogeneous	homogeneous	heterogeneous	heterogeneous
Mesophyll	Parenchyma	regular	regular	regular	regular	secretory and regular	secretory and regular
	Idioblasts	absent	absent	absent	present	present	absent
	with helical wall						
	thickenings						
Vascular bundles		1	2	3	3	3	5
Lateral	Ad	Cell shape	irregular	irregular	longitudinally	longitudinally	longitudinally

sepals		frontal view	convex	convex	convex (conical)	elongated	elongated	elongated
		Outer pw shape	convex	convex	convex (conical)	papillose	papillose	papillose
		Cell shape in frontal view	irregular	irregular	longitudinally elongated	longitudinally elongated	longitudinally elongated	longitudinally elongated
Ab		Outer pw shape	convex	convex	convex	convex	convex	straight
		Stomata	present	absent	present	present	present	present
Margin		Cell type	papillae	papillae	papillae	papillae	papillae/short trichome	unicellular trichome/papillae
		Type	homogeneous	heterogeneous	homogeneous	homogeneous	heterogeneous	heterogeneous
		Parenchyma	regular	secretory and regular	regular	regular	secretory and regular	secretory and regular
		Idioblasts	absent	absent	absent	present	present	absent
Mesophyll		with helical wall						
		thickenings						
		vascular bundles	1	3	3	3	3	3
		Surface ornamentation on pw	smooth	smooth	smooth	slightly striate	striate	smooth
Petal	Ad	Cell shape	irregular	irregular	longitudinally elongated	longitudinally elongated	longitudinally elongated	longitudinally elongated
		frontal view						
		Outer pw shape	convex	convex	papillose	papillose	papillose	papillose

Margin	Cell type	papillae	long trichomes	long trichomes	long trichomes	long trichomes	papillae and sparse long trichomes
	Surface ornamentation on pw	smooth	smooth	smooth	striate	striate	smooth
Ab	Cell shape frontal view	irregular	longitudinally elongated	longitudinally elongated	longitudinally elongated	longitudinally elongated	longitudinally elongated
	Outer pw shape	convex	convex	convex (conical)	papillose	papillose	papillose
	Surface ornamentation on pw	smooth	striate	striate	striate	striate	striate
Ad	Cell shape frontal view	scale-like papillae	isodiametric	longitudinally elongated	isodiametric	irregular/longitudinally elongate	longitudinally elongated
	Outer pw shape	papillose	papillose	papillose	convex	convex	convex
Margin	Cell type	papillose	smooth	long trichomes	smooth	smooth	smooth
	Surface ornamentation on pw	smooth	striate	striate	striate	striate	striate
Ab	Cell shape frontal view	isodiametric	isodiametric	longitudinally elongated	isodiametric	longitudinally elongated	longitudinally elongated
	Outer pw shape	papillose	papillose	papillose	convex	convex/papillose	convex
Mesophyll	Parenchyma	Regular	mostly secretory	regular	mostly secretory	spongy	spongy

Labellum:
epichile

	vascular bundles	2	3	3	3	3	3
Ad	Secretory sulcus	absent	present	absent	absent	absent	absent
Margin	Cell type	papillose	smooth	smooth	smooth	long trichomes	
	Surface ornamentation on pw	smooth	slightly striate	slightly striate	striate	slightly striate	smooth
Ab	Cell shape frontal view	longitudinally elongated	longitudinally elongated	isodiametric	isodiametric	isodiametric/longitudinally elongated	isodiametric/longitudinally elongated
	Outer pw shape	convex/papillose	convex	papillose	papillose	papillose	papillose
Labellum: hypochile	Type	homogeneous	homogeneous	homogeneous	heterogeneous	heterogeneous	homogeneous
	Parenchyma	regular	regular	regular	regular and secretory	Regular and secretory	regular
	Vascular bundles	7	5	3	5	7	5
Ginostemium	Surface ornamentation on pw	striate	striate	smooth	striate	smooth	smooth
	Stelidia	flattened	cylindric	cylindric	cylindric (bifid apex)	cylindric (bifid apex)	cylindric
	Column foot	striate	smooth	smooth	smooth	smooth	slightly striate

Outer pw		convex	papillose	papillose	papillose	convex	papillose
shape							
Anther	Beak size	long	short	short	short	short	short
Stigma	Shape	transversally	isodiametric	transversally	transversally	transversally	transversally
		elongated		elongated	elongated	elongated	elongated

Figures

Figure 1. Floral morphology of *Bulbophyllum* sect. *Xiphizusa*. A, I) *B. plumosum*; B, G) *B. manarae*; C, J) *B. gladiatum*; D, H) *B. bidentatum*; E, F) *B. ciluliae*. Lateral sepals coloured in red (in B, E, and F), dorsal sepal coloured in pink (in E and F), petals in purple (in E and F), labellum in green (in E and F), gynostemium in orange (in E and F), and anther in yellow (in E and F). cf, column foot, st, stelidia. Scale bars: 2mm in A–D, G–J; 1mm in E, F.

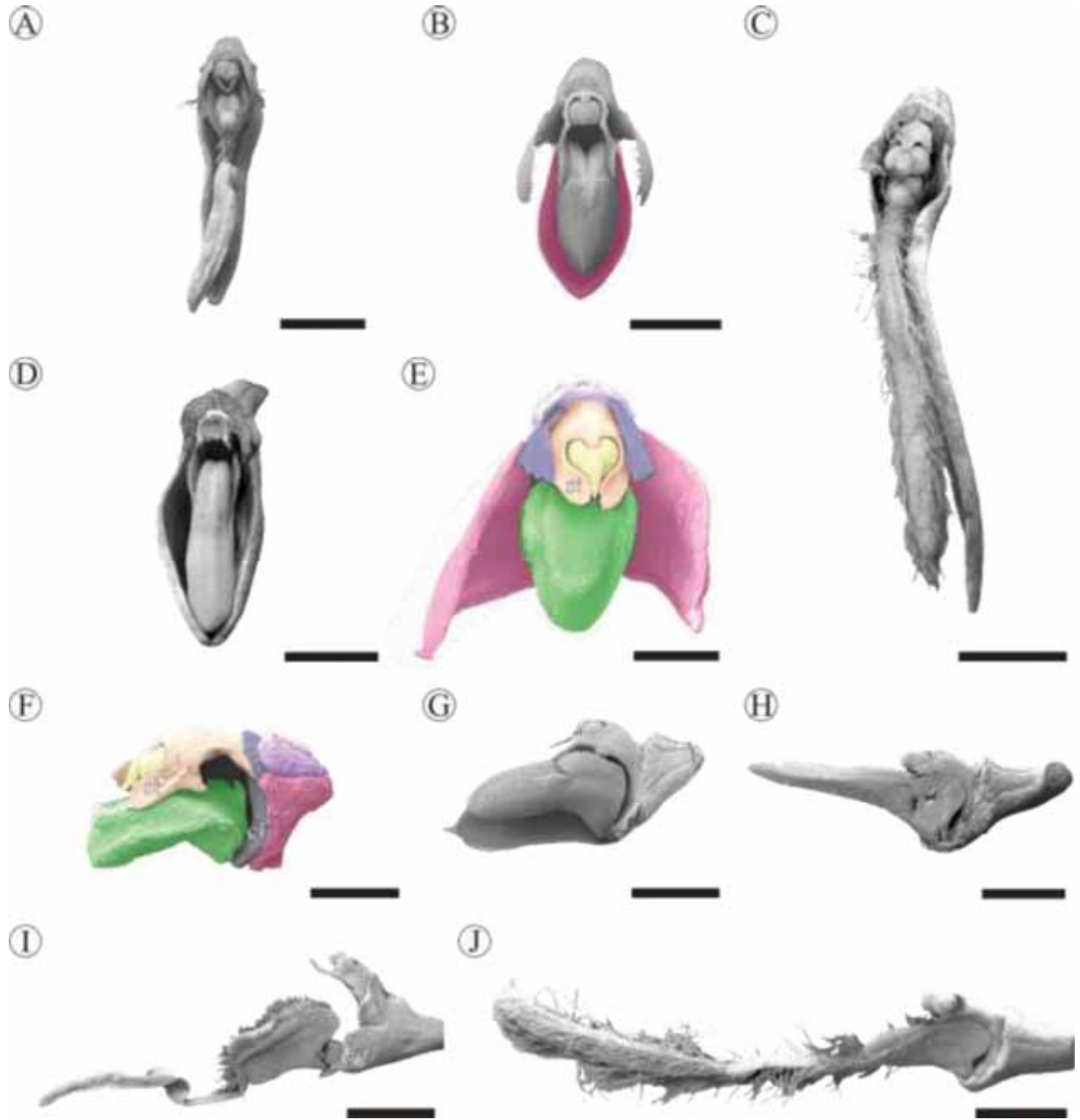


Figure 2. Micromorphology and anatomy of sepals of *Bulbophyllum* sect. *Xiphizusa*. A, H, L) *B. manarae*; B, G) *B. gladiatum*; C, K) *B. bidentatum*; D, F) *B. ciluliae*; E, I, J) *B. plumosum*. A–C) Dorsal sepal, adaxial surface; D, E, K) Dorsal sepal, transverse section (TS); F) Dorsal sepal, abaxial surface; G) Dorsal sepal, detail of secretory trichome at abaxial surface; H, I) Dorsal sepal, margin; J) Dorsal sepal, detail of the subepidermal secretory epithelium; L) Lateral sepal, adaxial surface. ih, idioblast of helical thickenings, ri, raphides idioblasts; red circles outlines, vascular bundles. Scale bars: 40µm in A–C, F, L; 200µm in D, E, J, K; 20µm in G; 100µm in H, I.

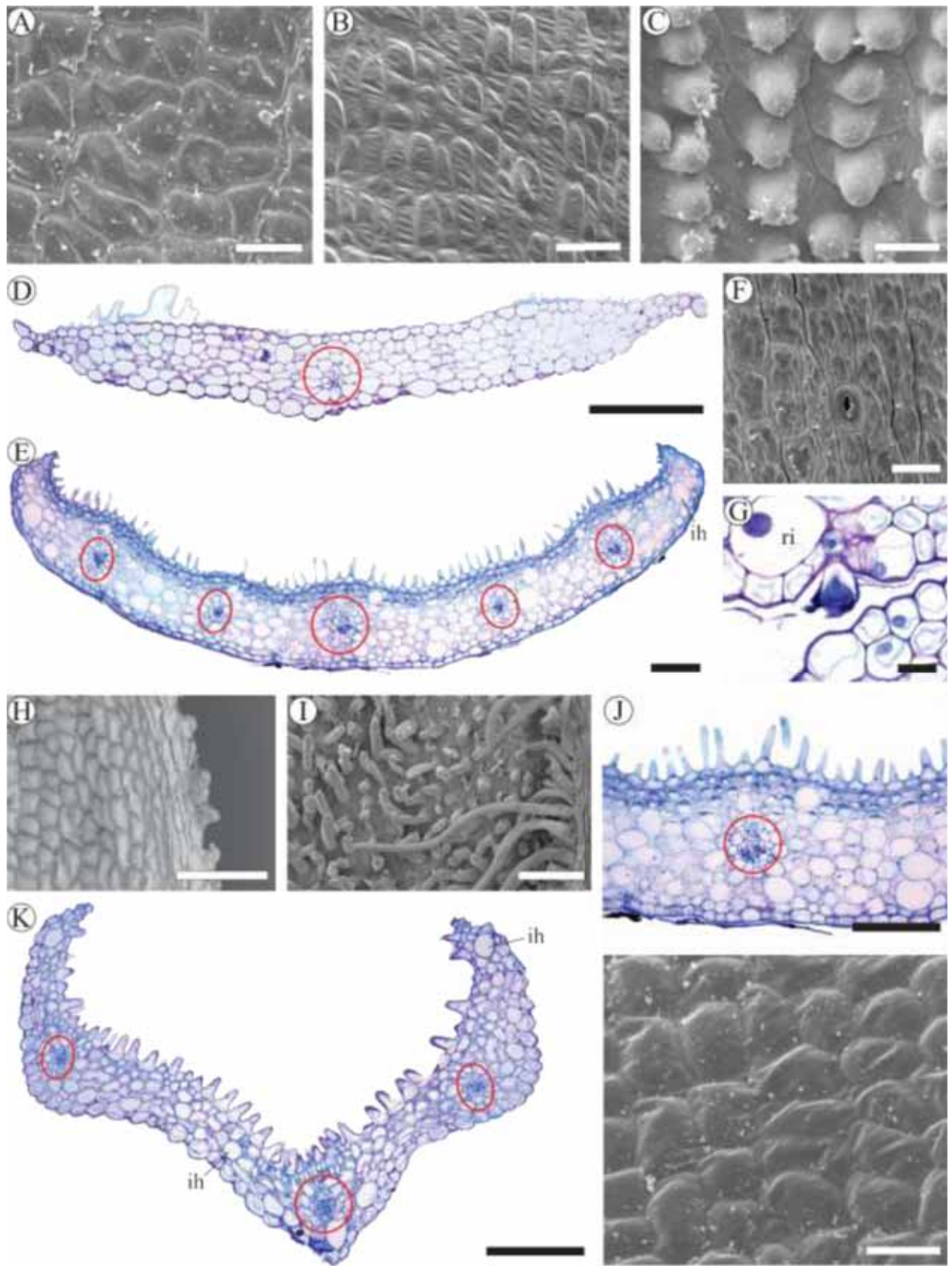


Figure 3. Micromorphology and anatomy of lateral sepals and petals of *Bulbophyllum* sect. *Xiphizusa*. A, N) *B. gladiatum*; B) *B. melloi*; C, E, H, O) *B. plumosum*; D, I, K, M) *B. ciluliae*; F) *B. manarae*; G, J, L) *B. bidentatum*. A, B) Lateral sepal, transverse section (TS); C–F) Lateral sepal, abaxial surface; G, H) Synsepal; I, J) Petal, adaxial surface; K, L) Petal, TS; M–O) Petal, margin. ih, idioblast of helical thickenings, ri, raphides idioblasts; *, synsepal, red circles outlines, vascular bundles. Scale bars: 200µm in A, L, M–O; 250µm in B; 50µm in C, E; 40µm in D, F, I, J; 100µm in G, H, K.

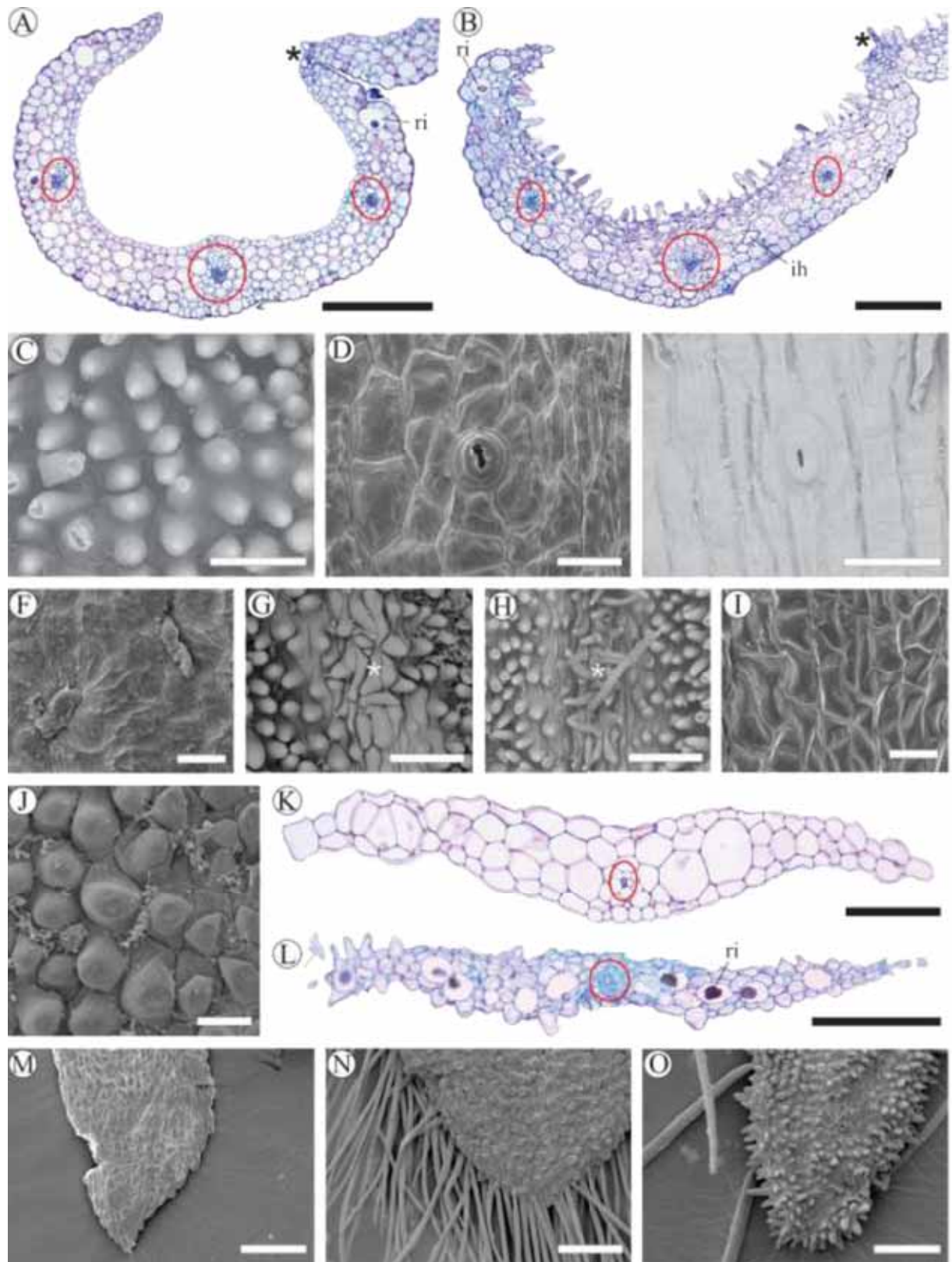


Figure 4. Micromorphology and anatomy of labella of *Bulbophyllum* sect. *Xiphizusa*. A, E) *B. bidentatum*; B, D, H) *B. ciluliae*; C, J) *B. plumosum*; F) *B. gladiatum*; G, I) *B. melloi*. A–C) Epichile, adaxial surface; D, E, G) Epichile, transverse section (TS); F) Epichile, margin; H, J) Callus, adaxial surface; I) Hypochile, TS. ri, raphides idioblasts; red circles outlines, vascular bundles. Scale bars: 40µm in A–C; 200µm in B, E, G; 400µm in F; 50µm in H; 500µm in I; 100µm in J.

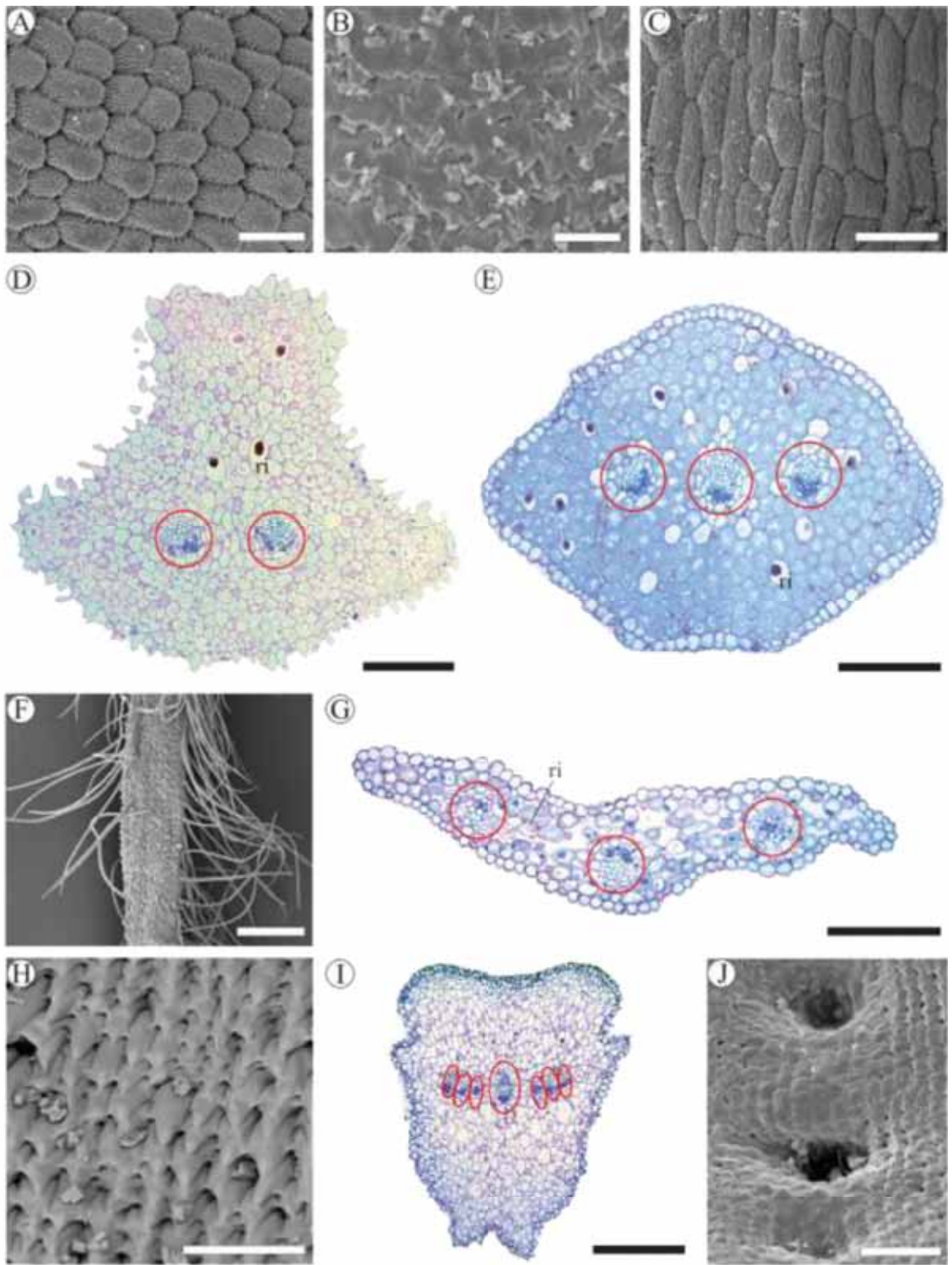


Figure 5. Micromorphology and anatomy of labella and gynostemium of *Bulbophyllum* sect. *Xiphizusa*. A–C, F, H) *B. plumosum*; D, E) *B. bidentatum*; G) *B. manarae*; I, K) *B. ciluliae*; J) *B. gladiatum*. A, E) Hypochile, transverse section (TS); B) Hypochile, longitudinal section (LS); C, D) Hypochile, abaxial surface; F, G) Lateral lobes, margin; H, I) Gynostemium surface; J) Gynostemium, TS at stigma level; K) Flower, LS. cf, column foot, ds, dorsal sepal, l, labellum, ov, ovary, ri, raphides idioblasts, si, stigma, vi, viscidium; red circles outlines, vascular bundles. Scale bars: 500µm in A, K; 200µm in B, E, G, J; 50µm in C; 40µm in D, H, I; 400µm in F.

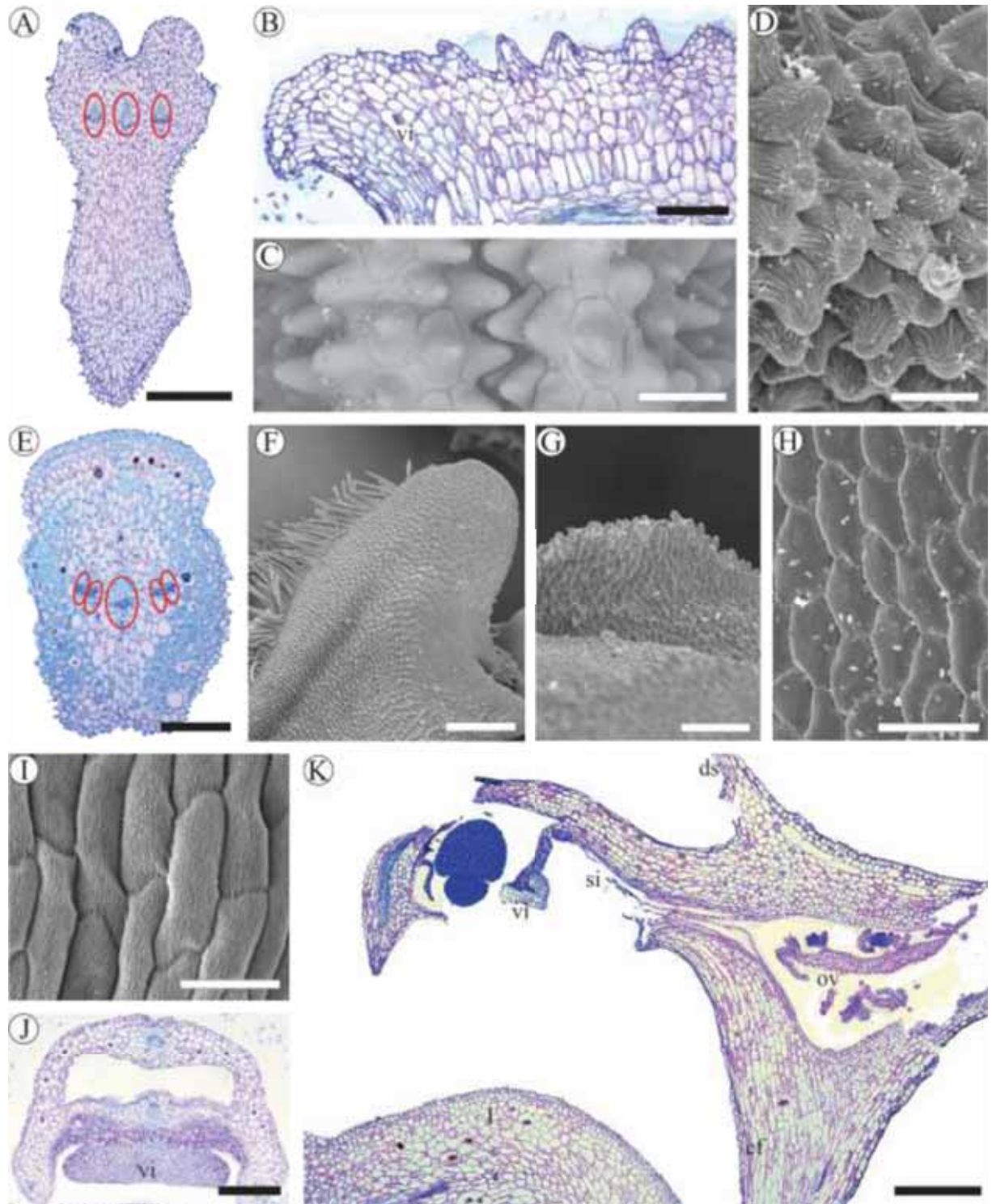
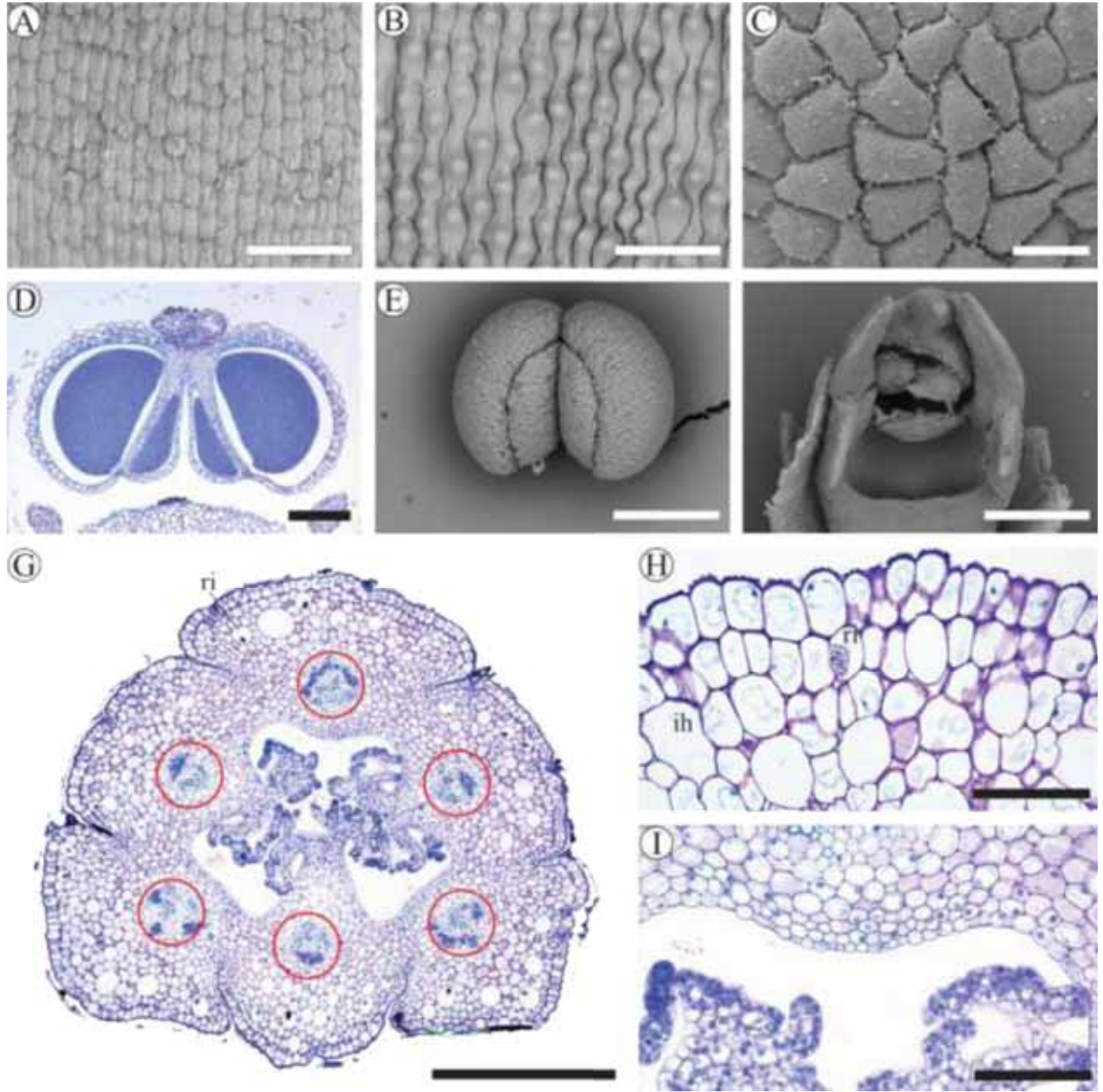


Figure 6. Micromorphology and anatomy of gynostemium of *Bulbophyllum* sect. *Xiphizusa*. A, F) *B. ciluliae*; B, E) *B. bidentatum*; C, H) *B. melloi*; D, G, I) *B. gladiatum*. A, B) Column foot surface; C) Anther surface; D) Anther, transverse section (TS); E) Pollinarium; F) Stigma; G) Ovary, TS; H) Ovary detail, outer epidermis, TS; I) Ovary detail, inner epidermis, TS. ih, idioblast of helical thickenings, ri, raphides idioblasts; red circles outlines, vascular bundles. Scale bars: 100µm in A, B, H, I; 40µm in C; 200µm in D; 250µm in E; 500µm in F, G.



CAPÍTULO V

Floral features of three species belonging to the Neotropical *Bulbophyllum* sect. *Furvescens* and *B.* sect. *Bulbophyllaria* (Orchidaceae, Asparagales)

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(A ser submetido ao periódico *Plant Systematics and Evolution* como *short communication*)

Abstract

Bulbophyllum sect. *Furvescens* and *B.* sect. *Bulbophyllaria* are sister groups and together considered the basalmost sections of Neotropical *Bulbophyllum*. Together, these sections comprise eight species mainly distributed in Central America and north of South America. The present study aims to describe floral micromorphology and anatomy of two species of *B.* sect. *Furvescens* (*B. setigerum* and *B. steyermarkii*) and one of *B.* sect. *Bulbophyllaria* (*B. bracteolatum*) to address the following questions: a) which features are common to the three species studied; b) which features are common to species belonging to *B.* sect. *Furvescens* and thus help better characterise it; and c) are there appreciable floral features distinguishing between both sections studied? To achieve that, usual techniques of light and scanning electron microscopies were used. From the ca. 100 floral characteristics analysed, 60 were homogeneous to all species and 74 were shared by species of *B.* sect. *Furvescens* only. Each species studied has a set of distinguishable characteristics. *Bulbophyllum setigerum* has sepals whose idioblasts with helical wall thickenings are preferably contiguous to the abaxial surface; petals with papillose margins; and elongate stelidia. *Bulbophyllum steyermarkii* has sepals with idioblasts with helical wall thickenings preferably contiguous to the adaxial surface; labellum with a developed secretory epithelium; and flattened and falcate stelidia. *Bulbophyllum bracteolatum* has sepals with idioblasts with raphides preferably contiguous to the abaxial surface; labellum with papillose cells and stomata on its abaxial surface; short stelidia; and anther with no beak. Similarities with *B. mentosum* (*B.* sect. *Micranthae*) and their implications are discussed.

Keywords *Bulbophyllum* • Floral anatomy • Micromorphology • Taxonomy

Introduction

Floral morphological characteristics have been extensively used to understand the origin, evolution, phylogenetic relationships and pollination of Orchidaceae (Stern et al. 1993; Freudenstein and Rasmussen 1999; Rudall 2002; Benitez-Vieyra 2006). In addition, anther characters, such pollinarium structure, have been traditionally used for classification of orchids (Dodson 1962; Romero 1990), even though such features are believed to be susceptible to selective pressure from pollinators and, therefore, likely to display high levels of parallelism or convergence (Dodson 1962; Atwood 1986).

In *Bulbophyllum*, the largest genus of Orchidaceae, with around 2200 species (Gravendeel et al. 2014), studies using floral anatomical and morphological data are rather scarce. The labellum anatomy of one Asian and six Brazilian species of *Bulbophyllum* was studied by Teixeira et al. (2004) and the authors suggest a link between the need of air currents to effective pollination (described by Borba and Semir 1998) and the presence of nectar in some species and also that labellum structure is homogeneous and highly conservative for the genus. Until recently, apart from that report, the only information available was on the morphology and development of the gynostemium of an Asian species, revealing the nature of its pollinium stalk (Rasmussen 1985).

Nunes et al. (2014) studied the floral micromorphology and anatomy of the Neotropical *Bulbophyllum* sect. *Didactyle*, investigating features that could be used to distinguish between species. Their data revealed many useful floral characteristics and also found out a greater diversity on labellum micromorphology than previously anticipated. The same authors conducted similar studies on Neotropical species belonging to *B.* sect. *Napelli* (Nunes et al. submitted – Capítulo II), *B.* sect. *Micranthae* (Nunes et al. in prep. – Capítulo III) and *B.* sect. *Xiphizusa* (Nunes et al. in prep. – Capítulo IV) and found even greater diversity not only in the labellum, but also in the other floral parts.

From the six sections currently recognised for Neotropical *Bulbophyllum*, *B.* sect. *Furvescens* E.C. Smidt, Borba & Van den Berg and *B.* sect. *Bulbophyllaria* (Rchb.f.) Griseb are sister groups and together are sister to the remaining Neotropical sections (Smidt et al. 2011) and the only groups whose floral anatomy and micromorphology are completely unknown.

Bulbophyllum sect. *Furvescens* was recently proposed (Smidt et al. 2011) to accommodate five species characterised by unifoliate pseudobulbs, inflorescence with a fleshy rachis and flowers spirally arranged, free sepals, erect petals and a column foot with entire apex and shorter than column length (Smidt et al. 2011). Species included in this section are distributed in Brazil, Ecuador, French Guiana, Guyana, Mexico, Peru, Suriname, and Venezuela (Smidt et al. 2011; Gravendeel et al. 2014).

Bulbophyllum sect. *Bulbophyllaria* comprises three species occurring in Cuba, Colombia, Costa Rica, Dominican Republic, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Panama, and Venezuela (Smidt et al. 2011; Gravendeel et al. 2014). Species belonging to this section are characterised by bifoliate pseudobulbs, inflorescence with a fleshy rachis and flowers spirally arranged, free sepals, erect petals and column foot with entire apex and shorter than column length (Smidt et al. 2011; Gravendeel et al. 2014).

The present study aims to describe the floral micromorphology and anatomy of two species currently included in *B. sect. Furvescens* and one included in *B. sect. Bulbophyllaria* to address the following questions: a) which features are common to the three species studied; b) which features are common to species belonging to *B. sect. Furvescens* and thus help better characterise this recently proposed section; and c) are there appreciable floral features distinguishing between *B. sect. Furvescens* and *B. sect. Bulbophyllaria*?

Materials and methods

Material from three species of *Bulbophyllum*, belonging to *B. sect. Furvescens* and *B. sect. Bulbophyllaria*, was collected on the field, fixed in FAA 50 (3.7% v/v formaldehyde, 50% ethanol, 5% acetic acid; Johansen 1940) and stored in 70% ethanol. The collection data is available on Table 1.

For floral micromorphological and anatomical data assessment, usual techniques of light microscopy (LM) and scanning electron microscopy (SEM) were used. For SEM analysis, the samples were transferred from 70% ethanol to a propanone series, then dehydrated through a critical point drier (CPD 030 - Bal-Tec AG, Balzers, Liechtenstein), and sputter coated with gold (SCD 050 - Bal-Tec AG, Balzers, Liechtenstein). Observations were made on a TM3000 (Hitachi, Tokyo, Japan) SEM and the images were acquired through its software.

For LM analysis, the samples were dehydrated through a graded ethanol series and embedded in historesin (Leica Historesin Embedding Kit, Nussloch, Germany). Sections (5-8 µm) were cut on a rotary microtome RM2245 (Leica Microsystems GmbH, Wetzlar, Germany) using disposable blades and stained with 0.05% Toluidine Blue O in 0.1 M sodium phosphate buffer pH 6.8 (O'Brien et al. 1965) for general analysis. The following histochemical tests were carried out on resin-embedded sections: Calcofluor White MR2 for cellulose (Hughes and McCully 1975), Coomassie Brilliant Blue for proteins (Southworth 1973), Ruthenium Red for pectin acids/mucilage (Johansen 1940), and Periodic Acid-Schiff reaction for total polysaccharides (Feder and O'Brien 1968). Autofluorescence was observed on a DMLB microscope (Leica Microsystems GmbH, Wetzlar, Germany) using filter cubes D, N2.1 and I3. Photomicrographies were obtained through a Leica DMLB microscope coupled with a digital camera and using the software LAS (Leica Application Suite V4.0).

Results

The description of the results is given according to the section the species studied belong. The results of histochemical tests performed on the three species studied are summarised in Table 2, and the variable characteristics in Table 3.

Bulbophyllum sect. *Furvescens*

The flowers of both *Bulbophyllum setigerum* and *B. steyermarkii* are characterised by free sepals with thickened bases (Fig. 1A–D, dorsal sepal in pink and lateral in red); glabrous petals smaller than the sepals (Fig. 1A–D, in purple); entire labellum, more or less fleshy and without lateral or basal appendices (Fig. 1A–D, in green); gynostemium (Fig. 1A–D, in orange) with conspicuous stelidia (cylindrical and elongate or flattened and falcate), inconspicuous teeth, foot and ovary short (Fig. 1A–D).

The dorsal sepal, on its adaxial surface, lacks conspicuous ornamentation on the outer periclinal walls; *B. steyermarkii* shows perpendicular stripes over the anticlinal walls (Fig. 1E). In frontal view, the epidermal cells are irregular (Fig. 1E) or isodiametric (Fig. 1F); the outer periclinal walls are papillose in *B. setigerum* (Fig. 1F–G) and straight in *B. steyermarkii* (Fig. 2A). The abaxial surface lacks conspicuous ornamentation, and shows irregular shaped epidermal cells (Fig. 2B) with convex outer periclinal walls (Figs 1G, 2A, C). At this surface, sunken glandular trichomes are observed in both species (Fig. 2B, C). The margin of the dorsal sepal is smooth (Fig. 2D) and composed of both epidermal and mesophyll cells in transverse section (Figs 1G, 2A, E).

The mesophyll is homogeneous, formed by regular parenchyma and with two types of idioblasts: with raphides and with helical wall thickenings (Figs 1G, 2A, C, E). In *B. setigerum*, most idioblasts with helical wall thickenings are distributed more or less symmetrically and contiguous to the abaxial surface (Fig. 1G); in *B. steyermarkii*, these idioblasts occur mainly contiguous to the adaxial surface, causing the rugose/wavy aspect (Fig. 2A). Both species show three collateral vascular bundles in the median third of the dorsal sepal (Figs 1G, 2A, E). *Bulbophyllum steyermarkii* shows notably fleshy sepals, especially near their base where the mesophyll shows more layers of regular-sized cells and also smaller adaxial subepidermal ones (Fig. 2E).

The lateral sepals are similar to the dorsal one, but shows longitudinally elongate epidermal cells on the abaxial surface.

The petals show similar adaxial and abaxial surfaces, with striate ornamentation in *B. setigerum* (Fig. 2F), and no conspicuous ornamentation in *B. steyermarkii* (Fig. 2G). In frontal view, the epidermal cells are longitudinally elongate (Fig. 2F, G) and possess convex outer periclinal walls (Fig. 2H). The margin is papillose in *B. setigerum* (Fig. 2H, I) and smooth in *B. steyermarkii* (Fig. 3A); in transverse section, it is formed by both epidermal and mesophyll cells (Fig. 2H). The mesophyll is homogeneous, formed by regular parenchyma with both idioblasts with raphides and with helical wall thickenings (Fig. 2H). There is a single collateral vascular bundle (Fig. 2H).

The distal portion of the labellum, on its adaxial surface, shows striate scale-like papillae (Fig. 3B); in *B. setigerum*, at the labellum apex, the epidermal cells become more elongate and give rise to trichomes (Fig. 3C). The abaxial surface is similar to the adaxial one, but lacks trichomes throughout (Fig. 3D). In *B. setigerum*, the mesophyll is homogeneous and formed by regular parenchyma (Fig. 3E) while in *B. steyermarkii* it also has a secretory epithelium at the periphery of the labellum (Fig. 3F); in both species, idioblasts with raphides and others with helical wall thickenings are present (Fig. 3E, F). There are two collateral vascular bundles in *B. setigerum* (Fig. 3E) and seven in *B. steyermarkii* (Fig. 3F).

The adaxial surface of the proximal portion of the labellum is similar to that of the distal one, but shows a differentiated sulcus (Fig. 3G, H). The sulcus is wide and located between two ridges in *B. setigerum* (Fig. 3G) while it is narrower and without ridges in *B. steyermarkii* (Fig. 3H). The abaxial surface lacks conspicuous ornamentation (Fig. 4A), its epidermal cells are irregular-shaped (Fig. 4A) and show either convex (Fig. 4A) or papillose (Fig. 4B) outer periclinal walls. There is a keel, more conspicuous in *B. steyermarkii* (Fig. 1D), where stomata are present (Fig. 4B). The mesophyll is similar to that of the distal portion of the labellum, but three vascular bundles are present in *B. setigerum* (Fig. 3G) and five in *B. steyermarkii* (Fig. 3H).

The gynostemium surface is striate *B. steyermarkii* (Fig. 4C) and smooth in *B. setigerum* (Fig. 4D). The epidermal cells, in frontal view, are longitudinally elongate (Fig. 4C, D). The mesophyll is homogeneous, with regular parenchyma that shows both idioblasts with raphides and with helical wall thickenings (Fig. 4E). Viscidium deriving from rostellum cells is present in both species (Fig. 4F). The column foot has striate surface and longitudinally elongate epidermal cells with convex outer periclinal walls (Fig. 4G); the mesophyll is similar to that of the gynostemium (Fig. 4E).

The anther shows a beak (Fig. 1A–D), which is long in *B. setigerum* (Fig. 1A, C) and

short in *B. steyermarkii* (Fig. 1B, D); its epidermal cells are papillose and shows striate ornamentation (Fig. 4H, I). The pollinarium is composed of four pollinia (Fig. 4I). The stigma is wide (Fig. 4J) and filled by elongate cells and mucilage (Fig. 4E, F, J). The ovary is six-lobed and unilocular (Fig. 4K), with the outer epidermis formed by rectangular to irregular-shaped cells (Fig. 4L) and the inner by irregular cells (Fig. 4M). The mesophyll is homogeneous, formed by regular parenchyma and with idioblast with raphides and with helical wall thickenings (Fig. 4K, L). There is a collateral vascular bundle in each lobe (Fig. 4K, L).

Bulbophyllum sect. *Bulbophyllaria*

Bulbophyllum bracteolatum possesses flowers with free sepals (Fig. 5A, B); glabrous petals smaller than the sepals (Fig. 5A); entire labellum, fleshy and with an abaxial keel (Fig. 5A, B); gynostemium (Fig. 5A, B) with short and acute stelidia and inconspicuous teeth and a short foot (Fig. 5A, B).

The dorsal sepal, on its adaxial surface, has smooth to slightly striate ornamentation on the outer periclinal walls (Fig. 5C). In frontal view, the epidermal cells are irregular (Fig. 5C) and the outer periclinal walls are convex (Fig. 5D, E). The abaxial surface shows striate ornamentation and irregular shaped epidermal cells (Fig. 5F) with convex outer periclinal walls (Fig. 5D, E). Both surfaces of the epidermis are uniseriate and, on the abaxial one, sunken glandular trichomes are present (Fig. 5F). The margin of the dorsal sepal is smooth (Fig. 5G) and composed of both epidermal and mesophyll cells in transverse section (Fig. 5H). The mesophyll is homogeneous, formed by regular parenchyma and with two types of idioblasts: with raphides and with helical wall thickenings (Fig. 5D, E, H); idioblasts with raphides are distributed more or less symmetrically and contiguous to the abaxial epidermis, causing the rugose/wavy aspect of this surface (Fig. 5D). Three collateral vascular bundles are present in the median third of the dorsal sepal (Fig. 5D).

The lateral sepals are similar to the dorsal one, but the adaxial epidermal cells are papillose (Fig. 5I) and two collateral vascular bundles are present (Fig. 5J).

The adaxial surface of the petal is smooth and shows irregular-shaped epidermal cells, in frontal view (Fig. 6A); in transverse section, their outer periclinal walls are convex (Fig. 6B). The abaxial surface shows striate ornamentation and epidermal cells more longitudinally elongate (Fig. 6C). The margin is smooth, and formed by epidermal and mesophyll cells in transverse section (Fig. 6B). At the median third of the petal, the mesophyll is homogeneous,

formed by regular parenchyma with both idioblasts with raphides and with helical wall thickenings (Fig. 6B); there is a single collateral vascular bundle (Fig. 6B). Near the petal apex, only idioblasts with raphides are present, and the epidermal cells towards the centre of the abaxial surface are papillose (Fig. 6D).

The distal portion of the labellum, on the adaxial surface, shows striate scale-like papillae (Fig. 6E); at the labellum apex, the epidermal cells become more elongate (Fig. 6F, G – in detail). The abaxial surface is similar to the adaxial one, but with papillose cells (Fig. 6H) and some stomata in the middle line (Fig. 6I). The margin is smooth (Fig. 6F). The mesophyll is homogeneous and formed by regular parenchyma (Fig. 6J, K), with both idioblasts with raphides and with helical wall thickenings (Fig. 6J, K); there are seven collateral vascular bundles (Fig. 6J).

The adaxial surface of the proximal portion of the labellum is similar to that of the distal one, but shows a differentiated sulcus between two ridges (Figs 6K, 7A). The abaxial surface is similar to that of the distal portion of the labellum, but shows a conspicuous keel, with stomata (Fig. 7B). The mesophyll is similar to that of the distal portion of the labellum, but with five vascular bundles.

The gynostemium surface lacks conspicuous ornamentation (Fig. 7C). The epidermal cells, in frontal view, are irregular-shaped (Fig. 7C). The mesophyll is homogeneous, with regular parenchyma that shows both idioblasts with raphides and with helical wall thickenings. Viscidium deriving from rostellum cells is present (Fig. 7D). The column foot has striate surface and longitudinally elongate epidermal cells with both convex and papillose outer periclinal walls (Fig. 7E); the mesophyll is similar to that of the gynostemium.

The anther does not show a conspicuous beak (Fig. 7F) and its epidermal cells are papillose with striate ornamentation (Fig. 7F, G); the pollinarium is composed of four pollinia (Fig. 7H). The stigma is wide (Fig. 7H) and filled by elongate cells and mucilage (Fig. 7I). The ovary is six-lobed and unilocular (Fig. 7J), with the outer epidermis formed by rectangular cells (Fig. 7K) and the inner by elliptic to irregular cells (Fig. 7L). The mesophyll is homogeneous, formed by regular parenchyma and with idioblast with raphides and with helical wall thickenings (Fig. 7J–L). There is a collateral vascular bundle in each lobe (Fig. 7J).

Discussion

Bulbophyllum setigerum and *B. steyermarkii* (*B. sect. Furvescens*) and *B.*

bracteolatum (*B. sect. Bulbophyllaria*) share ca. 60 floral characteristics out of 100 that were analysed, especially the presence of sunken glandular trichomes on the abaxial surface of the sepals; idioblasts with raphides and with helical wall thickenings on the mesophyll of the sepals; three vascular bundles on the dorsal sepal; a single vascular bundle on the petals; presence of secretory sulcus on the adaxial surface of the labellum; and a keel on the abaxial surface of the labellum.

Bulbophyllum setigerum and *B. steyermarkii*, representing *B. sect. Furvescens*, showed similarities in 74 characteristics out of ca. 100 here analysed; the remarkable are: sepals lacking conspicuous ornamentations on their epidermal walls, with sunken glandular trichomes on the abaxial surface and three collateral vascular bundles; petals with a single collateral vascular bundle; entire labellum with striate scale-like papillae on the adaxial surface, and smooth convex-walled cell on the abaxial surface; proximal portion of the labellum with a secretory sulcus on the adaxial surface and with a keel bearing stomata on the abaxial one; gynostemium with conspicuous stelidia and inconspicuous teeth; anther with a beak and striate papillose epidermal cells and bearing four pollinia; idioblasts with raphides and with helical wall thickenings present in all floral parts (except for the labellum of *B. steyermarkii*).

It is interesting to note that the similarity between the three species studied (60 shared characteristics – Table 3) may either reflect a close relationship between species currently included in *B. sect. Furvescens* and *B. sect. Bulbophyllaria*, or some degree of floral convergence mediated by environmental pressures and/or pollinators. To strengthen the first hypothesis, from the ca. 40 characteristics that showed variation between the three species (refer to Table 3), *B. setigerum* (*B. sect. Furvescens*) and *B. bracteolatum* (*B. sect. Bulbophyllaria*) are similar in 12 characteristics (including the presence of two ridges on the labellum, besides the overall shape of the latter – Table 3, shared states are highlighted in blue), while the latter shares with *B. steyermarkii* (*B. sect. Furvescens*) eight states of the analysed characteristics (Table 3, shared states are highlighted in pink).

In addition, molecular phylogenetic studies using maximum parsimony put the two sections as sister groups, albeit without bootstrap support (Smidt et al. 2011); *B. sect. Furvescens* itself also lacked bootstrap support. Considering our limited sampling and analysis centred only in floral data, besides the recognition that in some Orchidaceae groups vegetative data are more congruent with genetic relatedness (van den Berg et al. 2000; Cameron 2005; Chase et al. 2009), it is advisable to both broaden the sampling and include vegetative characters to deepen systematic discussions on these sections. Interestingly, from

the vegetative characteristics pointed out by Smidt et al. (2011) as diagnostic for *B.* sect. *Furvescens*, most match with *B.* sect. *Bulbophyllaria*, with the main differences being the presence of two leaves on the bulb and the shape of the rachis of species included within the latter section.

To account for the convergence hypothesis, in the Bayesian analysis, *B.* sect. *Furvescens* appears as sister to a clade that includes the remaining Neotropical *Bulbophyllum* (with *B.* sect. *Bulbophyllaria* as sister to the other four sections) with posterior probability of 0.51 (Smidt et al. 2011). Regarding the reproductive biology, information is available only for *B. setigerum*, whose flowers offer labellar nectar as reward and are pollinated by Milichiidae flies (Braga 1977), similar to *B.* sect. *Didactyle* – the latter with wind assistance (Borba and Semir 1998). Environmental pressures cannot be ruled out as driver of floral similarities between the studied species, especially *B. bracteolatum* and *B. setigerum* that occur as epiphytes in Amazonia (Barros et al. 2014), even though they have a broader distribution (Smidt 2007).

Differently from the other Neotropical sections (Smidt et al. 2011; Nunes et al. 2014), the basal species studied here are easily differentiable from the floral morphology standpoint, but floral micromorphology and anatomy add more information and some unique features. *Bulbophyllum setigerum* (*B.* sect. *Furvescens*) can be recognized by the sepals with a mesophyll whose idioblasts with helical wall thickenings are preferably distributed contiguous to the abaxial surface; petals with papillose margins; distal portion of the labellum clothed with papillose cells and with two vascular bundles on the mesophyll; proximal portion of the labellum with three vascular bundles; elongate and cylindrical stelidia; and anther with a prominent beak. *Bulbophyllum steyermarkii* (*Bulbophyllum* sect. *Furvescens*) shows sepals with perpendicular stripes on the epidermal anticlinal walls, besides a subepidermal tissue on the mesophyll (on the sepal base) and idioblasts with helical wall thickenings preferably distributed contiguous to the adaxial epidermis; labellum with a developed secretory epithelium and a narrow secretory sulcus; flattened and falcate stelidia; and anther with a short beak. *Bulbophyllum bracteolatum* (*B.* sect. *Bulbophyllaria*) shows sepals with idioblasts with raphides preferably distributed contiguous to the abaxial surface; lateral sepals with 2 vascular bundles (on the median third); labellum with papillose cells and stomata on the abaxial surface; stelidia short and acute; column foot with both convex-walled and papillose epidermal cells; and anther without a distinctive beak.

While some of the aforementioned characteristics were found to be exclusive to a single species in this study, some of them are notably shared with *B. mentosum* (Nunes et al.

in prep. – Capítulo III). This species is currently included in *B.* sect. *Micranthae* (Smidt et al. 2011), but with incongruence between the plastid and nrITS data. Morphologically, *B. mentosum* shares with the species studied here the fleshy sepals; the short, fleshy and entire labellum; and short column foot and ovary (Nunes et al. in prep. – Capítulo III). In spite of the sepal thickness of *B. mentosum* superficially resemble that of *B. steyermarkii*, the former does not show a subepidermal mesophyll tissue on the basal third of the sepals (E.L.P. Nunes, unpublished results), and thus the thickness is due to the amount of common mesophyll cells only, as seen in *B. setigerum* and *B. bracteolatum*. In addition, *B. mentosum* and *B. setigerum* also share the abaxial distribution of idioblasts with helical wall thickenings on the sepals (Nunes et al. in prep. – Capítulo III).

Despite the similarities highlighted before between the species studied here and *B. mentosum*, this latter species shows both different habit and habitat – lithophyte in Cerrado and Campos Rupestres (Barros et al. 2014), thus convergent or parallel evolution on floral traits due primarily to environmental pressures seems unlikely. As already stressed by Nunes et al. (in prep. – Capítulo III), the positioning of *B. mentosum* in *B.* sect. *Micranthae* based on floral characteristics is controverse and until more data is available, especially from reproductive biology, neither a closer relationship of this species with the basal species studied here nor its inclusion in *B.* sect. *Micranthae* (but with severe divergence on floral features) can be excluded.

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Table 1 List of species studied and collection data.

Section	Species	Voucher
<i>Furvescens</i>	<i>Bulbophyllum steyermarkii</i>	Spirit material (E. C. Smidt 780,
	Foldats	Cuenca, Ecuador)
	<i>B. setigerum</i> Lindl.	Spirit material (J. Batista s.n, São Gabriel da Cachoeira, AM, Brazil)
<i>Bulbophyllaria</i>	<i>B. bracteolatum</i> Lindl.	UFMT (A. Petini-Benelli 173, Paranaíta, Mato Grosso, Brazil)

UFMT: Universidade Federal de Mato Grosso.

Table 2 Histochemical tests performed and reaction sites

Histochemical test	Reaction site
Coomassie Brilliant Blue	Cytoplasmic proteins (epidermis and subjacent tissue on the labellum sulcus)
Calcofluor White MR2	All cellulosic cell walls (including those of the idioblast with helical wall thickenings)
Ruthenium Red	Sunken glandular trichomes
Intrinsic fluorescence under UV	Cuticle; xylem elements

Table 3 Variable characteristics between the studied species belonging to *B.* sect. *Furvescens* and *B.* sect. *Bulbophyllum*

Characteristic			<i>Bulbophyllum</i> sect. <i>Furvescens</i>		<i>B.</i> sect. <i>Bulbophyllum</i>
			<i>B. setigerum</i>	<i>B. steyermarkii</i>	<i>B. bracteolatum</i>
Dorsal Sepal	AD	Ornamentation on pw	smooth	smooth	smooth/slightly striate
		Ornamentation on aw	absent	perpendicular stripes	absent
		Cell shape in fv	isodiametric	irregular	irregular
		Outer pw shape in TS	papillose	convex	convex
	AB	Ornamentation on pw	smooth	smooth	striate
		Ornamentation on aw	absent	perpendicular stripes	absent
	Mesophyll (basal third)	Subepidermal layers with small cells	absent	present	absent
	Mesophyll (median third)	Localization of idioblasts with helical wall thickenings	contiguous to abaxial epidermis	contiguous to adaxial epidermis	disperse only
		Localization of idioblasts with raphides	disperse only	disperse only	contiguous to abaxial epidermis
Lateral sepal	AD	Ornamentation on pw	smooth	smooth	smooth/slightly striate
		Ornamentation on aw	absent	perpendicular stripes	absent
		Cell shape in fv	isodiametric	irregular	irregular
		Outer pw in TS	papillose	convex	papillose
	AB	Ornamentation on pw	smooth	smooth	striate
		Ornamentation on aw	absent	perpendicular stripes	absent
		Cell shape in fv	longitudinally elongate	longitudinally elongate	irregular
	Mesophyll (basal third)	Subepidermal layers with small cells	absent	present	absent
	Mesophyll (median third)	Localization of idioblasts with helical wall thickenings	contiguous to abaxial	contiguous to adaxial	disperse only

			epidermis	epidermis	contiguous to abaxial epidermis
			disperse only	disperse only	
		Localization of idioblasts with raphides			
		Vascular bundles	3	3	2
Petal	AD	Ornamentation on pw	striate	smooth	smooth
		Cell shape in fv	longitudinally elongate	longitudinally elongate	Irregular
		Margin	papillose	smooth	smooth
	AB	Ornamentation on pw	striate	smooth	striate
Labellum (distal half)	AD	Cell type	papillose	scale-like papillae	scale-like papillae
	AB	Stomata Presence	absent	absent	present
	Mesophyll	Parenchyma type	regular	regular, secretory	regular
		Vascular bundles	2	7	7
		Secretory sulcus shape in TS	wide	narrow	wide
Labellum (proximal half)	AD	Presence of two ridges	present	absent	present
		Cell type	convex	convex	papillose
	Mesophyll	Parenchyma type	regular	regular, secretory	regular
		Vascular bundles	3	5	5
		Ornamentation on pw	smooth	striate	smooth
Column	Gynostemium Epidermis	Cell shape in fv	longitudinally elongate	longitudinally elongate	irregular
	Foot Epidermis	Outer pw shape in TS	convex	convex	convex, papillose
	Stelidia	Shape	cylindrical, elongate	flattened, falcate	short, acute
	Anther	Beak presence	present	present	absent
		Beak size	long	short	-

Similar states for *B. setigerum* and *B. bracteolatum* are highlighted in blue, similar states for *B. bracteolatum* and *B. steyermarkii* are highlighted in pink, and exclusive states are in bold. AB: abaxial surface, AD: adaxial surface, aw: anticlinal walls, fv: frontal view, pw: periclinal walls, TS: transverse section

Figures

Figure 1. Micromorphology and anatomy of flowers and dorsal sepals of *Bulbophyllum* sect. *Furvescens*. A, C, F, G) *B. setigerum*. B, D, E) *B. steyermarkii*. A, B) Flower in frontal view. C, D) Flower in side view. E, F) Dorsal sepal, adaxial surface. G) Median third of dorsal sepal, transverse section. ih, idioblast with helical thickenings; st, stelidia; red circles outlines, vascular bundles. Scale bars: 500µm in A; 1mm in B–D; 15µm in E; 25µm in F; 100µm in G.

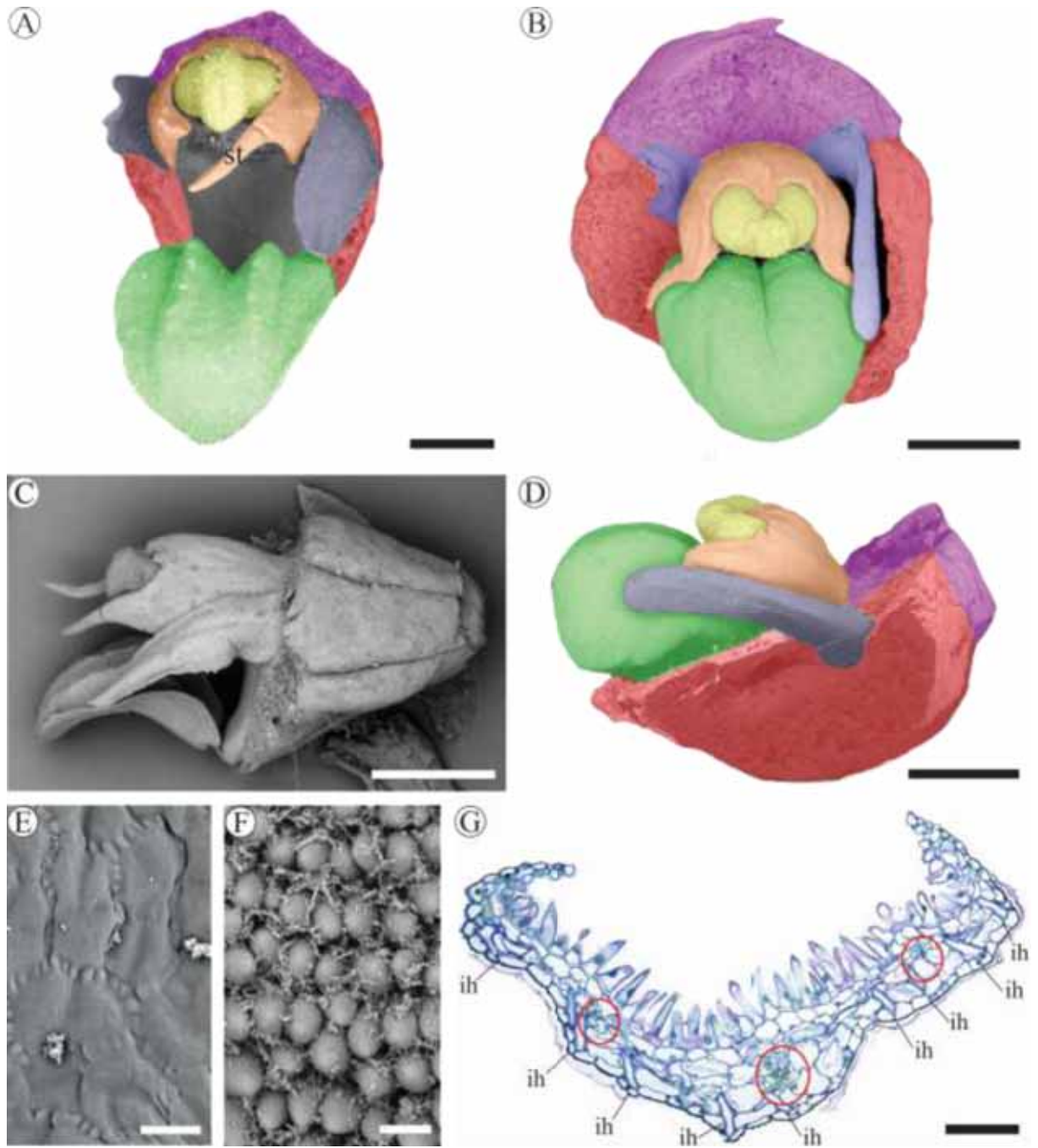


Figure 2. Micromorphology and anatomy of sepals and petals of *Bulbophyllum* sect. *Furvescens*. A, B, D, E, G) *B. steyermarkii*. C, F, H, I) *B. setigerum*. A, C) Dorsal sepal, transverse sections (TS). B) Dorsal sepal, abaxial surface. D) Dorsal sepal, margin. E) Basal third of dorsal sepal, in TS. F) Petal, adaxial surface. G) Petal, abaxial surface. H) Petal, in TS. I) Petal, margin. ih, idioblast with helical thickenings; red circles outlines, vascular bundles. Scale bars: 200µm in A, D; 25µm in B, F, G; 50µm in C, H, I; 250µm in E.

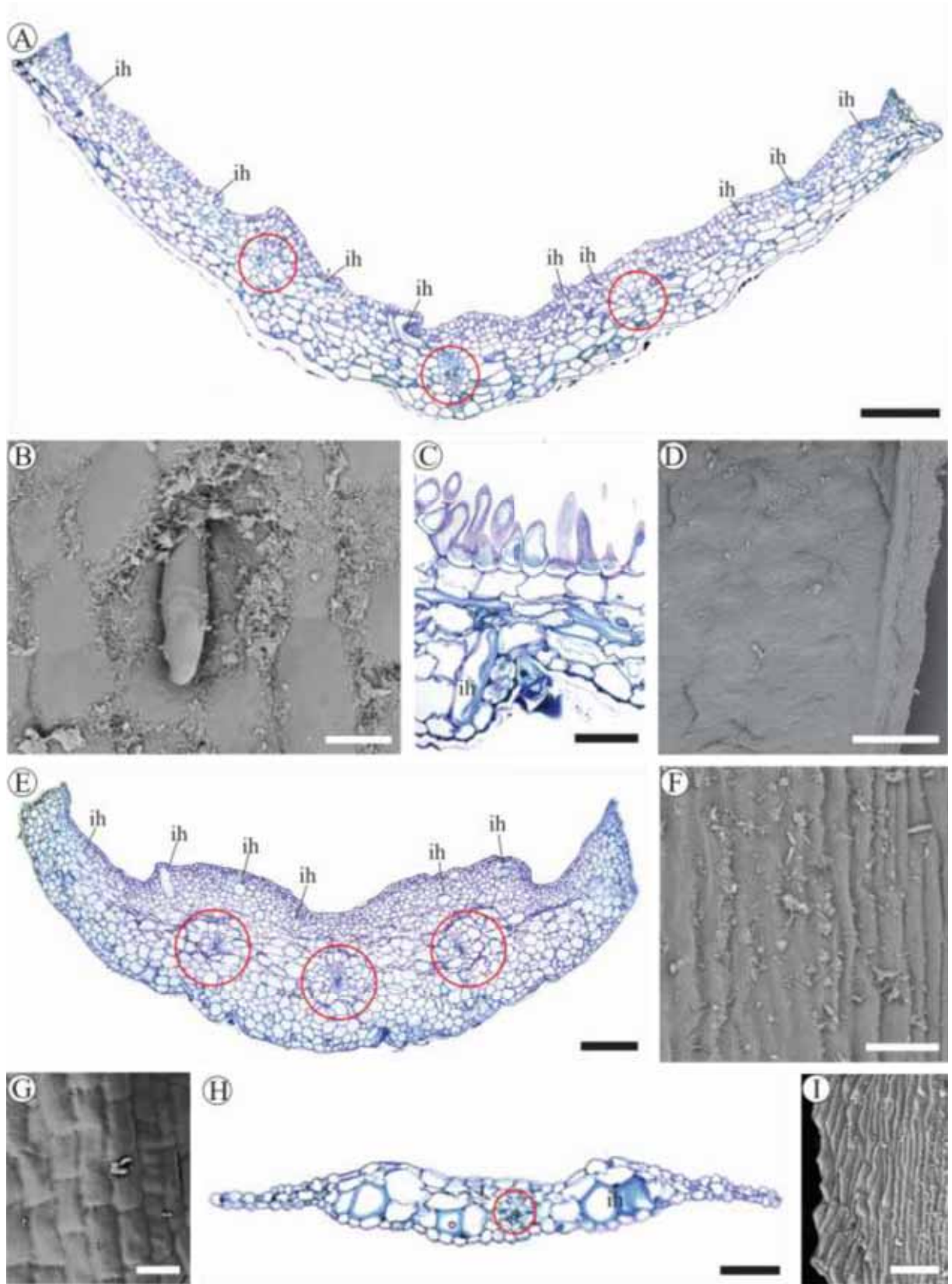


Figure 3. Micromorphology and anatomy of petals and labella of *Bulbophyllum* sect. *Furvescens*. A, C, D, F, H) *B. steyermarkii*. B, E, G) *B. setigerum*. A) Petal, margin. B, C) Distal thirds of labella, adaxial surfaces. D) Distal third of labellum, abaxial surface. E, F) Distal thirds of labella, transverse sections (TS). G, H) Proximal third of labella, TS. ih, idioblast with helical thickenings; ri, raphides idioblasts; red circles outlines, vascular bundles. Scale bars: 50µm in A, B, D; 250µm in C; 100µm in E, G; 200µm in F; 500µm in H.

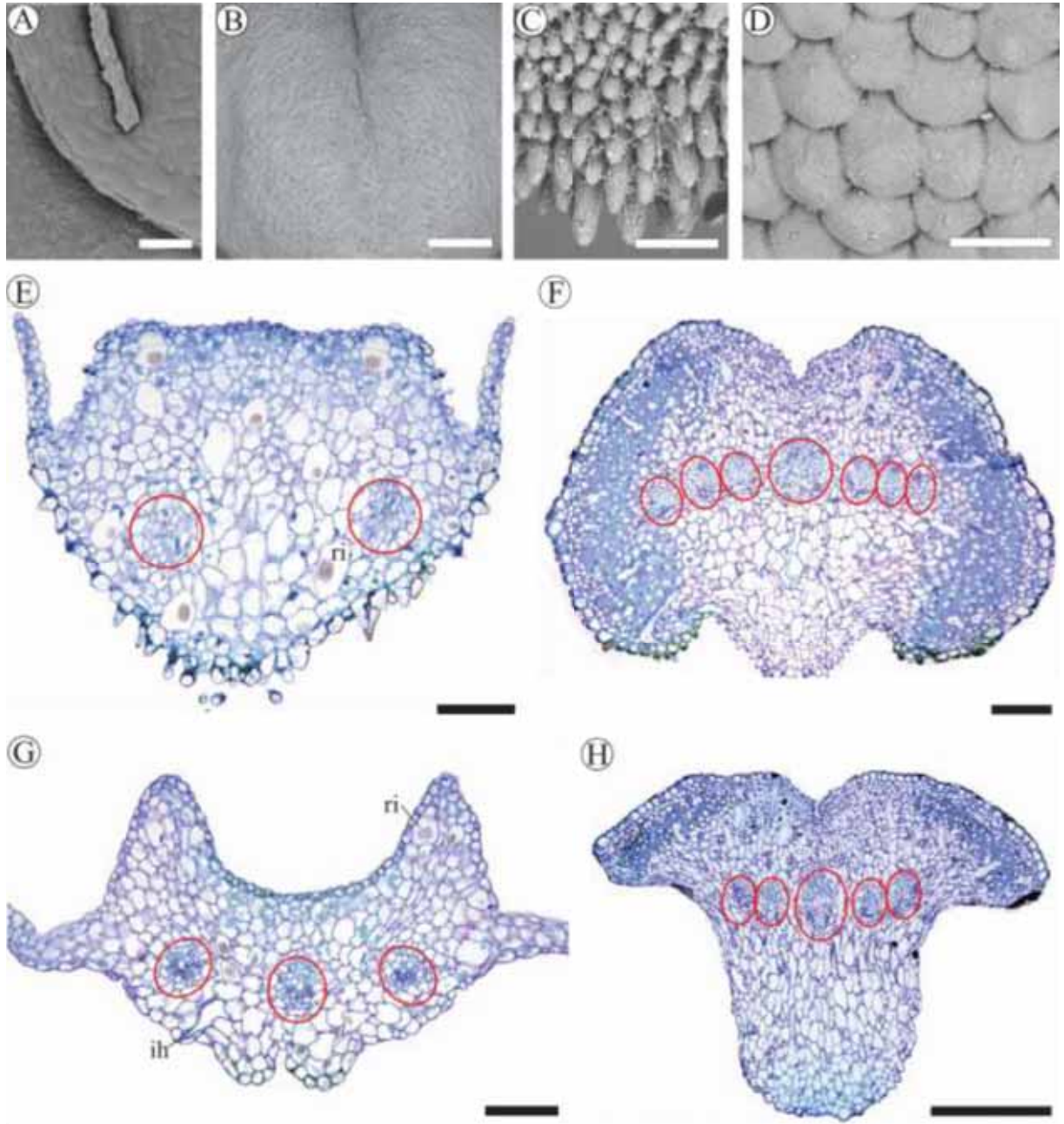


Figure 4. Micromorphology and anatomy of proximal thirds of labella and gynostemium of *Bulbophyllum* sect. *Furvescens*. A–C, G, H, J, L) *B. steyermarkii*. D–F, I, K, M) *B. setigerum*. A, B) Labella, abaxial surfaces. C, D) Gynostemium, surfaces. E) Flower, longitudinal section. F) Gynostemium at stigma level, transverse section (TS). G) Column foot, surface. H) Anther, surface. I) Anther, in TS. J) Stigma, frontal view. K) Ovary, in TS. L) Ovary, detail of the outer epidermis, in TS. M) Ovary, detail of the inner epidermis, in TS. cf, column foot; ds, dorsal sepal; ih, idioblast with helical thickenings; ls, lateral sepal; si, stigma; vi, viscidium; red circles outlines, vascular bundles. Scale bars: 100µm in A, B; 25µm in C, D; 500µm in E, H, J, M; 200µm in F, I, K, L; 50µm in G.

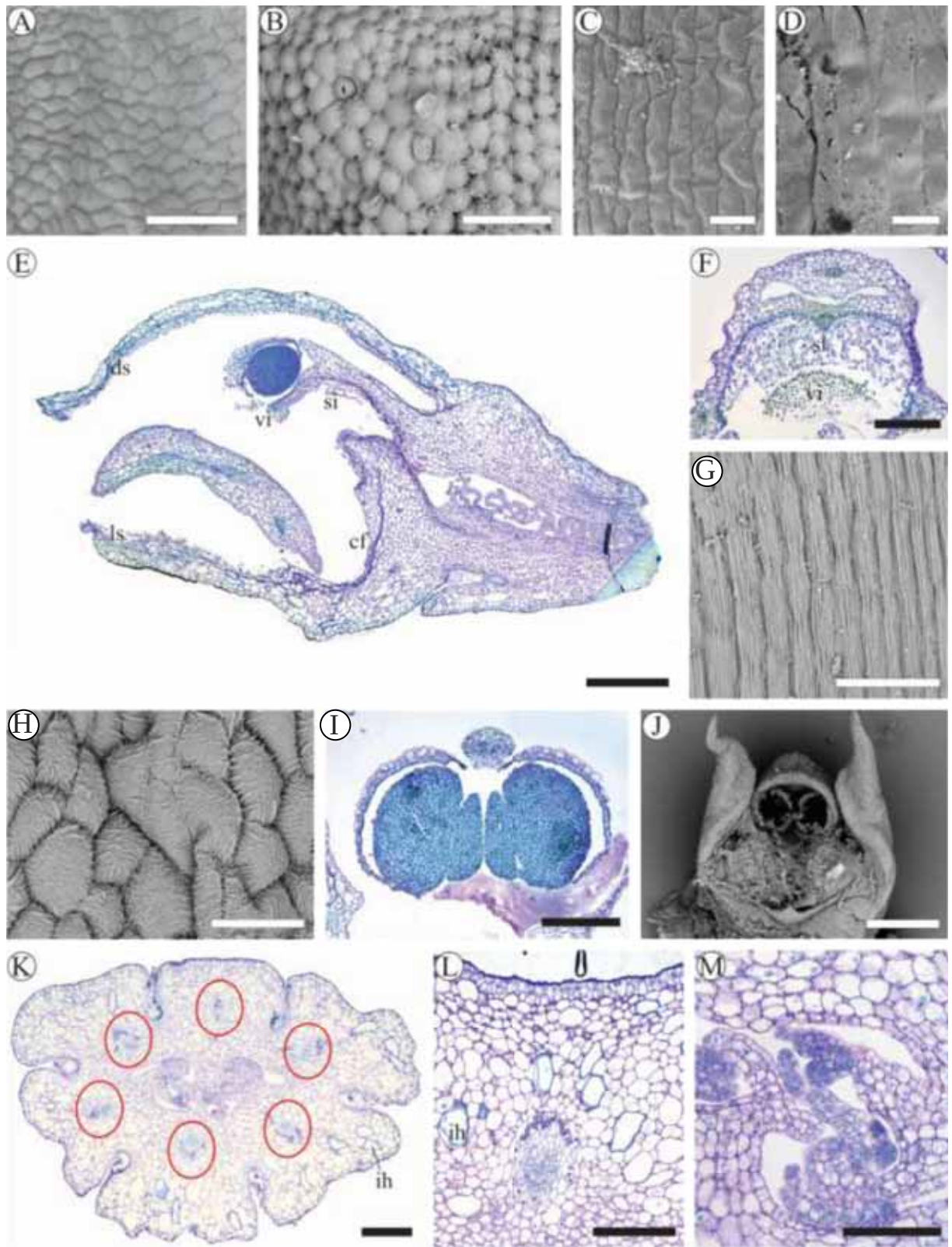


Figure 5. Micromorphology and anatomy of flower and sepals of *Bulbophyllum bracteolatum* (*B. sect. Bulbophyllaria*). A) Flower, frontal view. B) Flower, side view. C) Dorsal sepal, adaxial surface. D, E, H) Dorsal sepal, transverse sections (TS). F) Dorsal sepal, abaxial surface. G) Dorsal sepal, margin. I) Lateral sepal, adaxial surface. J) Lateral sepal, TS. ih, idioblast with helical thickenings; ri, raphides idioblasts; red circles outlines, vascular bundles. Scale bars: 1mm in A, B; 50µm in C, E; 100µm in D, F, H, I, J; 250µm in G.

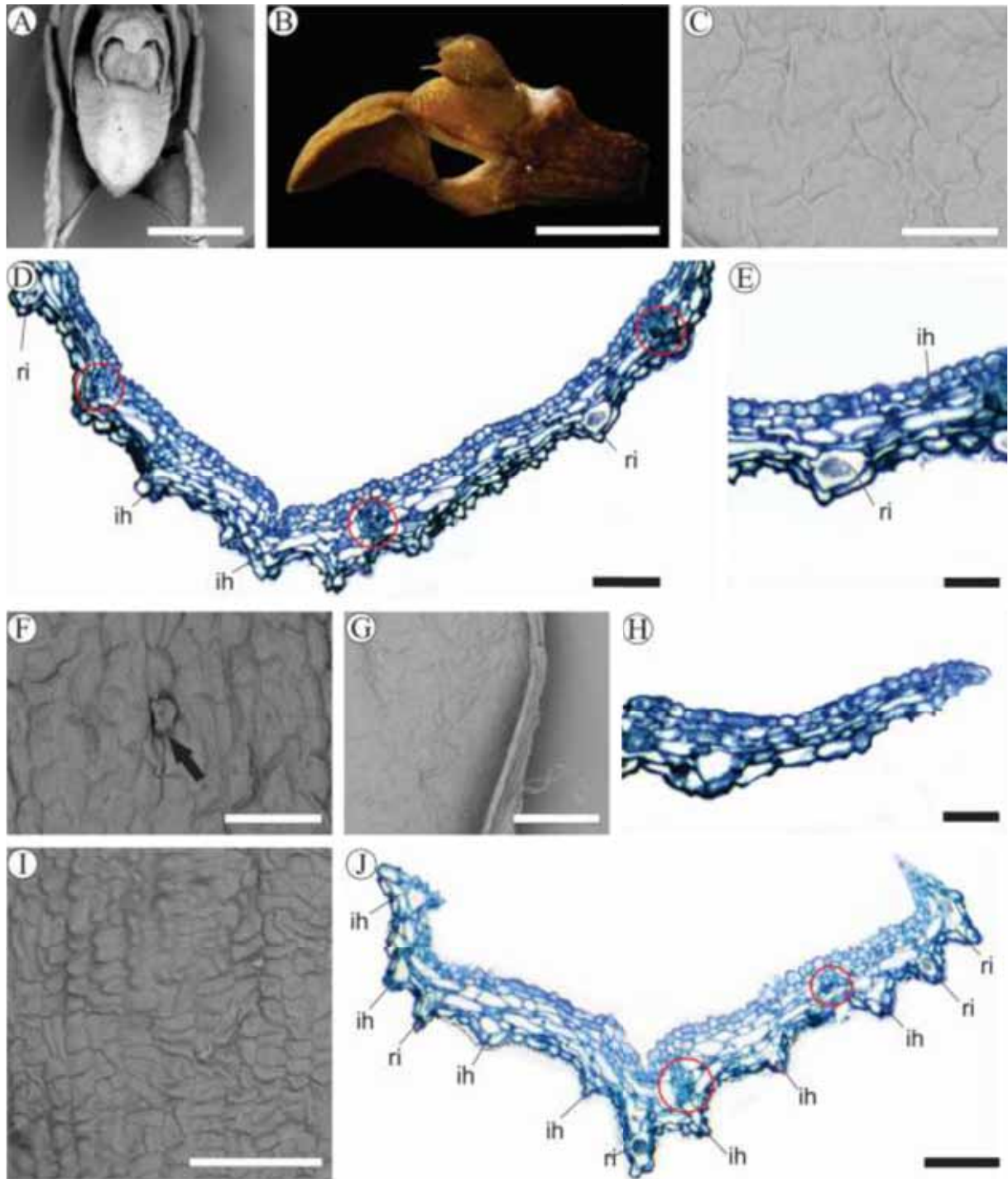


Figure 6. Micromorphology and anatomy of petal and labellum of *Bulbophyllum bracteolatum* (*B. sect. Bulbophyllaria*). A) Petal, adaxial surface. B, D) Petal, transverse section (TS). C) Petal, abaxial surface. E) Distal third of labellum, frontal view. F) Labellum, adaxial surface. G) Labellum apex, adaxial surface. H) Labellum, abaxial surface (detail in I). J) Distal third of labellum, TS. K) Proximal third of labellum, TS. ih, idioblast with helical thickenings; ri, raphides idioblasts; red circles outlines, vascular bundles. Scale bars: 25µm in A; 200µm in B, K; 50µm in C–E; 500µm in F, H; 100µm in G, I; 80µm in J.

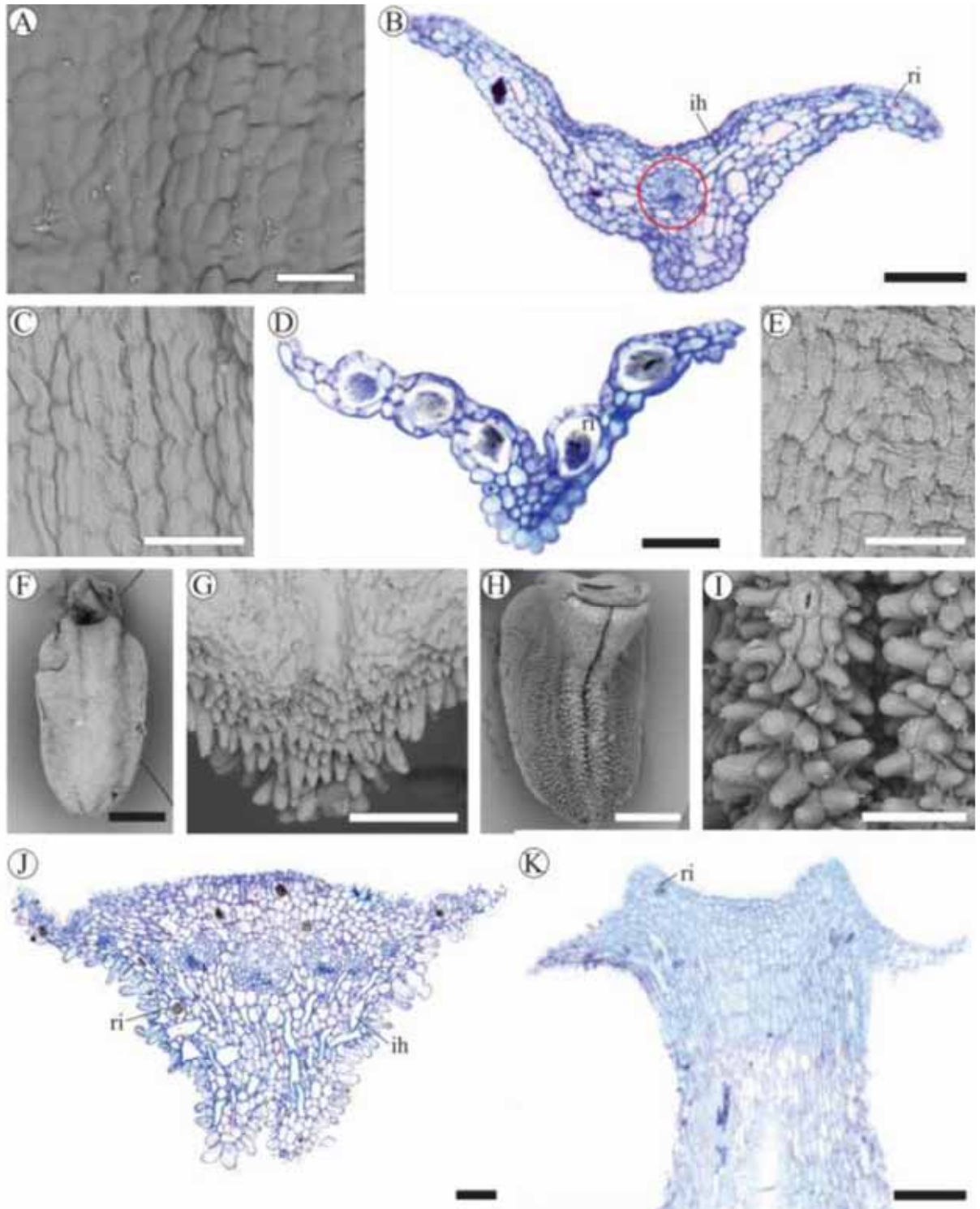
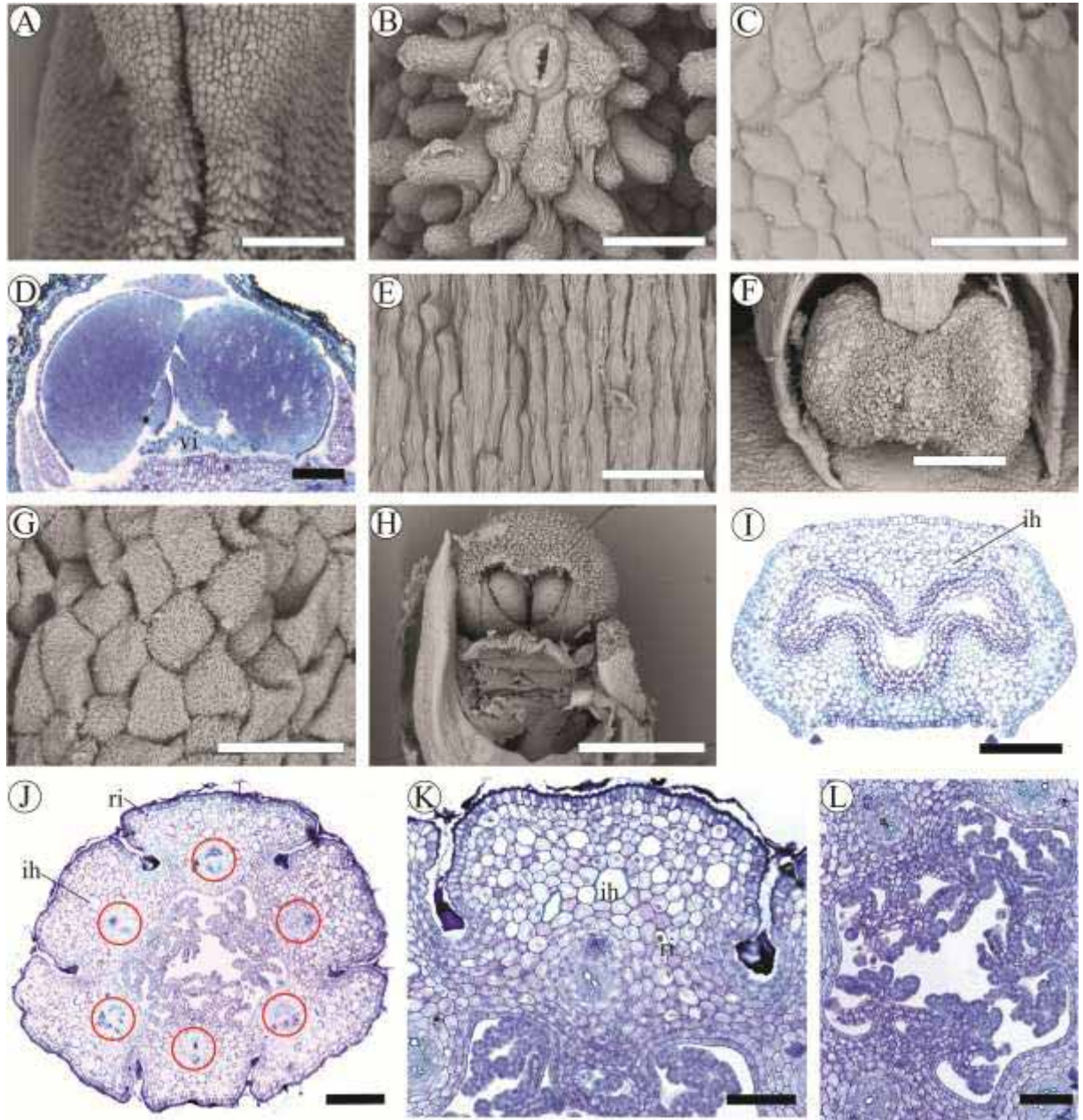


Figure 7. Micromorphology and anatomy of labellum and gynostemium of *Bulbophyllum bracteolatum* (*B. sect. Bulbophyllaria*). A) Proximal third of labellum, sulcus at adaxial surface. B) Proximal third of labellum, keel at abaxial surface. C) Gynostemium surface. D) Pollinia, transverse section (TS). E) Column foot surface. F) Anther, frontal view (detail in G). H) Gynostemium, frontal view. I) Gynostemium, TS. J) Ovary, in TS. K) Ovary, detail of the outer epidermis, in TS. L) Ovary, detail of the inner epidermis, in TS. ih, idioblast with helical thickenings; ri, raphides idioblasts; vi, viscidium; red circles outlines, vascular bundles. Scale bars: 250µm in A, F; 50µm in B, C, E; 200µm in D, I, J; 30µm in G; 500µm in H; 100µm in K, L.



CAPÍTULO VI

Evolution of floral characters in Neotropical *Bulbophyllum* (Orchidaceae: Bulbophyllinae)

Elaine Lopes Pereira Nunes, Eric de Camargo Smidt, Alessandra Ike Coan

INTRODUCTION

Orchidaceae comprises more than 800 genera (Govaerts *et al.*, 2014), from which seven are listed amongst the 57 genera with more than 500 species (Frodin, 2004). Species-rich genera comprise up to a quarter of all vascular plants and are often poorly known systematically (Frodin, 2004). The understanding of the processes causing the richness of big genera is one of the most challenging problems in Angiosperm evolution. In addition to the taxonomic challenges, these big genera offer a great opportunity to study phenomena such as rapid speciation, adaptative radiation, character evolution, key-innovation and others (Berry *et al.*, 2005). Studying such phenomena is of great interest, although attempts to consistently study big genera are rare, once a detailed knowledge of the group is an essential condition and this is unlikely in short term projects (Samain *et al.*, 2009).

Bulbophyllum Thouars, with about species estimation ranging from ca. 1900-2200 species (Govaerts *et al.*, 2014; Gravendeel *et al.*, 2014), is the largest genus within Orchidaceae (Govaerts *et al.*, 2014) and the second largest within Angiosperms (Frodin, 2004). The genus is widely distributed with main centres of diversity in Madagascar (about 200 species) and New Guinea (about 600 species) (Gravendeel *et al.*, 2014). The infrageneric classification is complicated and has been recently revised, grouping species in 17 African sections, 15 Malagasy sections, 66 Asian sections, and six Neotropical sections (Gravendeel *et al.*, 2014).

Floral morphology is quite diverse within the small Neotropical group that comprises ca. 60 species (Smidt *et al.*, 2011) and the role of both environmental and pollinator specificity has been suggested to explain the floral diversity (Borba & Semir, 1998; Verola, 2002; Teixeira *et al.*, 2004). Specifically, Verola (2002) after studying the reproductive biology of seven species suggested that different pollination mechanisms might be related to the occupation of different environments, with a sequence of adaptation from forestal habitats to open areas such as “campos rupestres” (rocky outcrops). However, no phylogenetic framework was available at the point those assumptions were made, being published only in 2011 by Smidt *et al.*, and it is possible that some similarities observed were caused by common ancestry before radiation to a different environment.

Much is still needed to be learned on the reproductive biology of this group, but in the light of the recent molecular phylogeny and the floral characteristics assessed by Nunes *et al.* (2014, submitted Capítulo III, Capítulo IV, Capítulo V), the present work aimed to: a) search for floral characters that could represent synapomorphies of the clades delimited in

phylogenetic studies of the Neotropical *Bulbophyllum*, and b) discuss the significance of some other floral characters regarding environment and pollination.

MATERIALS AND METHODS

Morphological, micromorphological and anatomical data

Floral morphological, micromorphological and anatomical data of species included in the Neotropical *Bulbophyllum* sect. *Didactyle*, *B.* sect. *Napelli*, *B.* sect. *Micranthae*, *B.* sect. *Furvescens*, *B.* sect. *Bulbophyllaria*, and *B.* sect. *Xiphizusa* were obtained from Nunes *et al.* (2014, submitted and in prep. – Capítulos I, II, III, IV and V). Additional unpublished data from these groups were obtained with the authors and vouchers are the same as indicated in those papers. Information regarding gross morphology of vegetative organs, flowers and inflorescences, besides habit, were obtained of an unpublished dataset of Smidt (2007).

Six non-Neotropical *Bulbophyllum* and one *Dendrobium* Sw. species were obtained from orchid nurseries (vouchers were deposited at Herbário Rioclarense – HRCB). Mature flowers from these species were prepared according to the methods described in Nunes *et al.* (in prep. – Capítulo V) for both light and scanning electron microscopies analysis. Additional information on vegetative and floral morphology, besides habit, was obtained from observations on plants kept in greenhouse and also in the literature when available.

The details of species sampled, with the data source are listed in Table 1.

Morphological data analysis

A total of 195 characters (15 constant and 151 parsimony-informative characters) were included in the analysis for 36 *Bulbophyllum* species and one *Dendrobium* (Appendix 2), the sister group of the former (van den Berg *et al.*, 2005). A parsimony (MP) analysis with all characters equally weighted was conducted in PAUP* v4.0b10 (Swofford, 2003). The resulting strict consensus tree was then used to reconstruct the history of morphological characters using a maximum parsimony approach implemented in Mesquite v2.75 (<http://mesquiteproject.org/mesquite/mesquite.html>, Maddison & Maddison, 2011). Only characters which could potentially be considered as synapomorphies were then selected and mapped in this tree.

Since the morphological strict consensus tree showed a low consistency index, the nrITS Bayesian tree of Smidt *et al.* (2011) was modified, excluding taxa to which floral micromorphological and anatomical information was not available, and used to reconstruct the history of morphological characters as aforementioned. Besides potential synapomorphies mapped on the Bayesian tree, some other characters were selected and discussed.

RESULTS

Potential floral synapomorphies

The resulting MP tree (Fig. 1) has a tree length of 816 steps, a consistency index (CI) of 0.3811 (0.3575 excluding uninformative characters), a homoplasy index of 0.6189 (0.6425 excluding uninformative characters) and a retention index of 0.5874 (rescaled = 0.2239). Eighteen characters that appeared only once (or when appeared more than once, occurred only in terminal taxa and seemed autapomorphic) on the tree were mapped on it as possible synapomorphies (Fig. 1).

For *Dendrobium* and *Bulbophyllum* two characters were recognised: presence of sunken glandular trichomes on the abaxial surface of the lateral sepals (character 46) and a sulcus on the adaxial surface of the labellum (on the hypochile, character 83). For the African *Bulbophyllum* and the Neotropical clade, ornamented abaxial surface on the lateral sepals (character 38) and one vascular bundle on the petals (character 73) were assigned as potential synapomorphies.

No characters could be assigned to major clades inside Neotropical *Bulbophyllum*, except for *B.* sect. *Napelli*, which show two pollinia as potential synapomorphy (character 114). Still regarding *B.* sect. *Napelli*, a double labellar epidermis (character 86) appears as potential synapomorphy for a clade consisting of *B. granulosum*, *B. atropurpureum* and *B. campos-portoi*.

In *Bulbophyllum* sect. *Micranthae*, only the clade consisting of *B. adiamantinum* and *B. insectiferum* showed potential floral synapomorphies: reticulate ornamentation of the adaxial surface of the dorsal sepal (character 6), the lateral sepals (character 32) and the petal (character 57), besides reticulate ornamentation of the abaxial surface of the dorsal sepal (character 17), clawed petal insertion (character 54), and the presence of unicellular trichomes on the column foot (character 118).

In *Bulbophyllum* sect. *Xiphizusa*, the clade containing all studied species, except *B. ciluliae*, was supported by one potential synapomorphy: total connation of lateral sepals (character 161).

Two potential synapomorphies were identified for *B.* sect. *Didactyle*: the presence of a secretory cavity in the labellum callus (character 78) and the presence of idioblasts with helical thickenings in the labellum epichile (character 105). For the *core* clade of *B.* sect. *Didactyle*, the presence of unicellular trichomes as the main epidermal component of the adaxial surface of the labellum hypochile (character 82), the epichile (character 99) and of the abaxial surface of the epichile (character 102) were considered as potential synapomorphies.

Using the nrITS Bayesian tree topology, 19 potential floral synapomorphies were identified, being 17 identical to those of the morphological MP tree (Fig. 2). The differences are regarding the clade consisting of *B.* sect. *Xiphizusa* + *B.* sect. *Didactyle*, that is supported by the presence of a developed callus in the labellum (character 77) and the median lobe of the labellum differentiated in hypochile and epichile (character 97). An epichile is present also in *B. saltatorium* (not shown), an African species, but was here regarded as not homolog to that of the Neotropical sections.

Character evolution

The presence of idioblasts with helical wall thickenings was reconstructed in different floral organs, since it showed variation in previous studies. In general, this cell type was present in African and Neotropical *Bulbophyllum* species, but showed several different reversals according to each floral part (Fig. 3A–D). Its presence was consistent in *B.* sect. *Didactyle* and its absence was consistent in *B.* sect. *Xiphizusa*, albeit with two reversals.

The surface ornamentation of the floral organs was divided in several binary characters in the morphological matrix (Appendix 2), and only the smooth vs. ornamented is being considered for dorsal sepal, lateral sepal and petal. The adaxial surface is highly variable across the floral organs, the dorsal sepal showing more similarity with the petal than with the lateral sepals (Fig. 4A–C). The abaxial surface of the dorsal sepal is smooth in *Bulbophyllum* in general (Fig. 5A), while the lateral sepal and petal show ornamented surfaces (Fig. 5B, C), although the latter show some variation.

The labellum surface ornamentation was scored as a single binary character, since the only state besides “smooth” was striate to all 37 species studied. In general, both hypochile and epichile show striate surfaces, but several reversals are present across taxa (Fig. 6A–D).

A sulcus is present in the labellum hypochile in all species studied (Fig. 7A), except in those from *B. sect. Didactyle*, where a secretory cavity occurs (Figs 1, 2). A secretory activity of this sulcus was proved for both the Asian and the Neotropical *Bulbophyllum*, although reversals occur in *B. rupicolum* (*B. sect. Micranthae*) and species of *B. sect. Xiphizusa* (Fig. 7B).

The mesophyll of the labellum hypochile is generally composed of regular parenchyma only, but a mesophyll composed mostly of spongy parenchyma is present in Asian and African species (Fig. 7C). A mesophyll with both regular and secretory parenchyma appeared three times in the Neotropical species, at least with two independent origins (Fig. 7C).

The mesophyll of the epichile showed only spongy parenchyma in *B. saltatorium* (African), *B. sect. Xiphizusa* and *B. weddellii* (*B. sect. Didactyle*), while it is composed by regular parenchyma in the remainder of *B. sect. Didactyle* (Fig. 7D). A mesophyll composed only by secretory parenchyma is present only in *B. sect. Xiphizusa* (Fig. 7D).

The mesophyll of the sepals is usually composed by regular parenchyma only (Fig. 8A, B), but secretory parenchyma appeared in *B. sect. Xiphizusa* (Fig. 8B) maybe independently in the lateral sepals (Fig. 8B).

DISCUSSION

Considering the two different phylogenetic trees used in this study, even with the limitations imposed by the use of morphological data (discussed in Swofford, 1991; Donoghue & Sanderson, 1992; Patterson *et al.*, 1993, and others), we found a good general agreement in both morphological and molecular results. In general, species grouping was congruent, in spite of a different general topology. Such differences did not affect significantly the search for possible floral synapomorphies, since most of them were present regardless of the tree topology. It is interesting to note that, regardless of the tree used, the number of synapomorphies was low, and usually were above or below section level. The only synapomorphies indicated for the Neotropical sections were the presence of two pollinia for *B. sect. Napelli*, already indicated by Nunes *et al.* (submitted – Capítulo II) and the presence of a secretory cavity in the labellum callus and of idioblasts with helical thickenings in the labellum epichile for *B. sect. Didactyle*.

Such difficulty in finding morphological (micromorphological and anatomical as well) synapomorphies is not only confined for Neotropical *Bulbophyllum*, but for the genus as a

whole. Fischer *et al.* (2007) and Gravendeel *et al.* (2014) pointed out that unique morphological synapomorphies characterizing clades are scarce, but those supporting combinations of characters are abundant. Gravendeel *et al.* (2014) stated that plotting morphological characters on the molecular phylogenetic tree is only successful for terminal branches, thus optimizing small diagnostic character sets for small clades that typically include 20-50 species. This statement seems to be true also for the Neotropical clade studied here, that includes less than 70 species (Smidt *et al.*, 2011). Our results highlight the difficulties faced when working with big plant genera as discussed elsewhere (Berry *et al.*, 2005; Samain *et al.*, 2009) and the need of more detailed studies and better sampling to try to understand the processes underlying the richness of a genus like *Bulbophyllum* – monophyletic (Gravendeel *et al.*, 2014) and with about 2200 species (Govaerts *et al.*, 2014). Moreover, it is necessary to point out that some of the floral synapomorphies showed here for *Dendrobium* + *Bulbophyllum* or Asian and African *Bulbophyllum* in relation to Neotropical species, will probably not be held if the framework is expanded.

Many puzzling characteristics that we, in general, could not explain solely by phylogenetic relationships were found here and will be outlined below with some considerations. One of such characteristics is the variation of wall/cuticle ornamentation between different floral parts (present in a less extent in the labellum) and both adaxial and abaxial surfaces in a single species and, to our knowledge, has never been verified for other groups. In some cases, it was known that the differential surface ornamentation was on the outer periclinal walls of the floral organs (*Bulbophyllum* sect. *Napelli* – Capítulo II, most species of *Bulbophyllum* sect. *Micranthae* – Capítulo III), while in others it seems to be a cuticle feature (*B. rupicolum* – Capítulo III), but an interaction between both wall ornamentation and cuticle ornamentation creating the final pattern cannot be excluded. Since sometimes such ornamentation patterns are regarded as systematically useful (Newton, 1972; Ehler, 1975, 1976; Barthlott, 1981; Gale & Owens, 1983; Sanso *et al.*, 2014), it is interesting to better understand the nature of such sculpturing as well the processes by they arise in different species and are maintained, especially when they are present in the cell wall itself, because cell wall production and folding must be a costly process and seems unlikely to be kept under neutral selection. We could not preliminarily relate such features neither to pollination nor to environmental pressures, and a deeper analysis will be needed to approach this character(s) variation. Interestingly, labellar surfaces are more commonly striate, regardless of the surface side.

Here we have found evidence that a labellar sulcus or cavity (we have considered the cavity consistently found in *B. sect. Didactyle* by Nunes *et al.* (2014) as a different character than the sulcus itself, but that remains to be verified), that is presumably secretory and most likely work as nectary, is present throughout Neotropical *Bulbophyllum* (this study; Capítulos II, III, IV and V) and also in African and Asian species (this study; Teixeira *et al.* 2004; Davies & Stpiczyńska, *in press*). The implications for fly pollination will be discussed later.

Secretory tissues were also found in species belonging to *B. sect. Xiphizusa* (Nunes *et al.*, Capítulo IV), but on the sepals and are the sole case within Neotropical *Bulbophyllum* (this study); variation was found also in the presence of labellar secretory tissues between species (even with a restricted sampling). *Bulbophyllum sect. Xiphizusa* is the most diverse section in the Neotropics, and from the analysed characters, the fusion of the lateral sepals may be regarded as the major innovation in the Neotropical group. According to Soltis *et al.* (2005), sympetaly is a prominent key innovation in Angiosperm evolution, once it changes the petal structure, allows the formation of floral tubes whose size is easily modulated, and also increases the flower architecture stability. Since petals are usually reduced in Neotropical *Bulbophyllum* (briefly discussed in Nunes *et al.*, submitted – Capítulo II), but sepals are bigger and showy, it is reasonable to consider that synsepaly (even partial) may have played a similar role in *B. sect. Xiphizusa*.

Species belonging to *B. sect. Xiphizusa* are also noteworthy on the almost complete absence of idioblasts with helical wall thickenings in their flowers, a cell type that is regarded as important for water storage and structural support of mesophyll in drought situations (Haberlandt, 1924; Whitner *et al.*, 1974; Olatunji & Nengim, 1980; Pridgeon, 1981, 1982; Burr & Barthlott, 1991), and was expected to be found in this group that occur in “campos rupestres” (rocky outcrops), especially because its sister group, *B. sect. Didactyle*, occur in similar habitats and consistently show this cell type (Nunes *et al.*, 2014).

Speciation in Angiosperms, specially Orchidaceae, is regarded as caused by divergence in floral characters reflecting adaptation to pollinators (Stebbins, 1970, 1974; Armbruster, 1996; Johnson, 1996; Johnson *et al.*, 1998; Kay & Sargent, 2009; Schiestl & Schüter, 2009; van der Niet & Johnson, 2012), although sometimes, it may primarily occur through divergence in vegetative characters reflecting adaptation to the physical environment, as is Euphorbiaceae (Stebbins, 1970, 1974). In spite of the many nice examples of floral diversification due to major pollinator shifts in Orchidaceae (e.g., Chase & Palmer, 1997; Hapeman & Inoue, 1997; Johnson *et al.*, 1998), nothing similar has been described for *Bulbophyllum*, that is consistently fly-pollinated (van der Pijl & Dodson, 1966; Christensen,

1994; Borba & Semir, 1998; Verola, 2002; Gravendeel *et al.*, 2014), with the exception of *B. ambrosia* being pollinated by *Apis cerana* bees (Chen & Gao, 2011).

Diptera are the second most important insect group of pollinators for angiosperms even though the majority of flies are not dependent on flowers for food (Endress, 1994). The fly pollination syndrome, also known as myiophily, is usually classified into two categories, namely, micromyiophily and sapromyiophily (Meve & Liede, 1994); the first is characterized by flowers that emit odours reminiscent of decaying plant matter or fruits (e.g., sweet scents, ripe fruit scents, fermentation scents), while the second is characterized by flowers that emit odours reminiscent of decaying zoogenous substances such as rotting flesh and faeces, that attract flies that usually ovoposit or forage on such substances. In the micromyiophily (usually simply referred as myiophily) flowers are regarded as unspecialized, shallow, with dull colours, abundant nectar and promiscuous in their pollination relationships (Faegri & van der Pijl, 1966; Endress, 1994). The sapromyiophilous flowers, in turn, have dark floral colours, the presence of long filiform appendages or bracts, floral traps and the absence of nectar (Faegri & van der Pijl, 1966; Endress, 1994). A large number of sapromyiophilous and myiophilous flowers are believed to work by deceit, including *Bulbophyllum* species (Endress, 1994; Christensen, 1994).

From the current knowledge on floral morphology, anatomy, micromorphology and pollination of Neotropical *Bulbophyllum* species, they are best described as micromyiophilous, since nectar is usually present and the odours described are sweet. Even in *B. sect. Napelli*, where flowers have more unpleasant smell and darker colours, nectar is present (Nunes *et al.*, submitted – Capítulo II; Verola, 2002), and neither oviposition nor consumption of floral parts were observed (Verola, 2002). This way, deception is ruled out for Neotropical *Bulbophyllum*.

A more specialized system, dependent on wind to effectuate pollination by Milichiidae flies is known for species of *B. sect. Didactyle* and with some specificity (Sazima, 1978; Borba & Semir, 1998; Silva *et al.*, 1999; Azevedo *et al.*, 2006), where floral odour is the main cue in attracting these pollinators (Borba & Semir, 1998; Silva *et al.*, 1999) and the nectar presence is important to keep the flies on the flower for longer periods, until the wind, by chance, press the fly against the gynostemium (Teixeira *et al.*, 2004; Nunes *et al.*, 2014). A similar mechanism dependent upon the wind was described for one species of *B. sect. Xiphizusa*, although nectar presence or odour was not recorded (Verola, 2002) and even though secretory tissue was present in the sepals and/or labellum of some species, their function as osmophores or nectaries as still unclear (Capítulo IV).

Floral odour plays a critical role both in attracting pollinators and pollinator specificity in Orchidaceae (van der Pijl & Dodson, 1966; Hills, Williams & Dodson, 1972; Schiestl *et al.*, 2003; Schiestl & Ayasse, 2002; Mant *et al.*, 2005; Xu *et al.*, 2011), and is regarded as the primary attractants in fly pollination while specific colours and forms secondarily enhance attractiveness (Kugler, 1956). Glistening nectar or pseudonectaries seem to play an optic attractive role for flies as well for Hymenoptera (Kugler, 1955). Blue flowers are also preferred by bees and Bomblidae Diptera (Kevan, 1978), and apart from that, and some generalisations on dull or dark colours and supposed attraction by movable parts predicted by the myiophilous syndrome, little is known on visual cues related to fly pollination. Specially, less is known on the relationship between the many pigment types, distribution, surface effects and structural colours (briefly speculated for *B. sect. Napelli* – Capítulo II) and cell types related to the visual system of Diptera and their pollination preferences. The eyes of the Diptera are compound and complex structures, comparable to that of the Hymenopterans, but still little is known on their ability to view colours (Stavenga, 2002; Lunau, 2014) and also on why such a big plant genera would consistently rely on Diptera as pollinators, without major shifts to other pollinator groups, as seen in other Orchidaceae.

From this preliminary combination of floral characters with molecular phylogenetics and their discussion in the light of the available literature, three questions are raised as grounds for future studies regarding *Bulbophyllum*: a) what are the forces, if any, preventing pollinator shifts on *Bulbophyllum*; b) are variable floral epidermal surfaces of *Bulbophyllum* related in any way with fly pollination; and c) Did fly pollination, conceived in a broad pollination syndrome sense, evolved only once in *Bulbophyllum*?

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Table 1. Species studied and source of the data analysed.

Section (Gravendeel <i>et al.</i> , 2014)	Taxon	Source of data
Outgroup	<i>Dendrobium kingianum</i> Bidwill ex Lindl.	This study, Adams & Lawson (1993), Adams <i>et al.</i> (2006)
<i>B. sect. Leopardinae</i> Benth. & Hook. f.	<i>Bulbophyllum ambrosia</i> (Hance) Schltr.	This study, Chen & Gao (2011), Wood (1986)
<i>B. sect. Racemosae</i> Benth. & Hook. f.	<i>Bulbophyllum elassonotum</i> Summerh.	This study
<i>B. sect. Sestochilos</i> (Breda) Benth. & Hook. f.	<i>Bulbophyllum lobbii</i> Lindl.	This study, Stern <i>et al.</i> (1986)
<i>B. sect. Ptiloglossum</i> Lindl.	<i>Bulbophyllum saltatorium</i> Lindl.	This study, Vermeulen (1987)
<i>B. sect. Megaclinium</i> G.A.Fisher & JJ. Verm., in prep.	<i>Bulbophyllum falcatum</i> (Lindl.) Rchb.f. <i>Bulbophyllum scaberulum</i> (Rolfe) Bolus	This study, Vermeulen (1987) This study, Vermeulen (1987)
<i>B. sect.</i> <i>Bulbophyllaria</i> (Rchb.f.) Griseb	<i>Bulbophyllum bracteolatum</i> Lindl.	Nunes <i>et al.</i> (Capítulo V), Smidt (2007)
<i>B. sect. Furvescens</i> E.C. Smidt, Borba & van den Berg	<i>Bulbophyllum setigerum</i> Lindl. <i>Bulbophyllum steyermarkii</i> Foldats <i>Bulbophyllum atropurpureum</i> Barb.Rodr. <i>Bulbophyllum campos-portoi</i> Brade	Nunes <i>et al.</i> (Capítulo V), Smidt (2007) Nunes <i>et al.</i> (Capítulo V), Smidt (2007) Nunes <i>et al.</i> (Capítulo II), Smidt (2007) Nunes <i>et al.</i> (Capítulo II), Smidt (2007)
<i>B. sect. Napelli</i> Rchb.f.	<i>Bulbophyllum granulosum</i> Barb.Rodr. <i>Bulbophyllum malachadenia</i> Cogn. <i>Bulbophyllum napelli</i> Lindl. <i>Bulbophyllum regnellii</i> Rchb.f. <i>Bulbophyllum adiamantinum</i> Brade	Nunes <i>et al.</i> (Capítulo II), Smidt (2007) Nunes <i>et al.</i> (Capítulo II), Smidt (2007) Nunes <i>et al.</i> (Capítulo II), Smidt (2007) Nunes <i>et al.</i> (Capítulo II), Smidt (2007) Nunes <i>et al.</i> (Capítulo III), Smidt (2007)
<i>B. sect. Micranthae</i> Barb.Rodr.	<i>Bulbophyllum chloroglossum</i> Rchb.f. <i>Bulbophyllum epiphytum</i> Barb.Rodr. <i>Bulbophyllum insectiferum</i> Barb.Rodr.	Nunes <i>et al.</i> (Capítulo III), Smidt (2007) Nunes <i>et al.</i> (Capítulo III), Smidt (2007) Nunes <i>et al.</i> (Capítulo III),

	<i>Bulbophyllum mentosum</i> Barb.Rodr.	Smidt (2007) Nunes <i>et al.</i> (Capítulo III), Smidt (2007)
	<i>Bulbophyllum micranthum</i> Barb.Rodr.	Nunes <i>et al.</i> (Capítulo III), Smidt (2007)
	<i>Bulbophyllum mucronifolium</i> Rchb.f. & Warm.	Nunes <i>et al.</i> (Capítulo III), Smidt (2007)
	<i>Bulbophyllum rupicolum</i> Barb.Rodr.	Nunes <i>et al.</i> (Capítulo III), Smidt (2007)
	<i>Bulbophyllum exaltatum</i> Lindl.	Nunes <i>et al.</i> (2014, Capítulo I), Smidt (2007)
	<i>Bulbophyllum involutum</i> Borba, Semir & F.Barros	Nunes <i>et al.</i> (2014, Capítulo I), Smidt (2007)
	<i>Bulbophyllum meridense</i> Rchb.f.	Nunes <i>et al.</i> (2014, Capítulo I), Smidt (2007)
B. sect. <i>Didactyle</i> (Lindl.) Cogn.	<i>Bulbophyllum peri</i> Schltr.	Nunes <i>et al.</i> (2014, Capítulo I), Smidt (2007)
	<i>Bulbophyllum popayanense</i> Kraenzl.	Nunes <i>et al.</i> (2014, Capítulo I), Smidt (2007)
	<i>Bulbophyllum weddellii</i> (Lindl.) Rchb.f.	Nunes <i>et al.</i> (2014, Capítulo I), Smidt (2007)
	<i>Bulbophyllum tripetalum</i> Lindl.	Nunes <i>et al.</i> (2014, Capítulo I), Smidt (2007)
	<i>Bulbophyllum bidentatum</i> (Barb.Rodr.) Cogn.	Nunes <i>et al.</i> (Capítulo IV), Smidt (2007)
	<i>Bulbophyllum ciluliae</i> Bianch. & J.A.N.Bat.	Nunes <i>et al.</i> (Capítulo IV), Smidt (2007)
	<i>Bulbophyllum gladiatum</i> Lindl.	Nunes <i>et al.</i> (Capítulo IV), Smidt (2007)
B. sect. <i>Xiphizusa</i> Rchb.f.	<i>Bulbophyllum manarae</i> Foldats	Nunes <i>et al.</i> (Capítulo IV), Smidt (2007)
	<i>Bulbophyllum melloi</i> Pabst	Nunes <i>et al.</i> (Capítulo IV), Smidt (2007)
	<i>Bulbophyllum plumosum</i> (Barb.Rodr.) Cogn.	Nunes <i>et al.</i> (Capítulo IV), Smidt (2007)

FIGURES

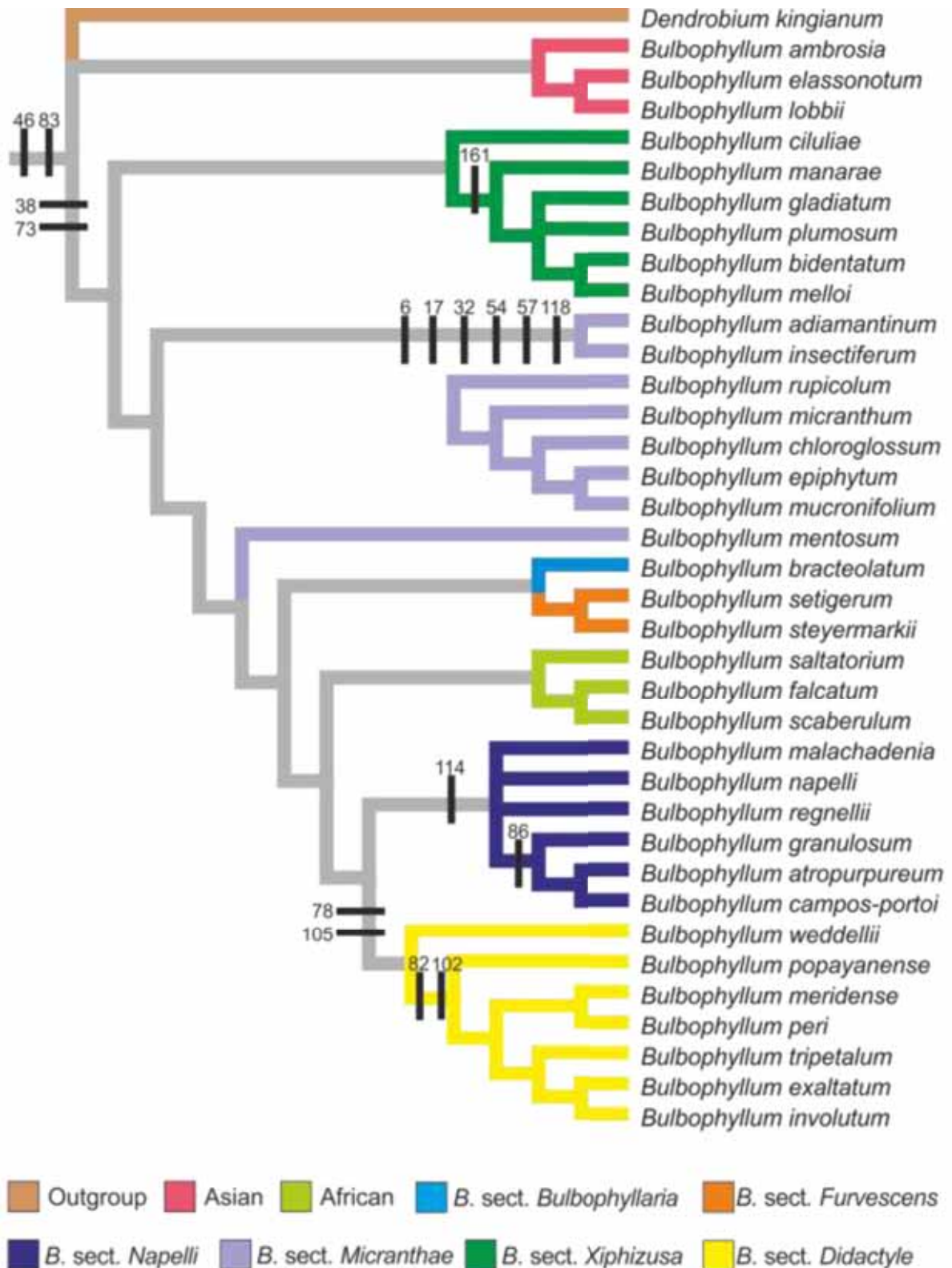


Figure 1. Distribution of potential floral synapomorphies on the MP morphological tree. The numbers close to the black bars indicate the number of the character within the morphological matrix. Other details of the tree: length = 816; consistency index (CI) = 0.3811; homoplasy index (HI) = 0.6189; CI excluding uninformative characters = 0.3575; HI excluding uninformative characters = 0.6425; retention index (RI) = 0.5874; rescaled consistency index (RC) = 0.2239.

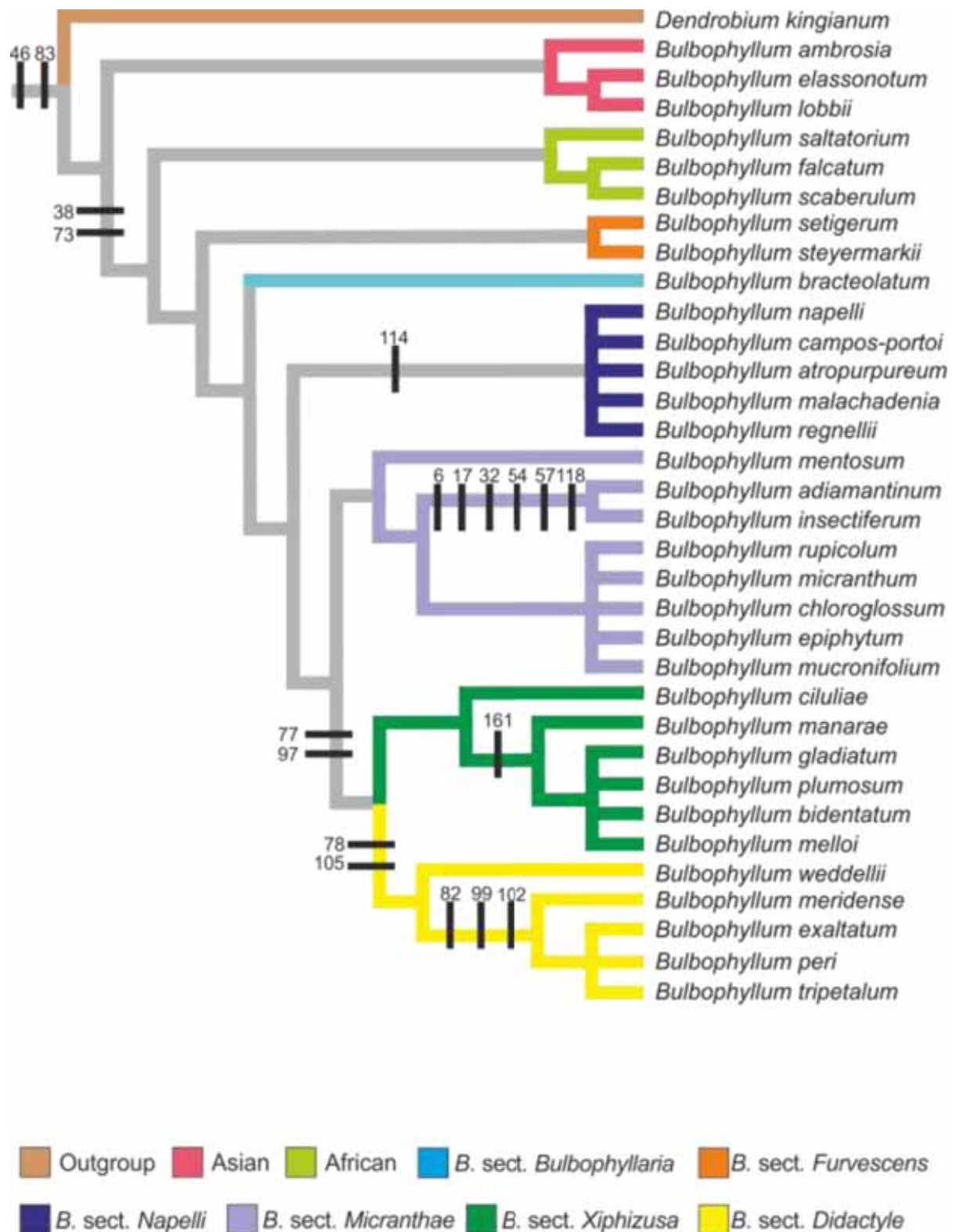


Figure 2. Distribution of potential floral synapomorphies on the nrITS Bayesian tree (modified from figure S2 of Smidt et al., 2011). The numbers close to the black bars indicate the number of the character within the morphological matrix.

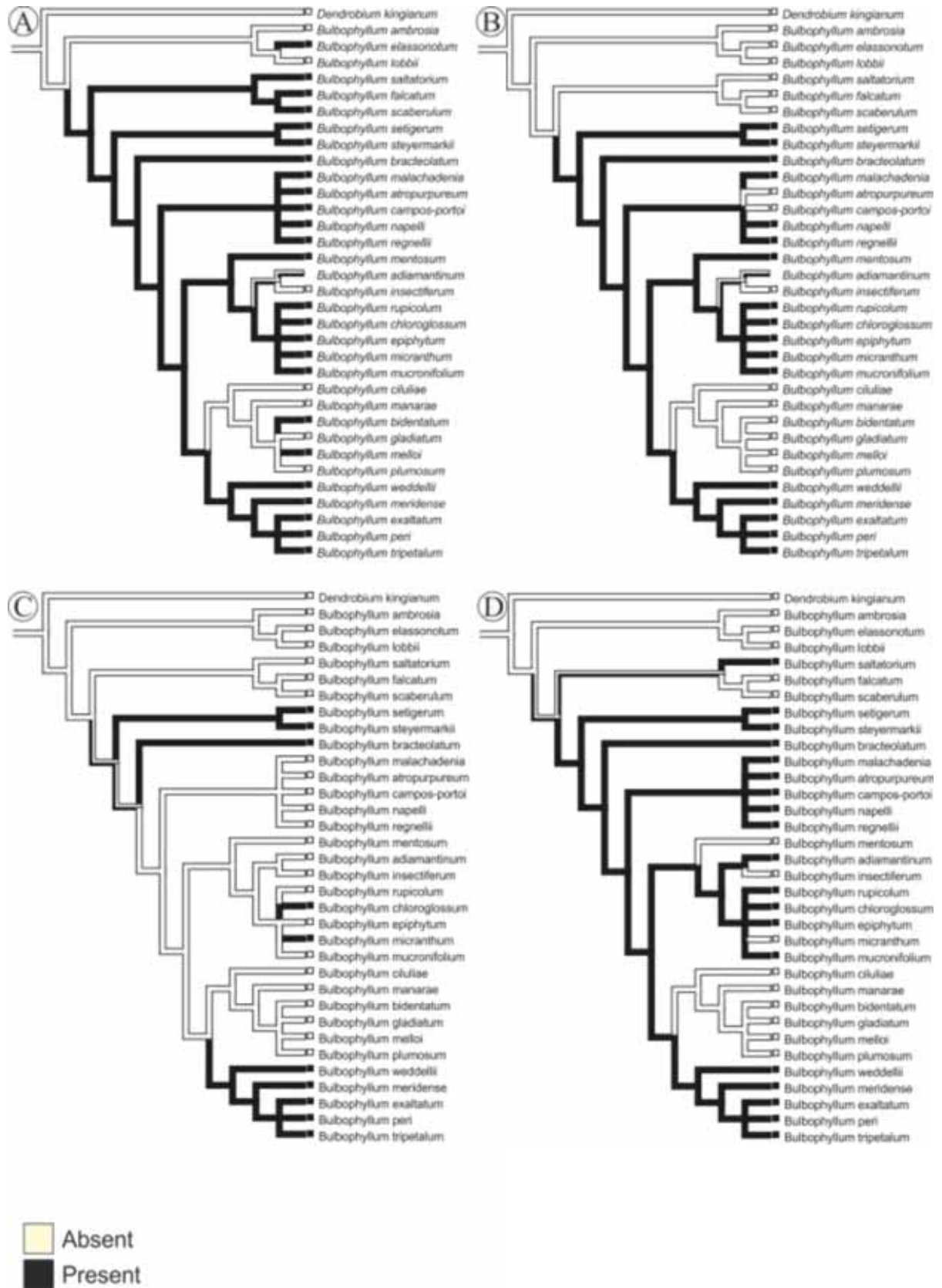


Figure 3. Reconstruction of the distribution of idioblasts with helical wall thickenings on the nrITS Bayesian tree across different floral organs. A) Sepals; B) Petals; C) Labellum; D) Column.

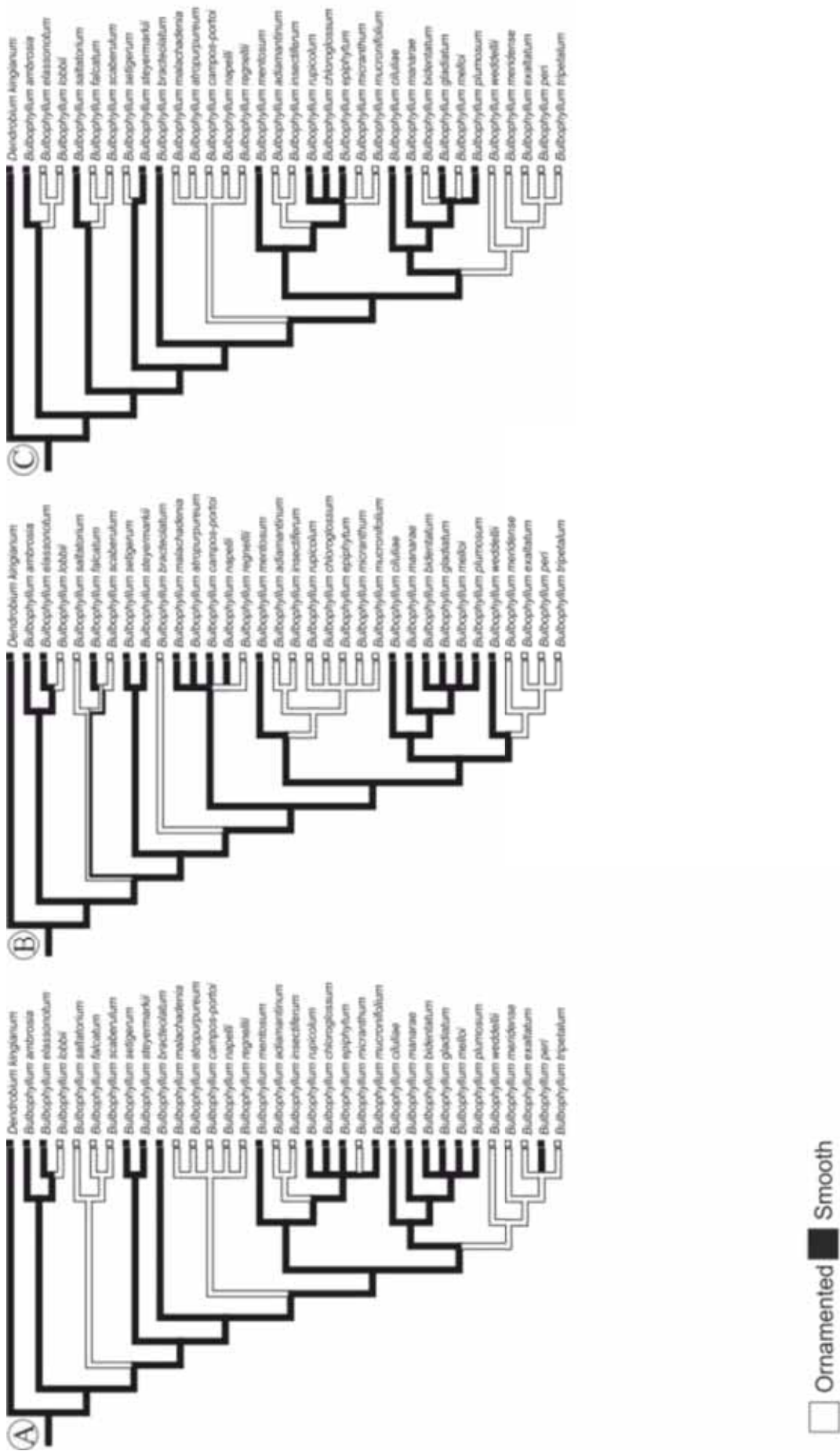


Figure 4. Reconstruction of the distribution of the adaxial surface ornamentation on the nrITS Bayesian tree across different floral organs. A) Dorsal sepal; B) Lateral sepals; C) Petals.

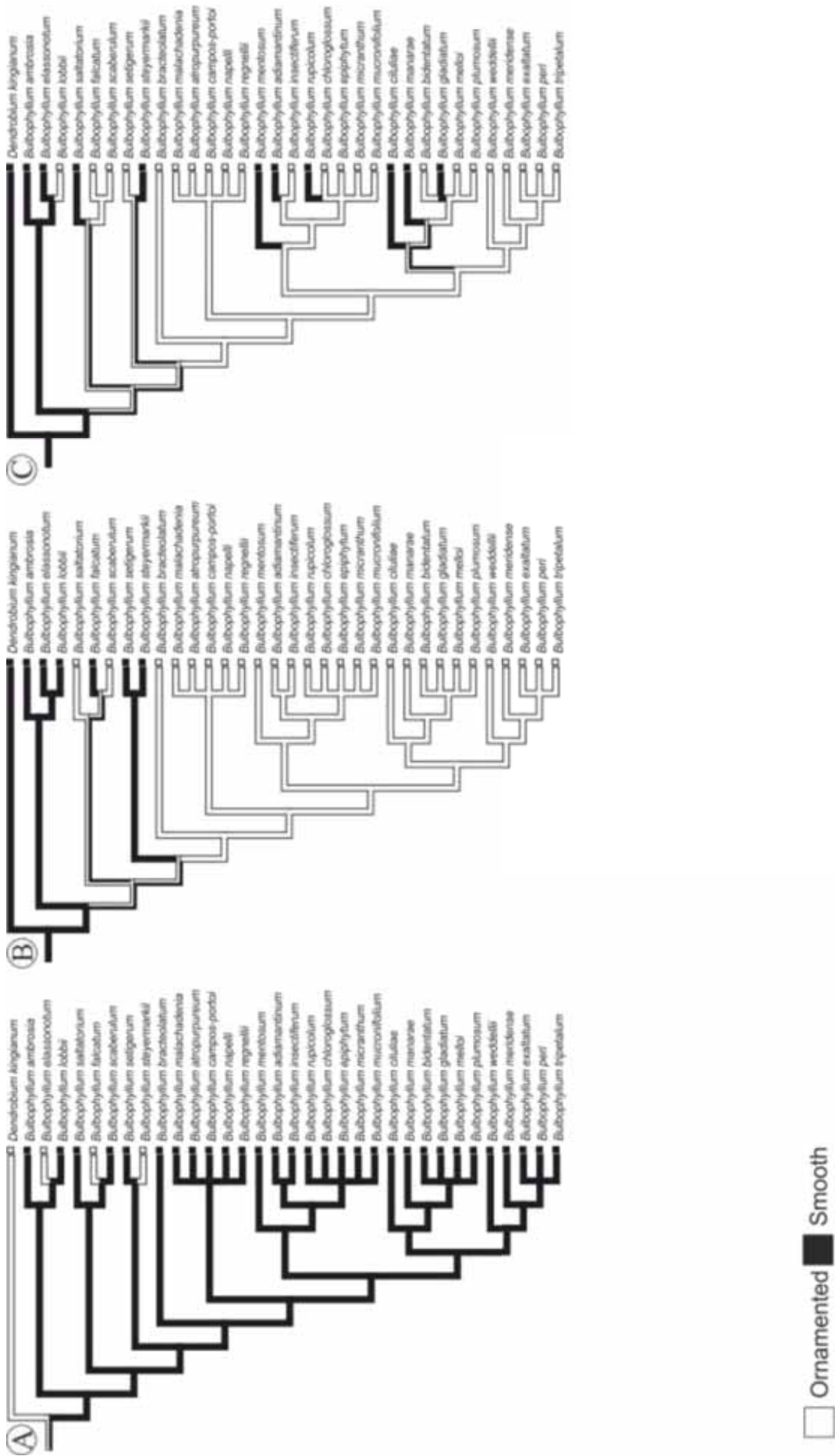


Figure 5. Reconstruction of the distribution of the abaxial surface ornamentation on the nrITS Bayesian tree across different floral organs. A) Dorsal sepal; B) Lateral sepals; C) Petals.

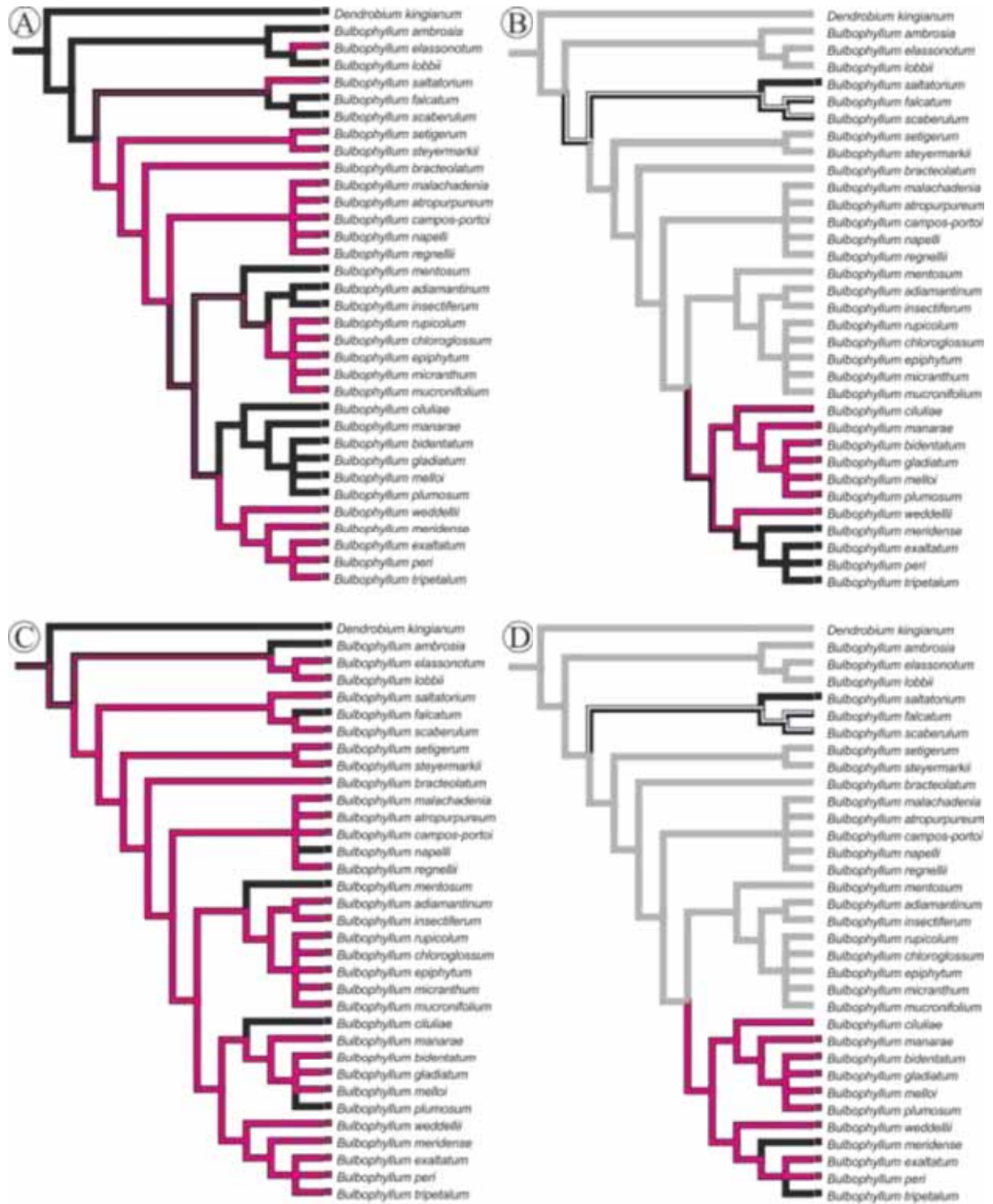


Figure 6. Reconstruction of the distribution of the surface ornamentation on the nrITS Bayesian tree. A) Labellum hypochile adaxial; B) Labellum epichile adaxial; C) Labellum hypochile abaxial; B) Labellum epichile abaxial.

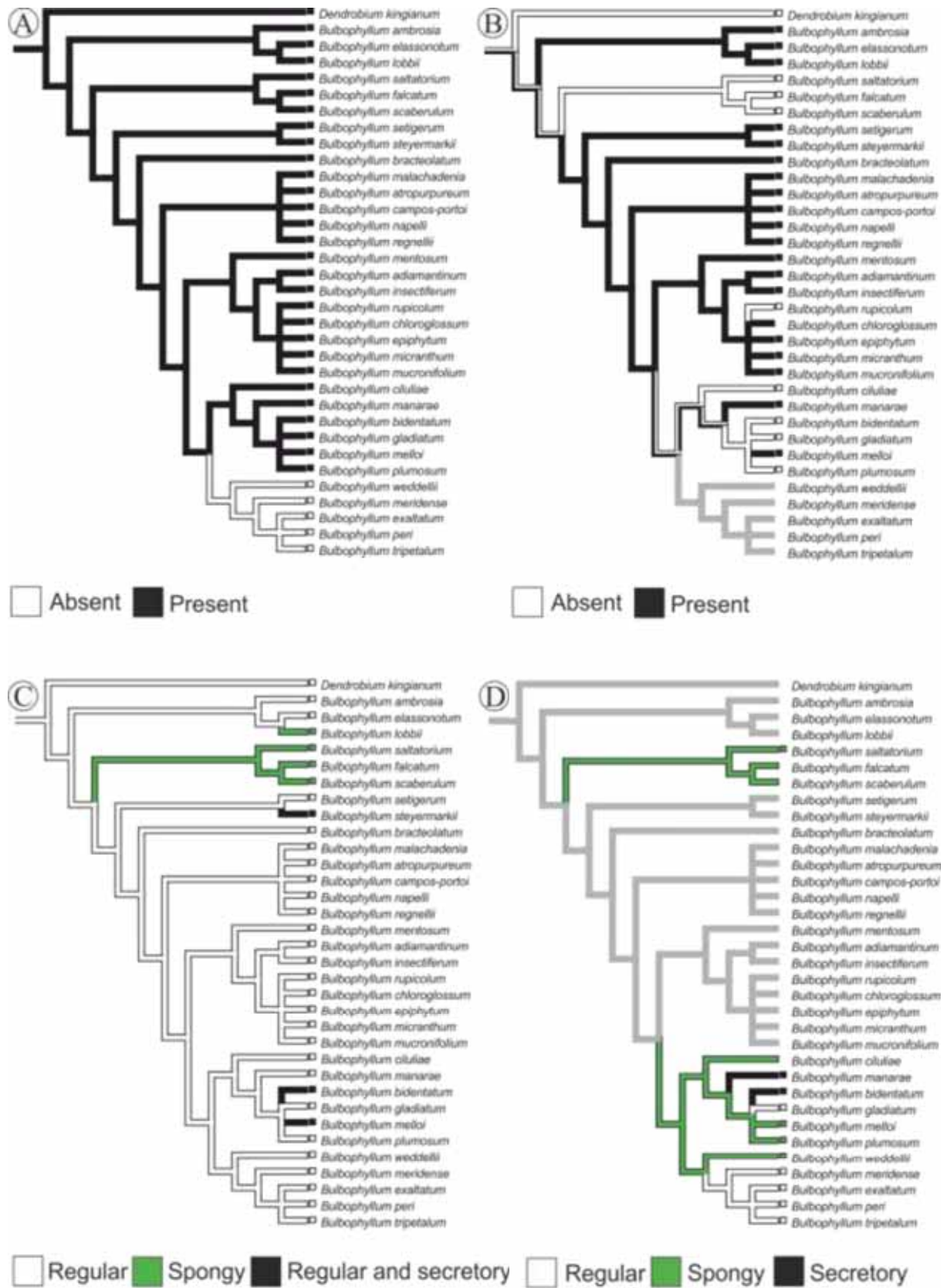


Figure 7. Reconstruction of the distribution of labellar secretory tissues on the nrITS Bayesian tree. A) Adaxial sulcus on the labellum hypochile ; B) Secretory activity of the adaxial sulcus on the hypochile ; C) Hypochile mesophyll; D) Epichile mesophyll.

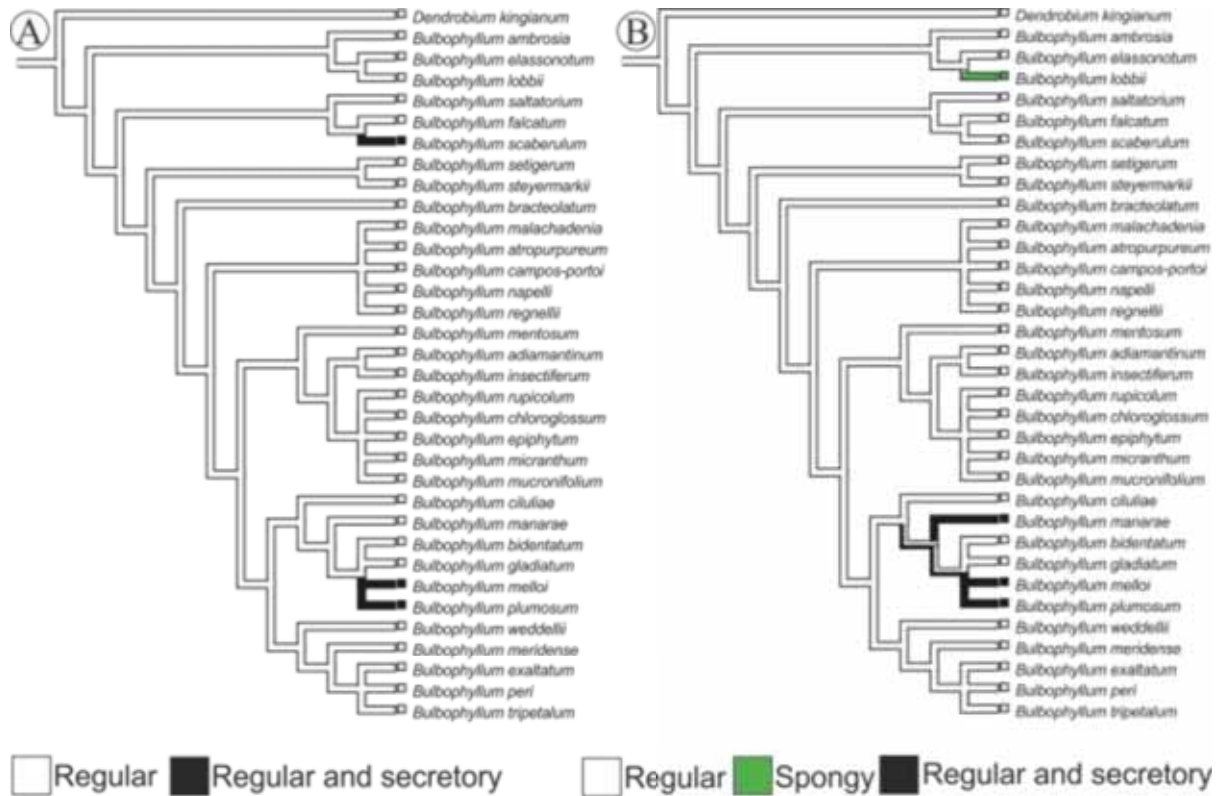


Figure 8. Reconstruction of the distribution of sepal secretory tissues on the nrITS Bayesian tree. A) Dorsal sepal ; B) Lateral sepal.

Considerações Finais

Após a análise de cerca de 50% das espécies Neotropicais de *Bulbophyllum*, pode-se afirmar que a anatomia e micromorfologia florais são mais diversas do que o previsto. Além disso, tecidos secretores foram evidenciados e também presentes de forma muito mais consistente e diversa do que o esperado, com base na literatura disponível.

De forma geral, as flores das espécies das seis seções Neotropicais do gênero compartilharam uma série de características que, em conjunto, auxiliam na caracterização do grupo, ainda que várias se repitam em grupos africanos e asiáticos. Nesse aspecto, poucas possíveis sinapomorfias florais puderam ser identificadas, o que era, de certa forma, esperado uma vez que isto se repete no gênero como um todo. Entretanto, é necessário ressaltar que mais análises precisam ser feitas utilizando o conjunto de dados levantados no presente trabalho, uma vez que as foram realizadas no Capítulo VI foram preliminares.

Vale ressaltar que foram encontrados dados micromorfológicos e anatômicos florais que permitem caracterizar cada seção, bem como diferenciar as espécies nelas inseridas.

Apesar do grande número de informações levantadas no presente trabalho, lacunas sobre a anatomia e micromorfologia florais de espécies Neotropicais e não-Neotropicais de *Bulbophyllum* ainda permanecem e direcionam possíveis trabalhos nesta área.

Para espécies Neotropicais, é necessário esclarecer a natureza do conteúdo dos tricomas secretores das sépalas para verificar se podem ser de fato osmóforos, bem como a dos tecidos secretores de espécies de *B.* sect. *Xiphizusa*, de *B.* sect. *Bulbophyllaria* e de *B.* sect. *Furvescens*. Além disso, estudos semelhantes são desejáveis em espécies não-Neotropicais, uma vez que a maior diversidade do gênero encontra-se fora dos Neotrópicos. Também são necessários estudos de biologia da polinização de espécies de *Bulbophyllum* como um todo, para, associados à anatomia e micromorfologia florais, tentar compreender a relação do maior gênero de Orchidaceae com a miofilia.

Apêndices



What do floral anatomy and micromorphology tell us about Neotropical *Bulbophyllum* section *Didactyle* (Orchidaceae: Bulbophyllinae)?

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Received 21 October 2013; revised 17 February 2014; accepted for publication 12 April 2014

Bulbophyllum section *Didactyle* comprises seven species, but distinction between these is often problematic. These species are pollinated by milichiid flies and air currents move the hinged labellum and press the pollinator against the gynostemium. The labellum structure is considered to be homogeneous and conservative for the genus. Therefore, the floral anatomy and micromorphology of *B.* section *Didactyle* were studied in order to identify characters useful for distinguishing the species. All species have sunken glandular trichomes on the abaxial surface of the sepals (possible osmophores) and a trilobed labellum, clothed with trichomes, with a secretory cavity in the callus that is bound by scale-like papillae. Of the c. 100 characters assessed, 25 varied between species, and each pair of species differed by at least four character states, mainly occurring on the labellum. The data presented allow for a distinction to be made between species and corroborates their grouping in *B.* section *Didactyle*, as proposed previously. The presence of osmophores and a nectary on the labellum is confirmed, although their structure is more diverse than anticipated. Moreover, structural differences between *B. weddellii* and the core of the section might be the result of the odour-mediated attraction of pollinators rather than flower morphology and thus phylogeny. © 2014 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2014, **175**, 438–452.

ADDITIONAL KEYWORDS: *Bulbophyllum exaltatum* – *Bulbophyllum involutum* – *Bulbophyllum meridense* – *Bulbophyllum perii* – *Bulbophyllum popayanense* – *Bulbophyllum tripetalum* – *Bulbophyllum weddellii* – myophilous – nectary – osmophore.

INTRODUCTION

Bulbophyllum Thouars comprises c. 1870 species (WCSP, 2013) and is the second largest genus of angiosperms (Frodin, 2004). Its distribution is pan-tropical, occurring in Australasia and the American tropics, but its greatest diversity is concentrated in South-East Asia (Vermeulen, 1987, 1993; Dressler, 1993; Siegerist, 2001). In the Neotropics, the genus is represented by 62 species (Smidt *et al.*, 2011) with a distribution ranging from northern Mexico to south-

ern Brazil (Smidt *et al.*, 2007). The greatest diversity of Neotropical *Bulbophyllum* is found in south-eastern Brazil in contact areas between the Atlantic rainforest and cerrado vegetation (Smidt *et al.*, 2007).

On the basis of molecular data, six monophyletic sections were recognized in Neotropical *Bulbophyllum*: *B.* section *Bulbophyllaria* (Rchb.f.) Griseb., *B.* section *Furvescens* E.C.Smidt, Borba & Van den Berg, *B.* section *Napelli* Rchb.f., *B.* section *Micranthae* Barb. Rodr., *B.* section *Didactyle* (Lindl.) Cogn. and *B.* section *Xiphizusa* (Rchb.f.) Cogn. (Smidt *et al.*, 2011). Of these sections, *B.* section *Furvescens* was first proposed by Smidt *et al.* (2011), and the remain-

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ing sections were recircumscribed to achieve monophyly (Smidt *et al.*, 2011).

Species of *B.* section *Didactyle* are mainly found in cerrado vegetation (Smidt *et al.*, 2011), growing directly on rocks and exposed to sunlight (Smidt, 2007). This section is characterized by unifoliate pseudobulbs, a racemose inflorescence with a thin rachis and distichously arranged flowers showing free lateral sepals, patent petals and a gynostemium foot with a bilobed apex that is shorter than the gynostemium length (Smidt *et al.*, 2011). The current circumscription of the section includes seven species based on the molecular phylogenetic analysis of five species and on the similar morphology of the other two (Smidt *et al.*, 2011).

This section shows great problems in specific delineation because of its highly variable morphology, and this has resulted in the naming of many geographically restricted 'species' (Smidt *et al.*, 2011), especially in the *B. exaltatum* species complex (Ribeiro *et al.*, 2008), with its broad distribution in the highland areas of South America and the presence of natural hybrids (Borba & Semir, 1998a; Mancinelli & Smidt, 2012).

The floral morphology of Neotropical *Bulbophyllum* is quite diverse, and some authors have suggested that this may be driven by environmental pressures and pollinator specificity (Borba & Semir, 1998b; Verola, 2002; Teixeira, Borba & Semir, 2004). The reproductive biology of three species from *B.* section *Didactyle* [*B. weddellii* (Lindl.) Rchb.f., *B. involutum* Borba, Semir & F.Barros and *B. exaltatum* Lindl. synonym *B. ipanemense* Hoehnel] is relatively well known. The main pollinators are Milichiidae flies, which show some specificity in pollinating *Bulbophyllum* spp., although the main pollinator of one species might be the secondary pollinator of another (Sazima, 1978; Borba & Semir, 1998b; Silva *et al.*, 1999; Azevedo, Borba & van den Berg, 2006). The pollination mechanism displayed by these species utilizes air currents to press the fly against the gynostemium (Sazima, 1978; Borba & Semir, 1998b), and it has been suggested that floral odour is the main cue in attracting these pollinators (Borba & Semir, 1998b; Silva *et al.*, 1999).

Studies of the flower morphology and anatomy of *Bulbophyllum* are scarce. Teixeira *et al.* (2004) studied the labellum anatomy of six Brazilian and one Asian species of *Bulbophyllum* with osmophores on their adaxial surface, and suggested that the labellum structure is homogeneous and highly conservative for the genus. Apart from this work, the only other report on the flower morphology for the genus is a study of the gynostemium of the Asian *B. ecornutum* J.J.Sm. (Rasmussen, 1985). Therefore, the aims of the present study are to describe the floral micromorphology and

Table 1. List of species studied and collection data

Species	Collection data
1. <i>B. exaltatum</i> Lindl.	BHCB (<i>BHMH</i> 479: Itabirito, Minas Gerais State, Brazil) HRCB (<i>E.L.P. Nunes</i> 13: Resende, Rio de Janeiro State, Brazil) HRCB (<i>A.I. Coan et al.</i> 195: São Gonçalo do Rio das Pedras, Minas Gerais State, Brazil)
2. <i>B. involutum</i> Borba, Semir & F.Barros	BHCB (<i>MHNJB</i> 918: Ouro Branco, Minas Gerais State, Brazil) HRCB (<i>E.L.P. Nunes</i> 12: Rio Pardo de Minas, Minas Gerais State, Brazil) HRCB (<i>A.I. Coan et al.</i> 134: São Gonçalo do Rio das Pedras, Minas Gerais State, Brazil)
3. <i>B. meridense</i> Rchb.f.	HRCB (<i>E.L.P. Nunes</i> 11: Caraguatatuba, São Paulo State, Brazil)
4. <i>B. perii</i> Schltr.	UPCB (<i>W.S. Mancinelli</i> 1143: Tibagi, Paraná State, Brazil)
5. <i>B. popayanense</i> Kraenzl.	HB (<i>W. Sweiden</i> 737: El Tambo, Cauca State, Colombia)
6. <i>B. tripetalum</i> Lindl.	UPCB (<i>W.S. Mancinelli</i> 1141: Tibagi, Paraná State, Brazil)
7. <i>B. weddellii</i> (Lindl.) Rchb.f.	HUEFS (<i>C. Azevedo</i> 188: Mucugê, Bahia State, Brazil) HRCB (<i>A.I. Coan et al.</i> 136: São Gonçalo do Rio das Pedras, Minas Gerais State, Brazil)

BHCB, Herbário Universidade Federal de Minas Gerais; BHMH, Museu de História Natural e Jardim Botânico da Universidade Federal de Minas Gerais; HB, Herbarium Bradeanum; HRCB, Herbário Rioclaresense, Universidade Estadual Paulista; HUEFS, Herbário Universidade Estadual de Feira de Santana; UPCB, Herbário Universidade Federal do Paraná.

anatomy of *B.* section *Didactyle* and to identify characters useful for both distinguishing between the species and circumscribing and diagnosing the entire section.

MATERIAL AND METHODS

Samples for this study were collected in the field or obtained from botanical gardens; all seven species currently assigned to *B.* section *Didactyle* were sampled (Table 1). Mature flowers were fixed in various fixatives chosen for their availability and suitability for use on field trips: FAA 50 (3.7% v/v formaldehyde, 50% ethanol, 5% acetic acid; Johansen,

1940), 1% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2 (McDowell & Trump, 1976), or 2% glutaraldehyde and 2% formaldehyde in 0.1 M phosphate buffer, pH 7.2 (Karnovsky, 1965). Micromorphological and anatomical characters were assessed using conventional means, such as light microscopy (LM) and scanning electron microscopy (SEM). At least four flowers from different specimens were analysed for each species, sourced, whenever possible, from different localities (Table 1).

The samples for LM analysis were embedded in historesin (Leica or Technovit), according to the instructions of the suppliers, and sectioned at 5–8 µm using disposable blades. Sections were stained with 0.05% toluidine blue O in 0.1 M sodium phosphate buffer, pH 6.8 (O'Brien, Feder & McCully, 1965), for general analysis. The following histochemical tests were performed on resin-embedded sections: Coomassie brilliant blue for proteins (Southworth, 1973), calcofluor white MR2 for cellulose (Hughes & McCully, 1975) and the periodic acid-Schiff reaction (PAS) for total insoluble polysaccharides (Feder & O'Brien, 1968).

Fresh material was hand sectioned and tested with iodine/potassium iodide solution (IKI) for starch (Johansen, 1940), Sudan III for lipids (O'Brien & McCully, 1981), Fehling's solutions A and B for reducing sugars (Purvis, Collier & Walls, 1964), neutral red under UV excitation for total lipids (Kirk, 1970), ruthenium red for pectic acids/mucilage (Johansen, 1940), ferric chloride for phenolic compounds (Johansen, 1940) and acidified phloroglucinol for lignin (Sass, 1951). Entire fresh flowers were immersed in neutral red for osmophore identification and distribution (Kearns & Inouye, 1993). Photomicrography was achieved by means of a Leica DMLB microscope coupled with a digital camera and using LAS (Leica Application Suite V3.3.0) software.

For SEM analysis, the samples were transferred from 70% ethanol to formaldehyde–dimethylacetal for 16–24 h (Gerstberger & Leins, 1978), further dehydrated by critical point drying (CPD 030, Balzers) and sputter coated with gold (SCD 050, Bal-Tec). SEM observations were made using a Zeiss DSM 950 and images were obtained using Digital Image Processing Software 2.2 (DIPS-Leipzig).

RESULTS

The flowers of *B.* section *Didactyle* studied have free sepals (Fig. 1A, B), reduced petals (Fig. 1A) and a movable labellum that is divided into three lobes (Fig. 1C, D). The median lobe is the largest (Fig. 1C, broken line) and is divided into two parts: the hypochile (the proximal and fleshy part) (Fig. 1C, D) and the epichile (the distal, less fleshy part) (Fig. 1C,

D). On the hypochile, there is a callus with a secretory cavity (Fig. 1D, arrow). The lateral lobes are smaller and ear shaped (Fig. 1C, D, full line). The gynostemium has a bilobed foot, two long stelidia (Fig. 1A, E–P) and two ventral teeth (Fig. 1A, J–P).

The dorsal sepal has, on its adaxial surface, conspicuous striate wall ornamentation (Fig. 2A), except for *B. popayanense* Kraenzl. and *B. meridense* Rchb.f. (Fig. 2B); the striae are pronounced in *B. perii* Schltr. and *B. tripetalum* Lindl. (Fig. 2C). In frontal view, the epidermal cells are transversely elongate (Fig. 2B, C), except for *B. weddellii*, in which the cells are more isodiametric (Fig. 2A). In transverse sections, the outer periclinal wall is flat (Fig. 2D). On the abaxial surface, the cuticle ornamentation is irregular (Fig. 2E) and the outer periclinal wall is flat (Fig. 2D), but usually striate (Fig. 2F). All species have abaxial stomata (Fig. 2E, arrow) and sunken multicellular glandular trichomes that react positively to an *in vivo* test for osmophore localization and faintly for phenolic compounds and pectic acids (Fig. 2E, arrowhead, G).

The margin of the dorsal sepal is smooth (Fig. 2H), except in *B. popayanense*, where it is papillose (Fig. 2I); in transverse sections of all species, the margin consists of both epidermal and mesophyll cells (Fig. 2H). The mesophyll is homogeneous (Fig. 2D, J) with regular parenchyma and shows two types of idioblast, one with cellulosic, helical wall thickenings (Fig. 2D, K–M) and the other with raphides (Fig. 2J, L, M). In transverse section, the central third of the dorsal sepal of most species contains three collateral vascular bundles (Fig. 2J), one of which is sometimes indistinct; *B. weddellii* and *B. popayanense* have five vascular bundles (Fig. 2M, 1–5), two of which are much reduced (Fig. 2M, 2 and 5).

The lateral sepals show obvious striate ornamentation on their adaxial surface (Fig. 2N), except for *B. weddellii*, in which it is inconspicuous (Fig. 2O); the striae are particularly conspicuous in *B. tripetalum*. In frontal view, the adaxial epidermal cells (Fig. 2N, O) and the outer periclinal wall in transverse sections (Fig. 2P) are similar to those of the dorsal sepal. On the abaxial surface, the ornamentation is also irregular (Fig. 2Q), but the outer periclinal wall, in transverse section, is slightly convex (Fig. 2P). On this surface, all species show stomata (Fig. 2Q, arrow) and multicellular glandular trichomes (Figs 2Q, arrowhead, 3A, B). The margin and mesophyll of the lateral sepals are similar to those of the dorsal sepal (Figs 2J, M, P, 3B), usually with three collateral vascular bundles, but there are five in *B. weddellii* (Fig. 3C) and six in *B. popayanense* (Fig. 3D).

The petals show striate ornamentation on the adaxial surface (Fig. 3E, F). In frontal view, the

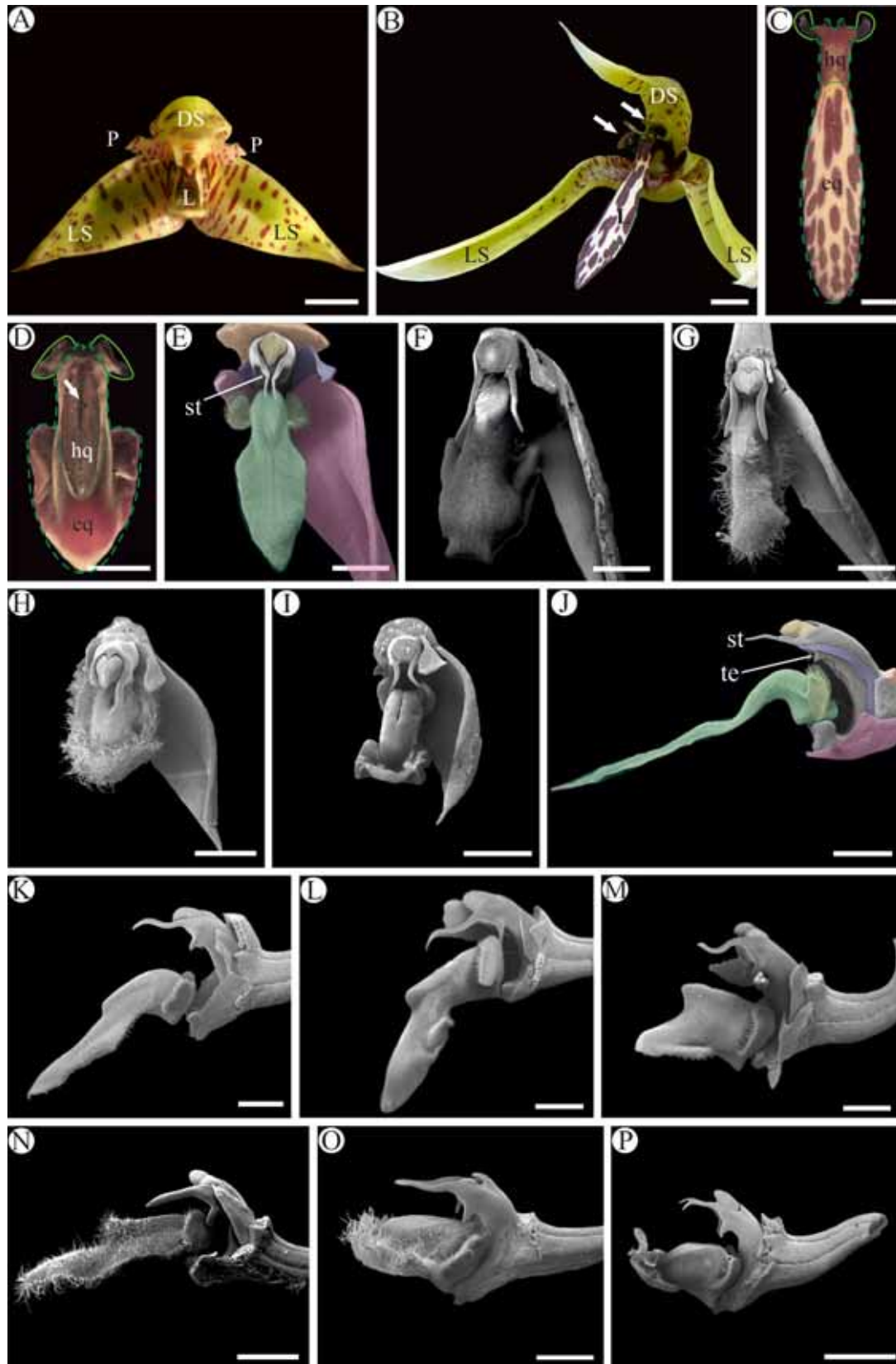


Figure 1. See caption on next page.

Figure 1. Floral morphology of *Bulbophyllum* section *Didactyle*. A, B, E–I, Flower in frontal view. J–P, Flower in side view. A, *Bulbophyllum exaltatum*. B, *Bulbophyllum weddellii* (arrows indicate flies visiting the flower). C, Labellum morphology of *B. weddellii* (middle lobe marked with a broken line and lateral lobes with a full line). D, Labellum morphology of *B. involutum* (middle lobe marked with a broken line and lateral lobes with a full line; arrow indicates the secretory cavity of the callus). E, *Bulbophyllum popayanense* (dorsal sepal in orange, lateral sepals in pink, petals in blue, middle lobe of the labellum in darker green and lateral lobes in light green, anther in yellow). F, *Bulbophyllum involutum*. G, Flower of *B. tripetalum*. H, *Bulbophyllum meridense*. I, *Bulbophyllum perii*. J, *Bulbophyllum weddellii*. K, *Bulbophyllum popayanense*. L, *Bulbophyllum involutum*. M, *Bulbophyllum exaltatum*. N, *Bulbophyllum tripetalum*. O, *Bulbophyllum meridense*. P, *Bulbophyllum perii*. DS, dorsal sepal; eq, labellum epichile; hq, labellum hypochile; L, labellum; LS, lateral sepal; P, petal; st, stelidium; te, tooth. Scale bars, 2 mm.

Figure 2. Micromorphology and anatomy of the sepals of *Bulbophyllum* section *Didactyle*. A–C, I, N, O, Adaxial surface. E, Q, Abaxial surface. D, F–H, J–M, P, Transverse section. A, *Bulbophyllum weddellii*. B, *Bulbophyllum meridense*. C, *Bulbophyllum tripetalum*. D, *Bulbophyllum exaltatum*. E, *Bulbophyllum involutum*. F, *Bulbophyllum weddellii*. G, *Bulbophyllum involutum*. H, *Bulbophyllum tripetalum*. I, *Bulbophyllum popayanense*. J, *Bulbophyllum involutum*. K, *Bulbophyllum perii*. L, *Bulbophyllum weddellii*. M, *Bulbophyllum popayanense*. N, *Bulbophyllum popayanense*. O–Q, *Bulbophyllum weddellii*. hi, idioblast with helical wall thickenings; ri, idioblast with raphides; vb, vascular bundle. Scale bars: 40 µm (A–C, K, N, O); 50 µm (D, L); 100 µm (E, M1–5, P, Q); 20 µm (F); 500 µm (G, J, M); 250 µm (H); 200 µm (I).

epidermal cells are more or less isodiametric (Fig. 3E), except for *B. weddellii*, in which they are longitudinally elongate (Fig. 3F). The outer periclinal wall, in transverse section, is flat in most species (Fig. 3G), but there are also papillose cells in *B. popayanense*, *B. exaltatum* and *B. involutum* (Fig. 3E, H, I). The ornamentation, cell shape and periclinal wall of abaxial epidermal cells are similar to those of their adaxial counterparts. The margin has unicellular trichomes (Fig. 3H), except for *B. weddellii* (Fig. 3I), *B. perii* and *B. tripetalum*. The mesophyll is homogeneous, with regular parenchyma and idioblasts with raphides and cellulosic helical wall thickenings (Fig. 3G, J). There is a single, central collateral vascular bundle (Fig. 3G, J).

The epichile shows striate ornamentation on its adaxial periclinal walls (Fig. 4A). Most epidermal cells are trichomes (Fig. 4B), except in *B. weddellii*, which has only longitudinally elongate cells (Fig. 4C); in *B. perii*, the trichomes are shorter and more regular in size than in the other species (Fig. 4D). Both abaxial and adaxial surfaces are similar (Fig. 4E). The margin bears larger trichomes with globose bases (Fig. 4B, arrows, F), except for *B. perii*, the trichomes of which are similar to those found on the surface of the epichile (Fig. 4D), and *B. weddellii*, in which they are lacking. The mesophyll is homogeneous, formed of regular parenchyma (Fig. 4B), with large intercellular spaces in *B. weddellii* (Fig. 4G), and containing idioblasts with raphides and cellulosic, helical wall thickenings (Fig. 4B, raphides not clearly visible).

The hypochile has a callus on its adaxial surface, which has scale-like papillose epidermal cells with striate ornamentation (Fig. 4H). These cells give a

positive reaction for lipids and form the boundary of the secretory cavity (Fig. 4I). The secretory cavity, in transverse sections, varies in size and shape between species (Fig. 4J–P) and is less conspicuous in *B. weddellii* (Fig. 4J, arrow). The epidermis usually has papillose cells (Fig. 4Q), especially at the base of the cavity (except for *B. popayanense* and *B. involutum*, which have short trichomes, Fig. 4R). With the epidermis, three to five subepidermal secretory layers (Fig. 4J–R) contain elevated levels of cytoplasmic proteins, as revealed by histochemical tests. At the callus periphery, the epidermis is similar to that of the epichile (Fig. 4D, E, J–P). The abaxial epidermis of the hypochile comprises only cells that have convex walls (Fig. 4S), papillae (Fig. 4T) or a combination of convex walls and trichomes (Fig. 4U). The mesophyll is similar to that of the epichile, but reacts positively for starch and lipids (Fig. 4J–P).

The lateral lobes of the labellum usually have trichomes on their adaxial surface (Fig. 2N), and papillae on the abaxial surface (Fig. 5A), whereas long trichomes occur at the margins and test positively for lipids (Figs 4U, 5A). The exceptions are *B. perii*, in which papillae clothe the entire surface of the lateral lobes (Fig. 5B), and *B. tripetalum*, which has flattened cells on its abaxial surface (Fig. 5C). The mesophyll is similar to that of the remainder of the labellum, but without vascularization (Fig. 5D).

The gynostemium lacks conspicuous ornamentation (Fig. 5E), and the epidermal cells, in frontal view, are longitudinally elongate (Fig. 5E). The mesophyll is homogeneous, with regular parenchyma containing idioblasts with raphides and cellulosic, helical wall thickenings (Fig. 5F). The gynostemium foot usually lacks conspicuous ornamentation (Fig. 5G), but it is

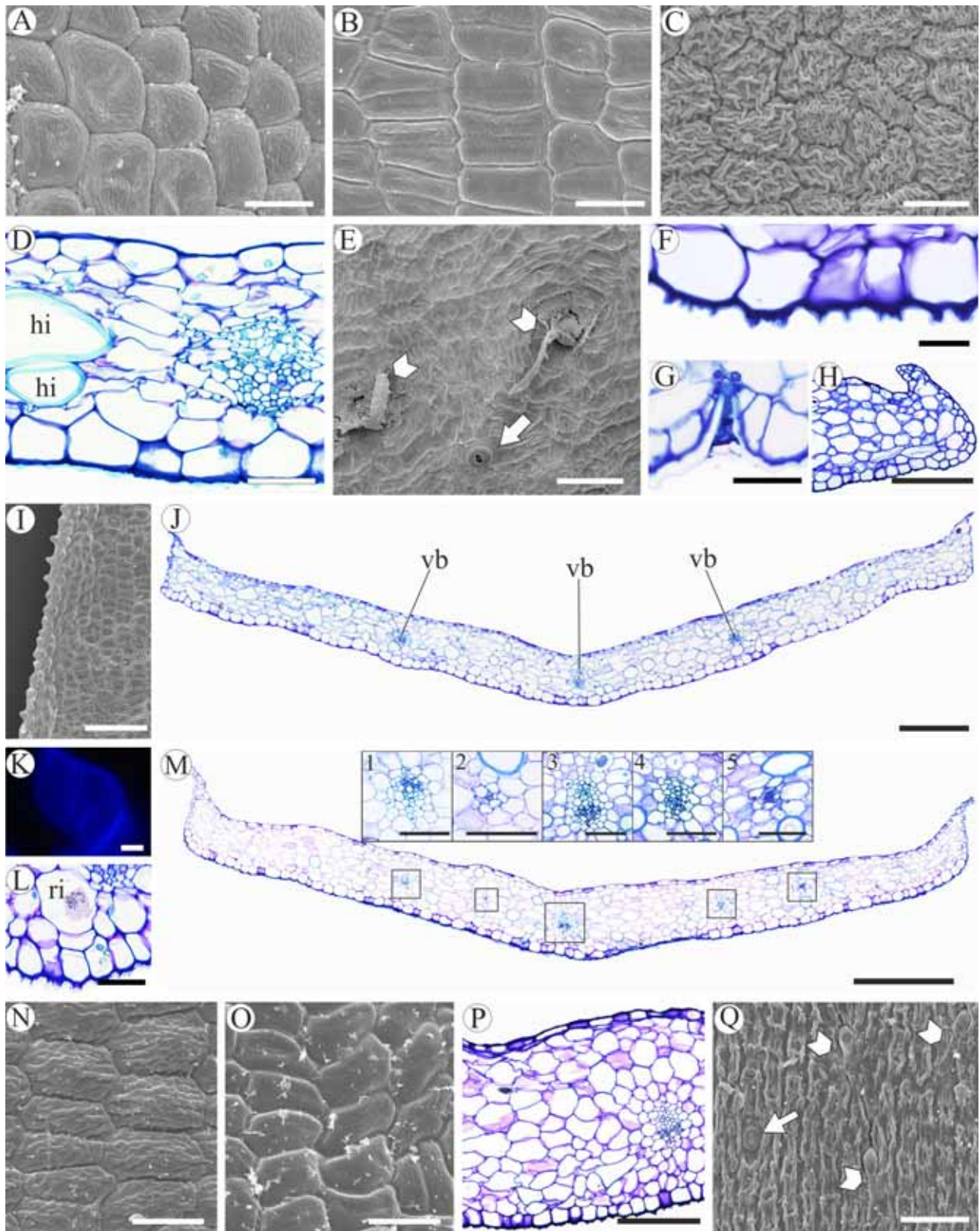


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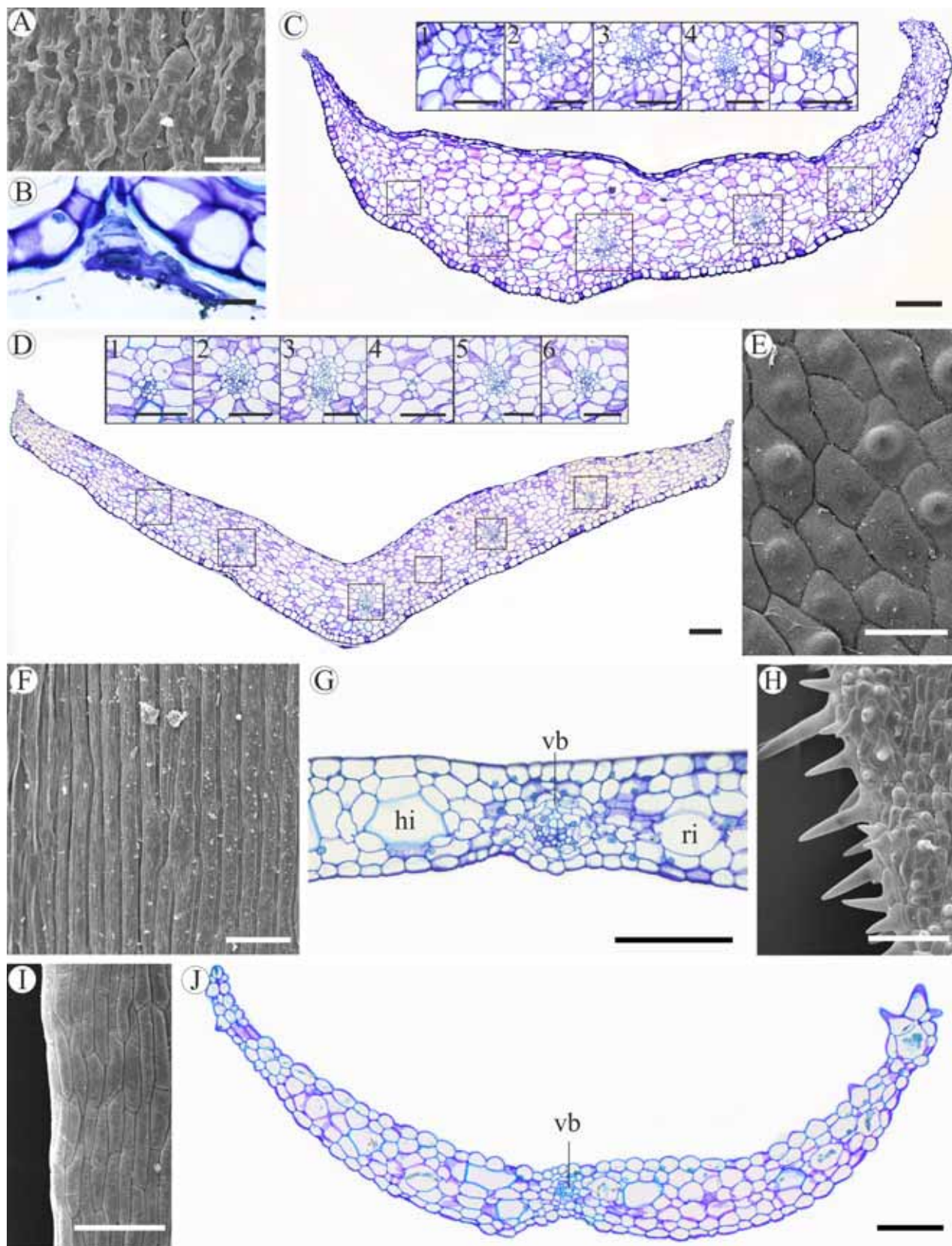


Figure 3. See caption on next page.

Figure 3. Micromorphology and anatomy of the lateral sepal and petals of *Bulbophyllum* section *Didactyle*. A, Scanning electron microscopy (SEM) detail of the abaxial surface of the lateral sepal of *B. weddellii* showing a glandular trichome. B, Light microscopy (LM) detail of a glandular trichome of *B. exaltatum*. C, Transverse section (TS) of the lateral sepal of *B. weddellii*. D, TS of the lateral sepal of *B. popayanense*. E, Adaxial surface of the petal of *B. involutum*. F, Adaxial surface of the petal of *B. weddellii*. G, TS of the petal of *B. meridense*. H, Detail of the margin of the petal of *B. meridense*. I, Detail of the margin of the petal of *B. weddellii*. J, TS of the petal of *B. popayanense*. hi, idioblast with helical wall thickenings; ri, idioblast with raphides; vb, vascular bundle. Scale bars: 40 µm (A, E, F); 20 µm (B); 200 µm (C, D); 100 µm (C1–5, D1–6, G, H, J); 60 µm (I).

striate in *B. exaltatum* and *B. tripetalum* (Fig. 5H). Its cells are longitudinally elongate from the frontal view (Fig. 5G, H).

The anther is papillose and its irregularly shaped cells have striate ornamentation (Fig. 5I). The pollinarium is composed of four pollinia (with smooth tetrads, the central two pollinia being smaller than the outer pollinia; Fig. 5J) and a viscidium formed by the disorganization of rostellar cells (Fig. 5K).

The stigma is longitudinally elongate (Fig. 5L), except in *B. weddellii*, which has a wider and isodiametric stigma (Fig. 5M); it comprises long, narrow cells embedded, but loosely arranged, in a mucilaginous matrix (Fig. 5K–M). The ovary is a complex, six-lobed structure, the lobes of which are arranged in two alternating whorls of three. The three outer lobes occur along the same radius as the sepals, and the three inner lobes occur along the same radius as the petals and carpels (Fig. 5N). In transverse section, the outer epidermal cells of the ovary appear columnar in shape, with convex outer periclinal walls (Fig. 5O). The inner epidermal cells are elliptic with convex outer walls (Fig. 5P). The ovary mesophyll is homogeneous and is formed of regular parenchyma containing idioblasts with raphides, with others having cellulosic, helical wall thickenings (Fig. 5N, O). Each lobe of the ovary contains a single collateral vascular bundle (Fig. 5N, O). Histochemical tests were performed on all species, and the results are summarized in Table 2. The characters that allow distinction between the species are summarized in Table 3.

DISCUSSION

In the present study, it was found that several floral micromorphological and anatomical characters were shared by most of the species of *B.* section *Didactyle*. The distinguishing features first described for the section were: multicellular glandular trichomes on the abaxial surface of the sepals; reduced petals with smooth or indumented margins, with a single collateral vascular bundle; a trilobed labellum, mostly clothed with trichomes, with a callus having a secretory cavity bound by scale-like papillae; a pollinarium formed of four pollinia with smooth tetrads; and

mesophyll with both crystalliferous and thickened idioblasts in all floral parts.

These data display better characterization and congruence when species considered to be representatives of *B.* section *Didactyle* are grouped according to Smidt *et al.* (2011). Although these authors used molecular techniques to study only five species, they speculated, on the basis of gross morphology, that the remaining two species also belong in this section. Prior to that, the most recent treatment of the group was that of Pabst & Dungs (1975), who considered only four species currently assigned to *B.* section *Didactyle*, with several species of *B.* section *Napelli* Rchb.f and a single species of *B.* section *Xiphisuza* E.C.Smidt, Borba & van den Berg.

Members of Orchidaceae display a great diversity of labellar osmophores (Stern, Curry & Pridgeon, 1987; Vogel, 1990; Ascensão *et al.*, 2005; Pansarin, Castro & Sazima, 2009; Wiemer *et al.*, 2009; Lumaga *et al.*, 2012). The labella of all species of *B.* section *Didactyle* studied here bear epidermal cells which, on the basis of their structure and cellular content, can be identified as osmophores. Similar data were obtained by Teixeira *et al.* (2004) for representatives of the same section, and the present results confirm and build upon their findings.

A feature first described here for *Bulbophyllum* flowers is the presence of sunken multicellular glandular trichomes on the abaxial surface of the sepals. Despite several attempts to discover their contents, histochemical tests performed on fresh flowers were not conclusive, but some cellular components showed a strong affinity for neutral red and others stained weakly for phenolic compounds and pectic acids (Table 2). Although there is anatomical evidence to consider these trichomes to be osmophores, other evidence is lacking (Stern, Curry & Whitten, 1986). However, this is not entirely true as, based on the behaviour of the pollinators of three of the seven species assigned to this section, there is indeed evidence that these structures are osmophores. For example, according to Borba & Semir (1998b), pollinating flies are more frequently attracted to (and alight on) the sepals than to other parts of the perianth. From here, they pass to the labellum and feed on the nectar. The presence of two possible types of

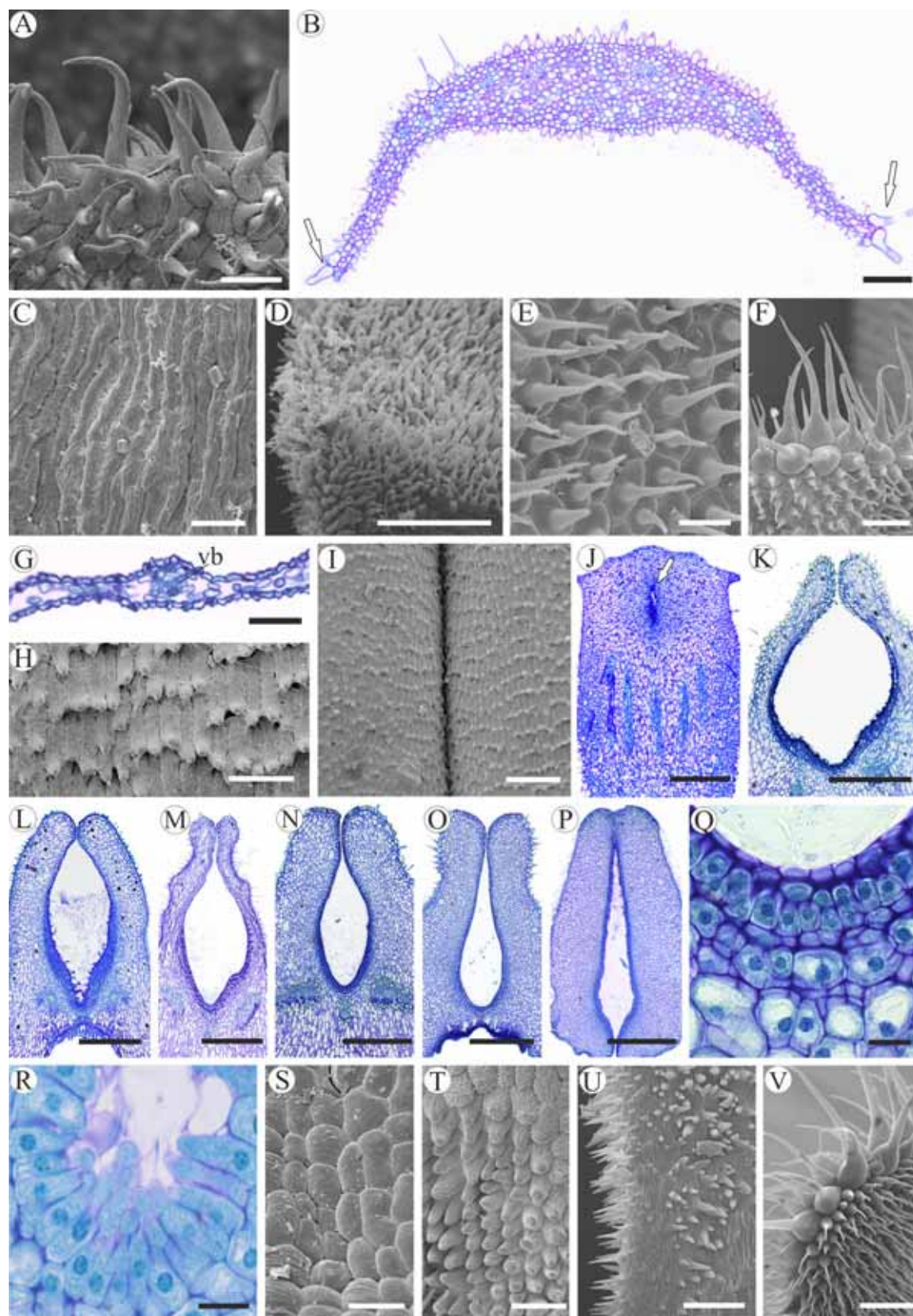


Figure 4. See caption on next page.

Figure 4. Micromorphology and anatomy of the labellum of *Bulbophyllum* section *Didactyle*. A, Adaxial surface of the epichile of *B. popayanense*. B, Transverse section (TS) of the epichile of *B. tripetalum*. C, Adaxial surface of the epichile of *B. weddellii*. D, Adaxial surface of the epichile of *B. perii*. E, Abaxial surface of the epichile of *B. exaltatum*. F, Detail of the margin trichomes of the epichile of *B. involutum*. G, TS of the epichile of *B. weddellii*. H, Adaxial surface of the labellum hypochile (callus) of *B. popayanense*. I, Detail of the callus adaxial surface showing the secretory cavity opening of *B. popayanense*. J, TS of the callus of *B. weddellii*. K, TS of the callus of *B. popayanense*. L, TS of the callus of *B. involutum*. M, TS of the callus of *B. exaltatum*. N, TS of the callus of *B. meridense*. O, TS of the callus of *B. tripetalum*. P, TS of the callus of *B. perii*. Q, Detail of the secretory cavity cells of *B. meridense*. R, Detail of the secretory cavity cells of *B. involutum*. S, Abaxial surface of the hypochile of *B. involutum*. T, Abaxial surface of the hypochile of *B. perii*. U, Abaxial surface of the hypochile of *B. weddellii*. V, Lateral lobe adaxial surface of *B. involutum*. vb, vascular bundle. Scale bars: 40 µm (A, E, H, S, T); 100 µm (B, D, F, I, V); 20 µm (C, G, Q, R); 500 µm (J–P); 200 µm (U).

trichome on two different floral parts indicates the importance of floral odour in attracting pollinators to these species. If confirmed, this is only the second report of this phenomenon in Orchidaceae. The first report of this kind is, to our knowledge, that of Stpiczyńska (1993), who investigated *Cymbidium tracyanum* L.Castle. However, osmophores occur at the tips of the petals and labellum in that species.

Somewhat similar sunken trichomes have also been recorded for the vegetative organs of Maxillariinae and Lycastinae (Stern, Judd & Carlswald, 2004), Pleurothallidinae (Pridgeon, 1981, 1982) and Dendrobiinae (Morris, Stern & Judd, 1996), and on the flower (tepals and labellum) of a single species of *Maxillaria* Ruiz & Pav. (Stpiczyńska & Davies, 2009). These floral hairs are thought to secrete resin. Mayer, Cardoso-Gustavson & Appezzato-da-Glória (2011) also found similar trichomes on both the vegetative and reproductive organs of *Cyrtorchilum flexuosum* Kunth [synonym *Oncidium flexuosum* (Kunth) Lindl.]. These hairs secreted a mixture of mucilage, lipophilic compounds and proteins, and were identified as colleters. Similar histochemical analyses should now be performed in order to determine the composition of the secretory products of the glandular sepal trichomes of this section of *Bulbophyllum*. The secretory cavity of the labellar callus was also present in all species and, based on anatomical and histochemical evidence, is here interpreted to be a nectary. This agrees with the findings of Teixeira *et al.* (2004) for certain species of Neotropical *Bulbophyllum*. One interesting discovery was that the secretory cavity of *B. weddellii*, sister species to the core of the section, was notably smaller than that of other species, which might indicate a tendency in this lineage to develop increasing amounts of nectariferous tissue during the course of evolution. Pollination in *B. weddellii*, *B. exaltatum* and *B. involutum* is, to a degree, dependent on air currents (Borba & Semir, 1998b), but the presence of nectar also appears to be important for this type of pollination mechanism to work, as has been suggested elsewhere (Borba & Semir, 1998b; Teixeira *et al.*, 2004). We speculate that there

is a trade-off of highly adapted floral morphology in wind-assisted species, where wind is involved in both the attraction of the pollinator and pressing it against the gynostemium, as in *B. weddellii* (pendulous inflorescence, several flowers open simultaneously, labellum is petaloid and spatulate), for simpler flowers that represent the core of the section (erect inflorescence, few flowers open simultaneously, sepals smaller and the labellum more fleshy), which are capable of producing more nectar. This results in flies being attracted to the flower for longer periods of time.

Other characters that aid in the better distinction between species were also present (Table 3). Of all the species of *B.* section *Didactyle* investigated, *B. weddellii* showed 11 exclusive character states, mostly on the labellum. *Bulbophyllum perii* was second in terms of the number of exclusive character states, with six character states occurring exclusively on the labellum. *Bulbophyllum popayanense* and *B. tripetalum* showed four exclusive character states, *B. involutum* and *B. exaltatum* showed only one, and *B. meridense* showed none. Despite the paucity of exclusive character states present in some species, other character states differed between similar species, thus allowing distinction between the latter. *Bulbophyllum exaltatum* and *B. involutum* can be distinguished on the basis of four characters (secretory cavity length, secretory cavity shape in transverse section, number of vascular bundles in the labellum hypochile and gynostemium foot surface ornamentation), whereas *B. meridense* and *B. tripetalum* differ in seven characters (ornamentation of the adaxial surface of the dorsal sepal, ornamentation of the adaxial surface of the lateral sepal, type of petal margin cell, secretory cavity length, secretory cavity shape in transverse section, cell type of abaxial surface of the lateral lobes and gynostemium foot surface ornamentation).

Moreover, *B. weddellii* also exhibits different floral morphological characters, such as falcate petals, a long spatula-like labellum with a small callus and a small secretory cavity on the labellum, which is why it was formerly assigned to *B.* section *Xiphizusa* by

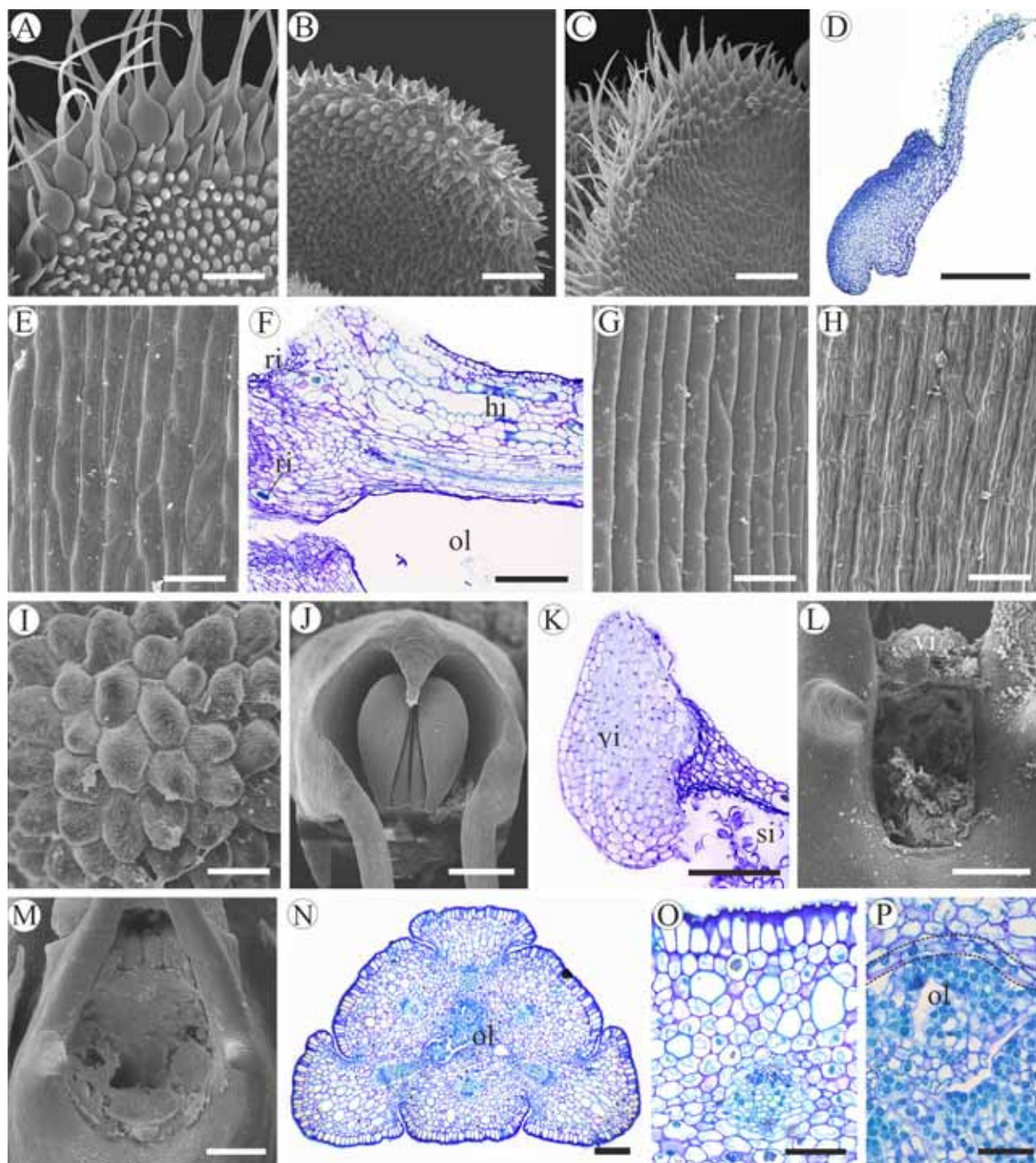


Figure 5. Micromorphology and anatomy of the labellum lateral lobes and gynostemium. A, Abaxial surface of the lateral lobe of *B. meridense*. B, Adaxial surface of the lateral lobe of *B. perii*. C, Abaxial surface of the lateral lobe of *B. tripetalum*. D, Transverse section (TS) of the lateral lobe of *B. meridense*. E, Gynostemium surface of *B. meridense*. F, Longitudinal section (LS) of the gynostemium of *B. exaltatum*. G, Gynostemium foot surface of *B. weddellii*. H, Gynostemium foot surface of *B. tripetalum*. I, Anther surface of *B. perii*. J, Frontal view of the gynostemium without the anther cap of *B. exaltatum*. K, LS of the developing viscidium of *B. involutum*. L, Detail of the longitudinally elongate stigma of *B. popayanense*. M, Detail of the broad stigma of *B. weddellii*. N, TS of the ovary of *B. involutum*. O, Detail of the ovary of *B. involutum* showing its outer epidermis. P, Detail of the ovary locule of *B. involutum* showing its inner epidermis (broken lines). hi, idioblast with helical wall thickenings; ol, ovary locule; ri, idioblast with raphides; S, sepal; si, stigma; vi, viscidium. Scale bars: 100 μ m (A, B, O); 200 μ m (C, K, N); 500 μ m (D, F); 40 μ m (E, G–I); 400 μ m (J, L, M); 50 μ m (P).

Table 2. List of histochemical tests performed on species of *Bulbophyllum* section *Didactyle* with common results and reaction sites

Reagent	Reaction	Location
Coomassie brilliant blue	Proteins stain blue	Labellum secretory epithelium
Calcofluor white MR2	Cellulosic cell walls fluoresce bright blue	Idioblasts with helical wall thickenings; epidermis striate wall
Periodic acid-Schiff	Polysaccharides stain purplish red	Cell walls, labellum mesophyll starch
Iodine/potassium iodide solution	Starch stains blue-black	Labellum mesophyll starch
Neutral red <i>in vivo</i>	Lipids/volatile compounds stain red	Sepal glandular trichomes; larger trichomes of the labellum margin and lateral lobes
Neutral red under UV light	Lipids fluoresce greenish blue	Larger trichomes of the labellum margin and lateral lobes, callus papillae
Sudan III	Lipids stain orange to red	Larger trichomes of the labellum margin and lateral lobes; small drops in the mesophyll cells
Fehling's solutions A and B	Aldehyde group (reducing sugars) is indicated by a red precipitate	Labellum secretory cavity cells – inconclusive
Ruthenium red	Pectic acids stain pink to red	Sepal glandular trichomes
Ferric chloride	Phenolic compounds stain dark grey or blue-black	Sepal glandular trichomes
Acidified phloroglucinol	Lignin stains red	Secondary walls of xylem elements

Pabst & Dungs (1975). However, despite these floral differences, *B. weddellii* also shares many characters with other species of the section (this study), shares the same pollinators as *B. involutum* (Borba & Semir, 1998b) and has a similar floral odour to that of *B. involutum* (Silva *et al.*, 1999), to which it is closely related (Azevedo, Borba & van den Berg, 2006). More recently, *B. weddellii* was placed in *B.* section *Didactyle* as sister group to the remaining six species, and *B.* section *Xiphisuzza* was placed as sister group to *B.* section *Didactyle* (Smidt *et al.*, 2011). As reported in other members of Orchidaceae (van der Pijl & Dodson, 1966; Hills, Williams & Dodson, 1972; Schiestl & Ayasse, 2002; Schiestl *et al.*, 2003; Mant, Peakall & Schiestl, 2005; Xu *et al.*, 2011) and other plant families (Proctor, Yeo & Lack, 1996), floral odour plays a critical role in both attracting pollinators and pollinator specificity. As suggested by Borba & Semir (1998b), this may be the most important signal for the attraction of a pollinator in *B. weddellii*, *B. involutum* and *B. exaltatum*. Attraction primarily by floral odours may allow for less rigid variation in floral morphology, micromorphology and anatomy, and this, in turn, probably explains the differences shown here between the flowers of *B. weddellii* and *B. involutum* (despite these species sharing the same two pollinators) and similarities between *B. involutum* and *B. exaltatum* (despite being pollinated by different species and possessing different floral odours; Borba & Semir, 1998b; Silva *et al.*, 1999).

Information on floral morphology and anatomy in Neotropical *Bulbophyllum* spp. is scarce, and the

variation found in this study was greater than anticipated, especially with regard to labellum micromorphology, which had only been studied previously for a small number of species (Teixeira *et al.*, 2004). We also found differences between the species that they studied, including greater distinction between the labellar surfaces of each taxon. For example, in *B. weddellii*, the abaxial surface of the hypochile possessed both convex-walled cells and short trichomes (referred to as papillae), whereas, on the lateral lobes, the papillae were restricted to the abaxial surface, the adaxial surface having trichomes, with much larger trichomes at the margins (referred to only as the presence of papillae and trichomes). Only trichomes were found on the epichile of *B. involutum* and *B. exaltatum*, whereas that of *B. weddellii* was glabrous (previous authors reported papillae and trichomes for *B. involutum* and a smooth surface for *B. exaltatum* and *B. weddellii*, referring to this part of the labellum as the 'limb'). These differences may solely be a result of differences in terminology, especially as the distinction between trichomes and papillae is somewhat subjective, and this may be exacerbated by the fact that papillae often mature to form trichomes. Moreover, a degree of intraspecific variation is to be expected. Although previous authors considered the organization of the epidermal surface to be homogeneous and conservative in *Bulbophyllum* (Teixeira *et al.*, 2004), the present study, merely by expanding taxon sampling in *B.* section *Didactyle*, demonstrated considerable variation, and it is anticipated that even greater variation will follow the

Table 3. List of variable characters between species of *Bulbophyllum* section *Didactyle*

Character		<i>B. weddellii</i>	<i>B. popayanense</i>	<i>B. involutum</i>	<i>B. exaltatum</i>	<i>B. meridense</i>	<i>B. tripetalum</i>	<i>B. perii</i>
Dorsal sepal	Adaxial	Surface ornamentation	Striate	Inconspicuous	Striate	Inconspicuous	Heavily striate	Heavily striate
		Cell shape in frontal view	± Isodiametric	Transverse elongate	Transverse elongate	Transverse elongate	Transverse elongate	Transverse elongate
	Margin	Type	Smooth	Papillose	Smooth	Smooth	Smooth	Smooth
	Mesophyll	Vascular bundles	5	5	3	3	3	3
Lateral sepal	Adaxial	Surface ornamentation	Inconspicuous	Striate	Striate	Striate	Heavily striate	Striate
		Cell shape in frontal view	Irregular	Transverse elongate	Transverse elongate	Transverse elongate	Transverse elongate	Transverse elongate
	Margin	Type	Smooth	Papillose	Smooth	Smooth	Smooth	Smooth
	Mesophyll	Vascular bundles	5	6	3	3	3	3
Petal	Adaxial + abaxial	Cell shape in frontal view	Longitudinally elongate	± Isodiametric	± Isodiametric	± Isodiametric	± Isodiametric	± Isodiametric
		Outer periclinal wall shape	Flat	Flat + papillose	Flat + papillose	Flat	Flat	Flat
	Margin	Margin cell type	Flat	Unicellular trichome	Unicellular trichome	Unicellular trichome	Flat	Flat
Labellum median lobe (epichile)	Adaxial + abaxial	Cell type	Longitudinally elongate	Unicellular trichome	Unicellular trichome	Unicellular trichome	Unicellular trichome	Unicellular trichome
	Margin	Trichome size	–	Long	Long	Long	Long	Short
		Trichomes	Absent	Larger	Larger	Larger	Larger	Similar to the remaining
Labellum median lobe (hypochile)	Mesophyll	Parenchyma	Loose	Compact	Compact	Compact	Compact	Compact
	Callus	Type	Inconspicuous	Conspicuous	Conspicuous	Conspicuous	Conspicuous	Conspicuous
		Secretory cavity length	1/7 of callus size	1/2 of callus size	Almost 1/2 of callus size	Longer than 1/2 of callus size	Less than 1/3 of callus size	Longer than 1/2 of callus size
		Secretory cavity shape in transverse section	Irregular	Ovate	Oblong-elliptic	Elliptic	Lanceolate	Subulate
	Abaxial	Cell type	Convex + short trichomes	Convex	Convex	Convex	Convex	Papillae
Labellum lateral lobe	Mesophyll	Vascular bundles	5	3	3	3	3	3
	Adaxial	Cell type	Trichome	Trichome	Trichome	Trichome	Trichome	Papillae
	Margin	Cell type	Larger trichomes	Larger trichomes	Larger trichomes	Larger trichomes	Larger trichomes	Papillae
	Abaxial	Cell type	Papillae	Papillae	Papillae	Papillae	Flat	Papillae
Gynostemium	Foot	Surface ornamentation	Inconspicuous	Inconspicuous	Striate	Inconspicuous	Striate	Inconspicuous
	Stigma	Shape	Almost circular	Longitudinally elongate	Longitudinally elongate	Longitudinally elongate	Longitudinally elongate	Longitudinally elongate

Character states in bold are exclusive to that species.

expansion of sampling to include other species of Neotropical *Bulbophyllum*.

ACKNOWLEDGEMENTS

We thank the São Paulo Research Foundation (FAPESP) for the Doctorate fellowship (Process 2011/11374-3) and Research Internship Abroad Fellowship (BEPE) (Process 2012/03433-2) to E.L.P.N., and the National Council of Technological and Scientific Development (CNPq) (Process 475212/2011-8 and 306498/2012-0 to A.I.C.). We are also very grateful to Sabine Adler (Ruhr Universität Bochum) for all the support and assistance with SEM, and the anonymous reviewers who helped to improve the manuscript.

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	1	2	3	4	5	6	7	8	9	0	1	1	2	3	4	5	6	7	8	9	0	1	2	2	2	2	2	2
<i>Bulbophyllum adiamantinum</i>	0	0	0	0	0	1	0	0	0	0	1	1	0	1	0	0	0	1	1	0	0	1	1	0	0	0	0	1
<i>Bulbophyllum ambrosia</i>	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1
<i>Bulbophyllum atropurpureum</i>	0	0	1	0	0	0	0	0	4	1	0	1	0	1	0	0	0	0	0	?	2	0	1	2	0	0	0	1
<i>Bulbophyllum bidentatum</i>	0	1	0	0	0	0	0	0	1	1	0	1	0	1	0	0	1	0	0	0	0	0	1	2	0	0	0	1
<i>Bulbophyllum bracteolatum</i>	0	1	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	1
<i>Bulbophyllum campos portoi</i>	0	0	0	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	?	2	0	1	0	0	0	0	1
<i>Bulbophyllum chloroglossum</i>	0	1	0	0	0	0	0	0	0	1	0	1	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0	1
<i>Bulbophyllum ciluliae</i>	0	1	0	0	0	0	0	0	0	1	0	1	0	1	0	0	1	0	0	0	0	1	1	2	0	0	0	1
<i>Bulbophyllum elassonotum</i>	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	1	0	?	0	0	1
<i>Bulbophyllum epiphytum</i>	0	1	0	0	0	0	0	0	2	1	0	1	0	1	0	1	0	0	0	0	1	1	1	0	0	0	0	1
<i>Bulbophyllum exaltatum</i>	0	0	0	1	0	0	0	0	3	4	1	0	1	0	0	0	0	0	0	3	2	1	1	0	0	0	0	1
<i>Bulbophyllum falcatum</i>	1	0	0	1	0	0	0	1	2	1	1	0	0	1	0	0	0	0	0	2	0	0	1	0	0	0	0	1
<i>Bulbophyllum gladiatum</i>	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	1	1	2	0	0	0	1
<i>Bulbophyllum granulosum</i>	0	0	0	1	0	0	0	0	3	0	1	0	1	0	0	0	0	0	?	2	0	1	0	0	0	0	0	1
<i>Bulbophyllum insectiferum</i>	0	0	0	0	0	1	0	0	2	0	1	0	1	0	0	1	0	0	1	0	1	1	0	0	0	0	0	1
<i>Bulbophyllum involutum</i>	0	0	0	1	0	0	0	0	3	4	1	0	1	0	0	0	0	0	3	2	1	1	0	0	0	0	0	1
<i>Bulbophyllum lobbii</i>	0	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1
<i>Bulbophyllum malachadenia</i>	0	0	0	1	0	0	0	0	2	0	1	0	1	0	0	1	0	0	?	0	0	1	0	0	0	0	0	1
<i>Bulbophyllum manarae</i>	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	1	2	0	0	0	0	1
<i>Bulbophyllum melloi</i>	0	1	0	0	0	0	0	1	0	1	1	0	1	0	0	0	1	0	0	0	0	0	1	2	0	0	1	1
<i>Bulbophyllum mentosum</i>	0	1	0	0	0	0	0	0	3	2	1	0	1	0	0	1	0	0	2	0	0	0	1	0	0	0	0	1
<i>Bulbophyllum meridense</i>	0	0	0	1	0	0	0	0	3	4	1	0	1	0	0	0	0	0	3	2	1	1	0	0	0	0	0	1
<i>Bulbophyllum micranthum</i>	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1
<i>Bulbophyllum mucronifolium</i>	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0	0	1
<i>Bulbophyllum napellii</i>	0	0	1	0	0	0	0	0	3	4	1	0	1	0	0	1	0	0	?	2	0	1	0	0	0	0	0	1
<i>Bulbophyllum peri</i>	0	1	0	0	0	0	0	0	3	4	1	0	1	0	0	0	0	0	3	2	1	1	0	0	0	0	0	1
<i>Bulbophyllum plumosum</i>	0	1	0	0	0	0	0	1	0	3	1	0	1	0	0	0	1	0	0	0	1	1	3	0	0	1	1	1
<i>Bulbophyllum popayanense</i>	0	1	0	0	0	0	0	0	3	4	1	0	1	0	0	0	0	0	3	2	1	1	2	0	0	0	0	1
<i>Bulbophyllum regnellii</i>	0	0	0	1	0	0	0	0	3	1	1	0	1	0	0	0	0	0	?	0	0	1	0	0	0	0	0	1
<i>Bulbophyllum rupicolum</i>	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1
<i>Bulbophyllum saltatorium</i>	0	0	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	1	0	0	1	1	0	0	0	0	1
<i>Bulbophyllum scaberulum</i>	0	0	0	1	0	0	0	1	2	2	1	0	1	0	0	0	0	0	1	1	0	1	0	0	0	1	0	1
<i>Bulbophyllum setigerum</i>	0	1	0	0	0	0	0	?	1	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1
<i>Bulbophyllum steyermarkii</i>	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1
<i>Bulbophyllum tripetalum</i>	0	0	0	1	0	0	0	0	3	4	1	0	1	0	0	0	0	0	3	2	1	1	0	0	0	0	0	1
<i>Bulbophyllum weddellii</i>	0	0	0	1	0	0	0	0	1	4	1	0	1	0	0	0	0	0	2	2	1	1	0	0	0	0	0	1
<i>Dendrobium kingianum</i>	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1

[illegible]

[illegible]

List of character and character states

1. DS apical glandule	0=absent	1=present	
2. DS smooth adaxial ornamentation	0=absent	1=present	
3. DS irregular adaxial ornamentation	0=absent	1=present	
4. DS striate adaxial ornamentation	0=absent	1=present	
5. DS granulose adaxial ornamentation	0=absent	1=present	
6. DS reticulate adaxial ornamentation	0=absent	1=present	
7. DS subepidermal tissue	0=absent	1=present	
8. DS adaxial secretory epidermis	0=absent	1=present	
9. DS adaxial cell shape fv	0=irregular	1=isodiametric	2=longitudinally elongate 3=transversely elongate
10. DS adaxial outer pw shape	0=convex	1=papillose	2=convex and papillose 3=unicellular trichome 4=flat
11. DS adaxial stomata	0=absent	1=present	
12. DS adaxial: sunken trichome	0=absent	1=present	
13. DS smooth abaxial ornamentation	0=absent	1=present	
14. DS irregular abaxial ornamentation	0=absent	1=present	
15. DS striate abaxial ornamentation	0=absent	1=present	
16. DS granulose abaxial ornamentation	0=absent	1=present	
17. DS reticulate abaxial ornamentation	0=absent	1=present	
18. DS abaxial cell shape fv	0=irregular	1=longitudinally elongate 2=isodiametric	3=transversely elongate
19. DS abaxial outer pw shape	0=convex	1=convex and papillose	2=flat
20. DS abaxial stomata	0=absent	1=present	
21. DS adaxial: sunken trichome	0=absent	1=present	
22. DS margin	0=smooth	1=unicellular trichome	2=papillose 3=papillose and unicellular trichomes
23. DS margin in ts	0=epidermis and mesophyll	1=epidermis only	
24. DS mesophyll parenchyma	0=regular	1=regular and secretory	
25. DS idioblast with raphides	0=absent	1=present	

26. DS idioblast with wall thickenings	0=absent	1=present				
27. DS mucilaginous idioblast with wall thickenings	0=absent	1=present				
28. DS number of vascular bundles	0=9	1=1 2=2	3=3	4=4	5=5	6=6 7=13
29. LS smooth adaxial ornamentation	0=absent	1=present				
30. LS striate adaxial ornamentation	0=absent	1=present				
31. LS granulose adaxial ornamentation	0=absent	1=present				
32. LS reticulate adaxial ornamentation	0=absent	1=present				
33. LS adaxial secretory epidermis	0=absent	1=present				
34. LS adaxial cell shape fv	0=irregular	1=isodiametric	2=longitudinally elongate	3=transversely elongate		
35. LS adaxial outer pw shape	0=convex	1=papillose	2=convex and papillose	3=flat		
36. LS adaxial stomata	0=absent	1=present				
37. LS adaxial sunken trichome	0=absent	1=present				
38. LS smooth abaxial ornamentation	0=absent	1=present				
39. LS irregular abaxial ornamentation	0=absent	1=present				
40. LS striate abaxial ornamentation	0=absent	1=present				
41. LS granulose abaxial ornamentation	0=absent	1=present				
42. LS reticulate abaxial ornamentation	0=absent	1=present				
43. LS abaxial cell shape fv	0=irregular	1=longitudinally elongate	2=transversely elongate			
44. LS abaxial outer pw shape	0=convex	1=papillose	2=convex and papillose	3=flat		
45. LS abaxial stomata	0=absent	1=present				
46. LS abaxial sunken trichome	0=absent	1=present				
47. LS margin	0=smooth	1=unicellular trichome	2=papillose	3=unicellular trichomes and papillae		
48. LS margin in ts	0=epidermis and mesophyll	1=epidermis only				
49. LS mesophyll parenchyma	0=regular	1=spongy	2=regular and secretory			
50. LS idioblast with raphides	0=absent	1=present				
51. LS idioblast with wall thickenings	0=absent	1=present				
52. DS mucilaginous idioblast with wall thickenings	0=absent	1=present				

53. LS number of vascular bundles	0=10	1=1	2=2	3=3	4=4	5=5	6=6	7=7	8=8
54. P insertion	0=sessile	1=clawed							
55. P smooth adaxial ornamentation	0=absent	1=present							
56. P striate adaxial ornamentation	0=absent	1=present							
57. P reticulate adaxial ornamentation	0=absent	1=present							
58. P secretory adaxial epidermis	0=absent	1=present							
59. P adaxial cell shape in fv	0=irregular	2=longitudinally elongate							
60. P adaxial outer pw shape	0=convex	1=papillose	2=convex and papillose	3=flat					
61. P adaxial stomata	0=absent	1=present							
62. P adaxial sunken trichome	0=absent	1=present							
63. P smooth abaxial ornamentation	0=absent	1=present							
64. P striate abaxial ornamentation	0=absent	1=present							
65. P reticulate abaxial ornamentation	0=absent	1=present							
66. P abaxial cell shape fv	0=irregular	2=longitudinally elongate							
67. P abaxial outer pw shape	0=convex	1=papillose	2=convex and papillose	3=flat					
68. P margin	0=smooth	1=papillose	2=unicellular trichomes	3=papillose and a few unicellular trichomes					
69. P margin in ts	0=epidermis and mesophyll	1=epidermis only							
70. P mesophyll parenchyma	0=regular	1=spongy	2=regular and secretory						
71. P idioblast with raphides	0=absent	1=present							
72. P idioblast with wall thickenings	0=absent	1=present							
73. P number of vascular bundles	0=7	1=1	2=2	3=3	4=0				
74. L lateral lobes presence	0=absent	1=present							
75. L lateral lobes vascularisation	0=absent	1=present							
76. L lateral lobes margin	0=smooth	1=papillose	2=unicellular trichomes						
77. L hypochile callus presence	0=absent	1=present							
78. L hypochile secretory cavity on the callus	0=absent	1=present							
79. L hypochile aligned wholes on the callus	0=absent	1=present							

		0=scale-like papillae	1=other cell type	
80. L hypochile callus cell type		0=smooth	1=striate	
81. L hypochile adaxial ornamentation		0=convex	1=papillose	2=scale-like papillae
82. L hypochile adaxial cell type (outside callus and sulcus)		0=absent	1=present	3=trichome
83. L hypochile adaxial sulcus presence		0=absent	1=present	
84. L hypochile adaxial secretory sulcus		0=absent	1=present	
85. L adaxial secretory epidermis		0=simple	1=double	
86. L epidermis simple/double		0=fused	1=unfused	
87. L double epidermis trichomes fused/unfused		0=absent	1=present	
88. L adaxial stomata		0=absent	1=present	
89. L abaxial keel		0=absent	1=present	
90. L abaxial keel stomata		0=absent	1=present	
91. L abaxial keel glandular trichome		0=absent	1=present	
92. L abaxial ornamentation		0=smooth	1=striate	
93. L abaxial stomata (outside keel)		0=absent	1=present	
94. L hypochile mesophyll parenchyma		0=regular	1=spongy	2=regular and secretory
95. L hypochile idioblast with raphides		0=absent	1=present	
96. L hypochile idioblast with wall thickenings		0=absent	1=present	
97. L epichile		0=absent	1=present	
98. L epichile adaxial ornamentation		0=smooth	1=striate	
99. L epichile adaxial cell type		0=convex	1=papillose	2=unicellular trichome
100. L epichile margin		0=smooth	1=unicellular trichome	
101. L epichile abaxial ornamentation		0=smooth	1=striate	
102. L epichile abaxial cell type		0=convex	1=papillose	2=unicellular trichome
103. L epichile mesophyll parenchyma		0=regular	1=spongy	2=secretory
104. L epichile idioblast with raphides		0=absent	1=present	
105. L epichile idioblast with wall thickenings		0=absent	1=present	
106. L number of vascular bundles (middle third)		0=13	1=9 2=2 3=3 4=11 5=5 6=7	

107. Gynostemium ornamentation	0=smooth	1=striate	
108. Gynostemium cell shape in fv	0=irregular	1=longitudinally elongate	
109. Anther beak presence	0=absent	1=present	
110. Anther beak size	0=short	1=long	
111. Anther ornamentation	0=smooth	1=striate	
112. Anther outer pw shape in ts	0=convex	1=papillose	
113. Anther stomata	0=absent	1=present	
114. Anther pollinia number	0=4	1=4(inner rudimentary)	2=2
115. Stigma shape	0=wide	1=long	2=isodiametric
116. Column foot ornamentation	0=smooth	1=striate	
117. Column foot cell shape in fv	0=longitudinally elongate	1=isodiametric	
118. Column foot outer pw shape in ts	0=convex	1=papillose	2=convex and papillose 3=unicellular trichome
119. Ovary outer epidermis cell shape in ts	0=rectangular	1=other	
120. Ovary inner epidermis cell shape in ts	0=rectangular	1=irregular	2=elliptic 3=elliptic and papillose
121. Ovary idioblast with raphides	0=absent	1=present	
122. Ovary idioblast with wall thickenings	0=absent	1=present	
123. Habit	0=preferably epiphyte	1=epiphyte and rupicolous	2=preferably rupicolous
124. Rhizome	0=conspicuous	1=inconspicuous	
125. Pseudobulb shape	0=deltoid	1=pyriform	2=ovoid fusiform cane-like
126. Pseudobulb shape in ts	0=elliptic	1=rhombic	2=orbicular
127. Pseudobulb colour	0=green	1=yellow	2=brown 3=red
128. Pseudobulb texture	0=smooth	1=rugose	
129. Number of leaves in the pseudobulb	0=1	1=two	2=more than 2
130. Leaf shape	0=aciculate, not sulcate	1=flat, linear	2=flat, lanceolate 3=flat, elliptic 4=flat, oblong 5=aciculate, sulcate
131. Leaf base	0=sessile	1=constrict	
132. Leaf apex	0=acute	1=obtuse	2=rounded
133. Leaf texture	0=membranaceous	1=coriaceous	2=fleshy

134. Inflorescence orientation	0=erect	1=pendent	
135. Inflorescence type	0=racemose	1=cymose	
136. Flower quantity	0=pauciflor	1=pluriflor	2=uniflor
137. Scape shape in TS	0=thin, cylindrical	1=thin, ribbon-like	2=thick, ribbon-like
138. Basal bract of the scape	0=conspicuous	1=inconspicuous	
139. Distribution of the remaining bracts of the scape	0=imbricate	1=non-imbricate	
140. Rachis consistency	0=thin	1=thickened	
141. Rachis orientation	0=erect	1=geniculate	2=pendent
142. Rachis bract	0=sessile	1=clawed	
143. Floral bracts distribution	0=imbricate	1=non-imbricate	
144. Inflorescence phyllotaxis	0=spiral	1=distichous	
145. Resupination	0=absent	1=present	
146. Resupination type	0=inflorescence bending	1=pedicel twisting	2=rachis bending
147. Anthesis	0=successive	1=simultaneous	
148. Flower texture	0=membranaceous	1=fleshy	
149. “Jugo” presence	0=absent	1=present	
150. “Jugo” type	0=inconspicuous	1=conspicuous	
151. Sepal positioning	0=erect	1=dorsal erect, lateral patent	2=dorsal patent, lateral erect 3=patent
152. Sepal surface	0=smooth	2=verrucose	
153. Sepal outer colour	0=white	1=green	2=yellow 3=white with purple dots and/or veins 4=purple
	5=brown	6=vinaceous	
154. Inner colour of the sepals	0=white	1=green	2=yellow 3=white with purple dots and/or veins 4=purple
	5=brown	6=vinaceous	
155. Dorsal sepal shape	0=linear	1=lanceolate	2=ovate 3=cordiform 4=spathulate 5=ovate-oblong
156. Dorsal sepal apex	0=acute, plane	1=acute, navicular	2=shortly caudate 3=long caudate
157. Number of veins in the sepals	0=1	1=3	
158. Lateral sepals shape	0=linear	1=lanceolate	2=ovate 3=cordiform 4=ovate-oblong 5=falcate

159.Lateral sepals base	0=adnate to the flower	1=forming a 90° keel	
160.Orientation of the apices of the sepals	0=parallel	1=convergent	2=divergent
161.Lateral sepals connation presence	0=absent	1=present	
162.Lateral sepals symmetry	0=symmetric	1=asymmetric	
163.Number of the veins of the lateral sepals	0=1	1=2	2=3
164.Apex shape of the lateral sepals	0=acute, plane	1=acute, navicular	2=obtuse
165.Petal shape	0=linear	1=lanceolate	2=ovate
166.petal positioning	0=erect	1=patent	2=revolute
167.Petal apex	0=acute, plane	1=obtuse	2=caudate
168.Petal predominant colour	0=green	1=white	2=yellow
169.Petal accessory colour	0=midvein purple	1=purple dots	2=purple stripes
170.Number of veins of the petal	0=0	1=1	2=2
171.Labellum apex	0=entire	1=trilobed	
172.Basal appendix presence on the labellum	0=erect	1=revolute	2=involute
173.Predominant colour of the labellum	0=absent	1=present	
174.Predominant colour of the labellum	0=white	1=green	2=yellow
175.Lateral lobes shape	0=purple centre	1=purple dots	2=purple stripes
		3=white centre	4=concolour
		5=green centre	6=white
176.Lateral lobes shape	0=absent	1=erect	2=orbicular
177.Epichile size	0=absent	1=larger than hypochile	2=smaller than the hypochile
178.Callus	0=between the lateral lobes	1=up to the middle of the labellum	2=up to the labellum apex
179.Callus apex	0=entire, acute	1=entire, obtuse	2=entire, truncate
180.Surface of the labellum disc	0=smooth	1=with a smooth sulcus	2=with transverse clogs
181.Epichile shape	0=lanceolate	1=ovate	2=spathulate
182.Epichile texture	0=membranaceous	1=fleshy	3=cordiform
183.Epichile apex	0=acuminate	1=obtuse	2=retuse
184.Epichile base	0=sessile	1=constricted	2=slender
		3=truncate	4=acute
		5=callous	6=papillose
		7=three-lobed	8=linear
		9=semi-elliptic	10=falcate
		11=reduced to a hypochile margin	12=three-lobed
		13=lamellate	14=papillose
		15=linear	16=cordiform
		17=retuse	18=acute
		19=slender	20=truncate
		21=constricted	22=slender

185. Stelidia size	0=absent	1=not surpassing the anther	2=surpassing the anther
186. Stelidia shape	0=acute apex, entire	1=acute apex, bidentate	2=obtuse apex 3=truncate apex
187. Stelidia positioning	0=erect 1=patent	2=revolute	
188. Teeth presence in the column	0=absent	1=present	
189. Column foot size	0=short	1=long	
190. Apex of the column foot	0=entire	1=lobulated	
191. Fruit surface	0=smooth	1=verrucose	
192. Pollination	0=bee	1=fly	
193. Pollinator	0=other	1=Milichiidae 2=Chloropidae	3=Sciaridae 4=Tachinidae
194. Reproductive system	0=self-compatible	1=self-incompatible	
195. Occurrence	0=Amazon 1=Andes	2=Cerrado	3=Atlantic Forest 4=Temperate forest
	5=Mesoamerican forest	6=Caatinga	7=African Forest 8=Australian dry forest