



**UNIVERSIDADE ESTADUAL PAULISTA**  
**“JÚLIO DE MESQUITA FILHO”**  
**INSTITUTO DE BIOCÊNCIAS - RIO CLARO**



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**PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS**  
**BIOLOGIA VEGETAL**

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# **Anatomia foliar de espécies de Poaceae (Poales) e sua importância na sistemática e filogenia**

**THALES HENRIQUE DIAS LEANDRO**



**Novembro - 2016**

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BIOLOGIA VEGETAL

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# Anatomia foliar de espécies de Poaceae (Poales) e sua importância na sistemática e filogenia

**THALES HENRIQUE DIAS LEANDRO**

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

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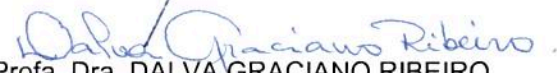
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# ÍNDICE

<b>1. RESUMO</b>	1
<b>2. ABSTRACT</b>	2
<b>3. INTRODUÇÃO GERAL</b>	3
<b>4. LITERATURA CITADA</b>	6
<b>ILUSTRAÇÕES</b>	11
<b>CAPÍTULO I. The utility of Bambusoideae (Poaceae, Poales) leaf blade anatomy for identification and systematics</b>	16
Abstract	17
Resumo	18
Introduction	19
Material and Methods	20
<i>Sampling area</i>	20
<i>Anatomical analysis</i>	21
Results	21
<i>Surface view</i>	21
<i>Cross section</i>	23
<i>Taxonomic treatment</i>	24
Discussion	26
Conclusion	28
Acknowledgments	29
References	29
Tables	35
Figures	39
<b>CAPÍTULO II. The contribution of foliar micromorphology and anatomy to the circumscription of species within <i>Chusquea ramosissima</i> Clade (Poaceae, Bambusoideae, Chusqueinae)</b>	44
Abstract	45
Introduction	46
Material and Methods	48
<i>Taxon sampling</i>	48

<i>Micromorphology (scanning electron microscopy)</i>	49
<i>Anatomy (light microscopy)</i>	50
<i>Images and terminology</i>	50
Results	51
<i>Epidermal surfaces (SEM and clearings)</i>	51
<i>Cross section</i>	53
Discussion	54
<i>Micromorphological and anatomical data</i>	54
<i>Implications for systematics</i>	55
<i>Taxonomic treatment</i>	58
Acknowledgments	62
References	62
Tables	67
Figures	70
<b>CAPÍTULO III. Fusoid cells in the grass family Poaceae (Poales): a developmental study reveals homologies and suggests insights into their functional role in young leaves</b>	79
Abstract	80
Introduction	82
Material and Methods	85
<i>Sampling</i>	85
<i>Developmental study (young leaves)</i>	85
<i>Anatomical study (mature leaf blades)</i>	86
<i>Description, observation, images, and tridimensional reconstruction</i>	87
Results	87
Discussion	91
<i>Fusoid cell origin and development</i>	91
<i>Phylogenetic implications of fusoid cells for the Graminid clade</i>	94
<i>Some evolutionary and functional insights on fusoid cells based on TEM data</i>	96
Conclusions	98
Acknowledgments	99
Literature Cited	99
Tables	106

Figures and Supplementary Documents	110
<b>CAPÍTULO IV. An update on comparative leaf blade anatomy in the systematics of Poaceae (Poales): the past thirty years since Ellis</b>	<b>123</b>
Abstract	124
Introduction	125
Leaf blade anatomical features revisited	128
<i>Plicate mesophyll parenchyma: arm and rosette cells</i>	128
<i>Midrib vasculature</i>	130
<i>Fusoid cells</i>	131
<i>Intercostal fibers</i>	133
<i>Refractive papillae</i>	133
<i>Stomatal apparatus bearing papillae on the subsidiary cells</i>	134
<i>Trichomes</i>	135
Subfamilial descriptions and general notes based on leaf blade anatomy data	137
<i>I. Anomochlooideae</i>	137
<i>II. Pharoideae</i>	138
<i>III. Puelioideae</i>	138
<i>IV. Oryzoideae</i>	139
<i>V. Bambusoideae</i>	139
<i>VI. Pooideae</i>	140
<i>VII. Aristidoideae</i>	141
<i>VIII. Panicoideae</i>	142
<i>IX. Arundinoideae</i>	142
<i>X. Micrairoideae</i>	143
<i>XI. Danthonioideae</i>	143
<i>XII. Chloridoideae</i>	144
Future prospects for investigating grass leaf blade anatomical anatomical features	144
Final considerations	145
Literature Cited	146
Tables	161
Figure	166





## 1. RESUMO

Buscando levantar caracteres da lâmina foliar que auxiliem na sistemática e filogenia de Poaceae foram realizados estudos anatômicos e ultraestruturais. Em Bambusoideae, caracteres como papilas dispersas na face abaxial e células buliformes organizadas paralelamente são úteis para o reconhecimento de Olyreae; enquanto papilas organizadas em colunas centrais e células buliformes em forma de leque são úteis para o reconhecimento de Bambuseae. Em Bambuseae, fibras intercostais e nervura central simples são caracteres diagnósticos de Arthrostylidiinae; enquanto complexo estomático portando duas papilas por célula subsidiária e nervura central complexa são caracteres diagnósticos de Chusqueinae. O levantamento de caracteres da lâmina foliar de espécies de *Chusquea* pertencentes ao clado II “*Chusquea ramosissima*” indica estruturas úteis para sua delimitação, tais como: complexo estomático, tipo de tricomas, tipo e arranjo de células silicosas, e tipo e número de camadas de células invaginantes; bem como sustenta o reconhecimento deste clado e de uma nova espécie para a Bolívia. O estudo do desenvolvimento foliar com ênfase nas células fusoides mostra sua origem proveniente do meristema fundamental e que a cavidade observada em secções transversais de lâminas foliares maduras é resultado do colapso de várias células fusoides. A origem proveniente do meristema fundamental é confirmada para toda a família e homologias são observadas dentre diferentes tipos de células parenquimáticas do mesofilo. O estudo ainda sugere que células fusoides evoluíram das células incolores observadas em Joinvilleaceae. São fornecidas descrições anatômicas gerais da lâmina foliar para as 12 subfamílias reconhecidas para Poaceae, bem como a definição estrutural e a reavaliação da importância de alguns caracteres na sistemática do grupo.

**PALAVRAS-CHAVE:** anatomia, clado BOP, clado PACMAD, clado Graminédeo, folha, gramíneas, linhagens basais, ontogenia.

## 2. ABSTRACT

To seek leaf blade features useful for systematics and phylogenetics of Poaceae, anatomical and ultrastructural studies were carried out. Within Bambusoideae, features such as papillae scattered on the abaxial surface and parallel-sided arrays of bulliform cells are useful for recognizing Olyreae; whereas centrally organized papillae and fan-shaped arrays of bulliform cells are useful for recognizing Bambuseae. Within Bambuseae, intercostal fibers and simple midrib are diagnostic features of Arthrostylidiinae; whereas stomatal apparatus bearing two papillae per subsidiary cell and complex midrib are diagnostic features of Chusqueinae. The survey of leaf blade anatomical features of *Chusquea* species that comprise the clade II “*Chusquea ramosissima*” shows structures for delimiting species, such as: stomatal apparatus, type of trichomes, type and arrangement of silica bodies, and type and number of layers of arm cells; as well as supports the recognition of this clade and of a new species from Bolivia. The foliar developmental study with emphasis on fusoid cells shows their meristematic origin from the ground meristem and that the cavity as seen in cross section in mature leaves is resulting from the collapse of several fusoid cells. Their origin from the ground meristem is confirmed throughout the family and homologies are observed among different types of mesophyll parenchymatous cells. The study also suggests that fusoid cells evolved from colourless cells of Joinvilleaceae. General leaf blade anatomical descriptions of the 12 recognized subfamilies of Poaceae are provided, as well as structural definition and reevaluation of the relevance of anatomical features in grass systematics.

KEY WORDS: anatomy, early-diverging lineages, BOP clade, Graminid clade, leaf, grasses, ontogeny, PACMAD clade.

### 3. INTRODUÇÃO GERAL

Todas as espécies estudadas nesta tese pertencem a Poales, que está inserida no clado das comelinídeas e atualmente compreende 14 famílias que compartilham características estruturais, embriológicas e moleculares (APG IV 2016; Stevens, 2001 onwards) (Fig. 1). São reconhecidos seis clados para o grupo, dentre eles o clado graminídeo, que compreende [Flagellariaceae (Joinvilleaceae + Ecdeicoleaceae) Poaceae] baseado no compartilhamento de características morfológicas e embriológicas (Linder e Rudall 2005) (Figs. 2 e 3).

Poaceae engloba aproximadamente 12.000 espécies que se caracterizam por apresentarem, de maneira geral, inflorescências bracteadas, perianto reduzido ou ausente, polén com exina ornamentada, embrião lateral diferenciado e cariopse (GPWG 2001; GPWG II 2012). Atualmente a circunscrição da família está estabilizada em 12 subfamílias (Soreng et al. 2015) (Fig. 4): Anomochlooideae, Pharoideae e Puelioideae, que constituem as linhagens basais; Oryzoideae, Bambusoideae e Pooideae, que compreendem o clado BOP; e Aristidoideae, Panicoideae, Arundinoideae, Micrairoideae, Danthonioideae e Chloridoideae, linhagens filogeneticamente mais derivadas que constituem o clado PACMAD. Embora seja uma família diversa e amplamente distribuída pelo mundo (Watson and Dallwitz 1992), sua circunscrição é bem sustentada por evidências estruturais, embriológicas, fisiológicas e moleculares (GPWG 2001; Stevens 2001 onwards; GPWG II 2012; Soreng et al. 2015). No Brasil, a diversidade da família está estimada em cerca de 1.478 espécies distribuídas em 11 subfamílias (Filgueiras et al. 2015) (e.g., Figs. 5-15), com exceção de Puelioideae, grupo que engloba espécies nativas de florestas tropicais da África (Clark et al. 2000). Poaceae desempenha relevante papel econômico e ecológico, contudo, a ampla diversidade de espécies torna sua sistemática por vezes um fator limitante.

Como a classificação da família é tradicionalmente baseada principalmente em características morfológicas de estruturas reprodutivas (Longhi-Wagner 2012), a dificuldade

na obtenção de material fértil ou a incongruência de evidências morfológicas e moleculares tornam a sistemática e filogenia do grupo ainda mais desafiadora. Nesse contexto, um clássico exemplo são as espécies de bambus lignificados, que apresentam ciclo de vida monocárpico plurianual e, portanto, florescem após vários anos investidos apenas em propagação vegetativa e morrem logo após a dispersão dos frutos. Dessa forma, a busca por características vegetativas que auxiliem na taxonomia de Poaceae tornou-se essencial como subsídio à identificação de espécies e à classificação mais natural do grupo, onde, historicamente, a anatomia da lâmina foliar ganhou destaque (Fig. 16).

Trabalhos como os de Duval-Jouve (1875), Schwendener (1890) e Avdulov (1931) foram pioneiros nesta linha e forneceram a base para estudos comparativos sobre a anatomia da lâmina foliar em Poaceae. Na década de 50, Brown (1958) estabeleceu características sistematicamente úteis para a família e determinou seis grandes grupos baseado em uma combinação única de caracteres da lâmina foliar examinados em secções transversais. Já Metcalfe, em 1960, forneceu descrições anatômicas gerais e diagnósticas da epiderme e mesofilo de vários táxons (e.g., Figs. 16-19), bem como ilustrações que foram um dos subsídios para a padronização terminológica estabelecida mais tarde por Ellis (1976, 1979). Anos depois, em 1987, Ellis apresentou uma revisão sobre a aplicabilidade das estruturas anatômicas da lâmina foliar na sistemática de Poaceae, demonstrando a importância desses caracteres para definir e delimitar as cinco subfamílias reconhecidas na época (Clayton e Renvoize 1986). Desde então, estudos anatômicos na família têm sido concentrados principalmente na lâmina foliar e são, em sua maioria, de cunho aplicado à sistemática e filogenia do grupo.

Estruturas anatômicas de Poaceae como mesofilo com células invaginantes (arm cells) e fibras intercostais são exemplos de caracteres anatômicos taxonomicamente informativos (ver Judziewicz et al. 1999; Viana et al. 2011, 2013; Leandro et al. 2016a) e frequentemente considerados em estudos filogenéticos (e.g., Clark et al. 2000; GPWG 2001; Sánchez-Ken et

al. 2007; GPWG II 2012). Por outro lado, algumas estruturas ainda apresentam dados conflitantes apesar do constante crescimento de trabalhos anatômicos envolvendo espécies do grupo. Este é o exemplo das células fusoides, consideradas características de espécies de Bambusoideae, mas também observadas em linhagens basais e em algumas espécies de Oryzoideae, Pooideae e Panicoideae (Tateoka 1963; GPWG 2001, Leandro et al. 2016b). Embora seja considerado um caráter sinapomórfico e importante para a definição da família, até então não há dados na literatura sobre sua origem, limitando, portanto, interpretações filogenéticas mais consistentes.

Considerando a importância da continuidade de estudos anatômicos da lâmina foliar em espécies de Poaceae, esta tese se apresenta estruturada em quatro capítulos:

(i) O primeiro capítulo “The utility of Bambusoideae (Poaceae, Poales) leaf blade anatomy for identification and systematics” abrange dados anatômicos sobre 13 espécies de bambus lignificados (Bambuseae) e de três espécies de bambus herbáceos (Olyreae), totalizando 16 espécies nativas estudadas. Este capítulo visa levantar estruturas anatômicas da lâmina foliar que auxiliem na identificação destas espécies simpátricas, principalmente aquelas monocárpicas plurianuais, ocorrentes no Parque Estadual das Fontes do Ipiranga (PEFI), São Paulo.

(ii) O segundo capítulo “The contribution of foliar micromorphology and anatomy to the circumscription of species within *Chusquea ramosissima* Clade (Poaceae, Bambusoideae, Chusqueinae)” visa fornecer dados não só para a diferenciação das espécies estudadas, mas também levantar características úteis ao reconhecimento do clado II “*Chusquea ramosissima*”, que compreende *Chusquea ramosissima* Lindman, *C. tenella* Nees e *C. longispiculata* L.G. Clark (Fisher et al. 2014). *Chusquea* Kunth é o grupo mais diverso de bambus lignificados e sua monofilia é sustentada principalmente por dados moleculares (Fisher et al. 2009; Kelchner

and BPG 2013; Fisher et al. 2014). O grupo é um exemplo da incongruência de evidências morfológicas e moleculares, reforçando a importância deste estudo.

(iii) O terceiro capítulo “Fusoid cells in the grass family Poaceae (Poales): a developmental study reveals homologies and suggests insights into their functional role in young leaves” fornece dados sobre o desenvolvimento de 16 espécies de Poaceae distribuídas em seis subfamílias, além de uma espécie pertencente a cada um dos grupos externos (Flagellariaceae e Joinvilleaceae). Este trabalho visa fornecer principalmente dados sobre a origem das células fusoides em Poaceae comparando seu desenvolvimento com células similares observadas em Joinvilleaceae, bem como traz importante contribuição para o entendimento estrutural deste caráter anatômico amplamente utilizado na sistemática e filogenia da família.

(iv) O quarto capítulo “An update on comparative leaf blade anatomy in the systematics of Poaceae (Poales): the past thirty years since Ellis” tem como referência o trabalho de Ellis (1987) e, a partir dele, fornece dados sobre o estado da arte e da importância da anatomia da lâmina foliar na sistemática de Poaceae. São apresentadas descrições anatômicas de cada subfamília e discussões sobre a aplicabilidade de caracteres anatômicos foliares na sistemática do grupo.

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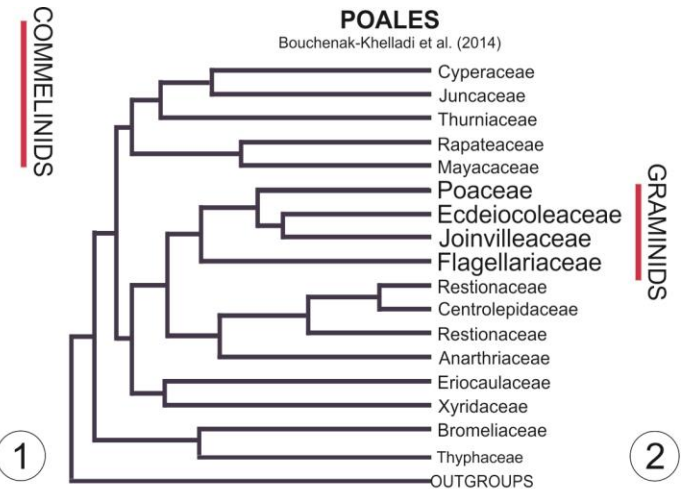
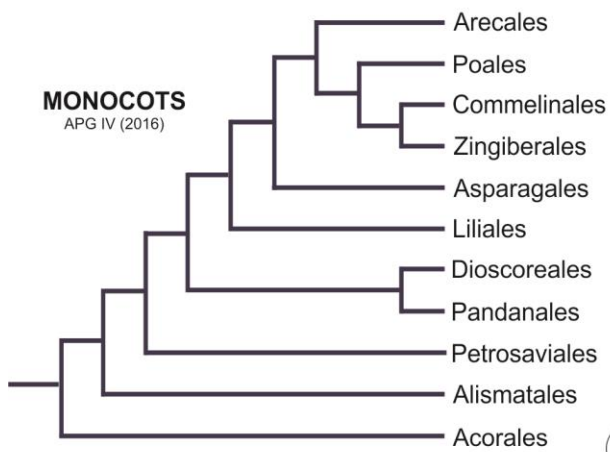
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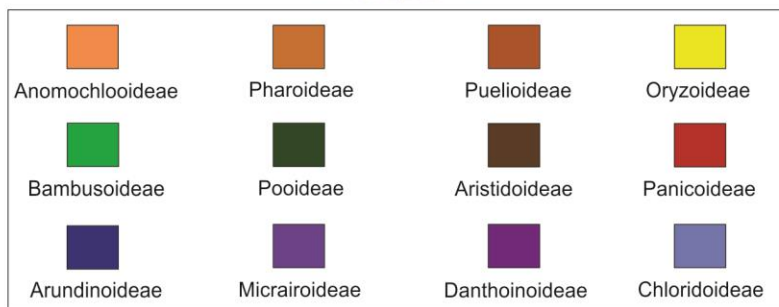
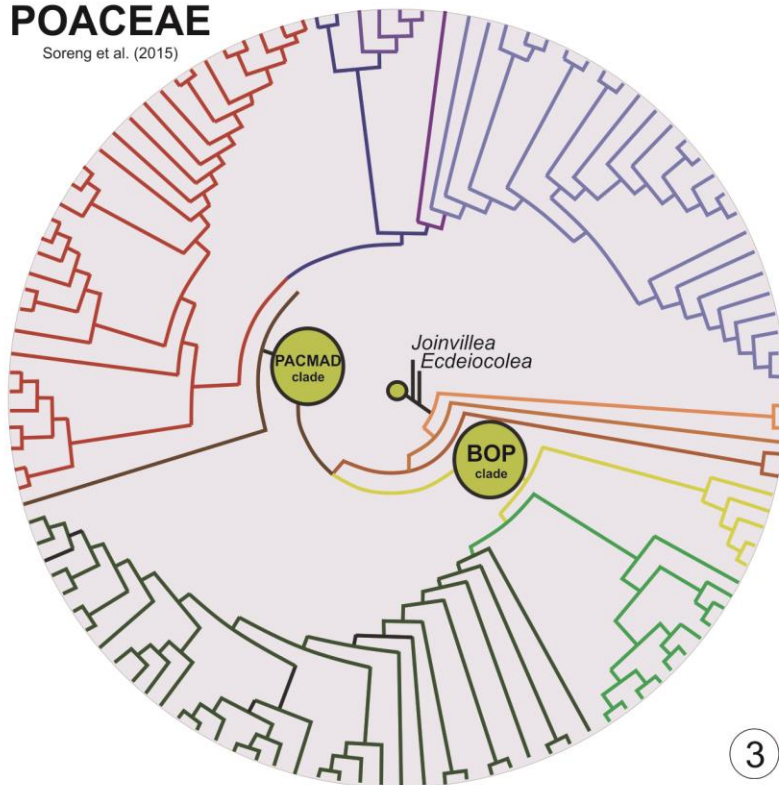


# ILUSTRAÇÕES

**Figuras 1-4.** Relações filogenéticas de monocotiledôneas, Poales e Poaceae; e distribuição geográfica das famílias pertencentes ao clado graminídeo. **1.** Relações filogenéticas das monocotiledôneas baseado em APG IV (2016). **2.** Relações filogenéticas de Poales baseado em Bouchenak-Khelladi et al. (2014). **3.** Relações filogenéticas de Poaceae baseado em Soreng et al. (2015). **4.** Distribuição geográfica das famílias pertencentes ao clado graminídeo (Poaceae, Ectopogonaceae, Joinvilleaceae e Flagellariaceae) retirado de Stevens (2001 onwards).



**POACEAE**  
Soreng et al. (2015)



1

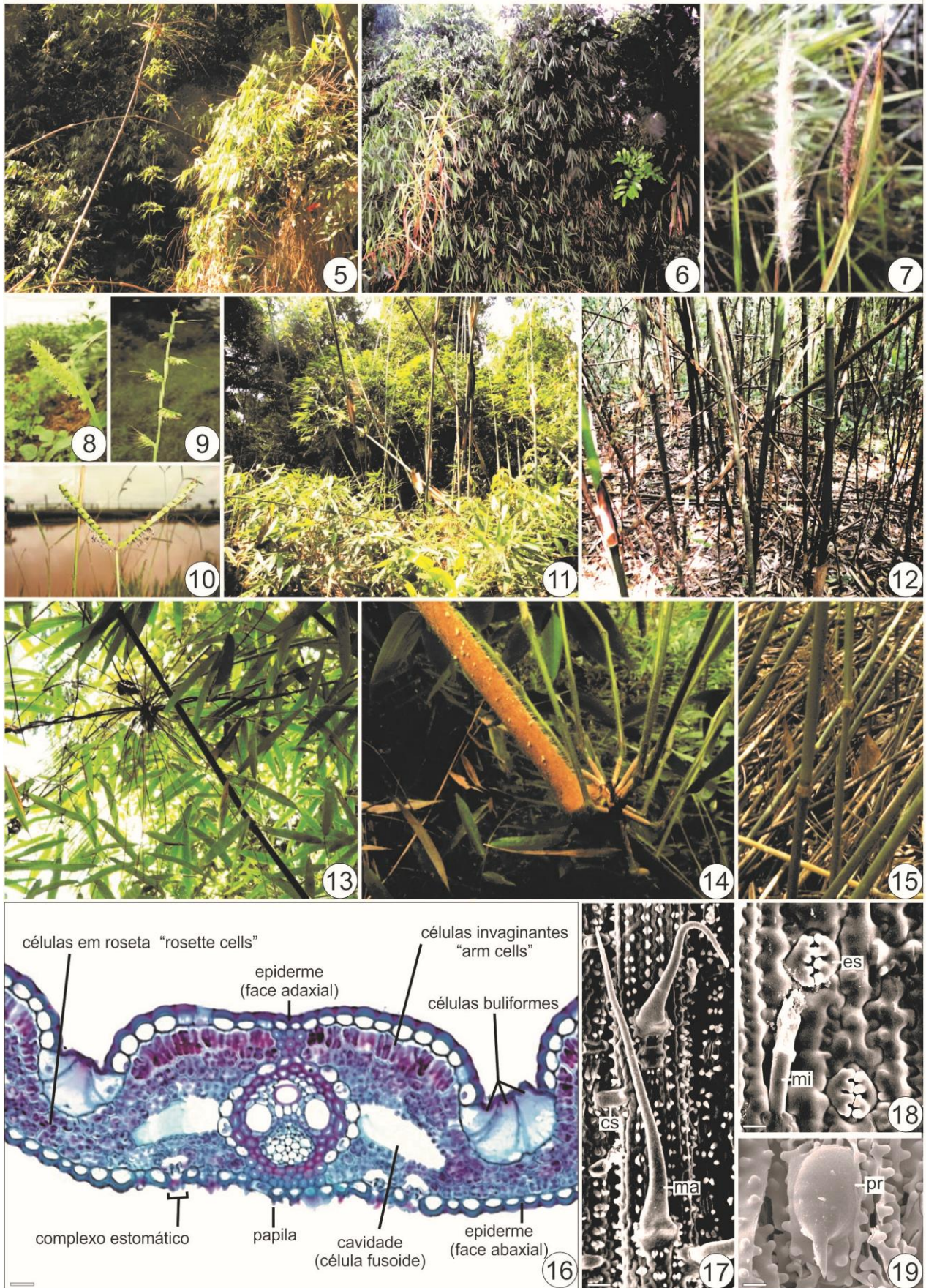
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**Figuras 5-19.** Diversidade de espécies e aspecto geral lâmina foliar evidenciando caracteres úteis à sistemática de Poaceae. (16) secção transversal em microscopia ótica; (17-19) vista frontal em microscopia eletrônica de varredura. **5.** *Merostachys argyronema* Lindm. **6.** *Merostachys pluriflora* Munro ex E.G. Camus. **7.** *Imperata brasiliensis* Trin. **8.** *Cenchrus echinatus* L. **9.** *Oplismenus hirtellus* (L.) P. Beauv. **10.** *Paspalum notatum* Flüggé. **11.** *Merostachys neesii* Rupr. **12.** *Merostachys riedeliana* Rupr. ex Doell. **13.** *Merostachys scandens* Send. **14.** *Chusquea capituliflora* Trin. var. *pubescens* McClure & L.B. Sm. **15.** *Merostachys scandens*. **16.** *Aulonemia pumila* L.G. Clark & Lodoño (Clark 382). **17.** *Chusquea attenuata* (Irwin et al. 29242). **18.** *Chusquea tenuiglumis* (Hatschbach 42746). **19.** *Luziola bahiensis* (Steud.) Hitchc. (Guglieri 1610). Imagens (5-6, 11-15) R T Shirasuna, (7-10) A. Guglieri-Caporal. *es* estômato; *cs* célula silicosa; *ma* macrohair; *mi* microhair; *pr* prickles. Barras de escala: 16 = 25 µm; 17 = 30 µm, 18 = 6 µm, 19 = 4 µm.





# CAPÍTULO I

## **The utility of Bambusoideae (Poaceae, Poales) leaf blade anatomy for identification and systematics**

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**Abstract**

Bambusoideae is a diverse subfamily that includes herbaceous (Olyreae) and woody (Arundinarieae and Bambuseae) bamboos. Species within Bambusae are particularly difficult to identify due to their monocarpic lifecycle and the often long durations between mass flowering events; whereas the herbaceous bamboos are pluricarpic, but often are found with no reproductive structures. The leaf blade anatomy of 16 sympatric species of native Brazilian bamboos (Olyreae and Bambuseae) from the Atlantic Rainforest was studied in order to detect useful features for their identification. All the studied species share the following features: epidermis with a single stratum of cells; adaxial bulliform cells; mesophyll with arm cells, rosette cells, and fusoid cells; and collateral vascular bundles. Herbaceous bamboos share two features: papillae scattered on the abaxial surface and parallel-sided arrays of bulliform cells; whereas woody bamboos share: centrally organized papillae and fan-shaped arrays of bulliform cells. Also within the woody bamboos, intercostal fibers and a midrib with only one vascular bundle (simple midrib) characterize the subtribe Arthrotylidiinae; whereas a midrib with more than one vascular bundle (complex midrib) and a stomatal apparatus with two papillae per subsidiary cell characterize the subtribe Chusqueinae. There are also diagnostic features for the sampled species, such as: papillae shape, and the outline and structure of the midrib. An identification key for all the studied species is provided based on the anatomical features.

*Keywords:* Arthrotylidiinae, Bambuseae, Chusqueinae, leaf blade, Olyreae.

## **Resumo**

Bambusoideae é uma subfamília que inclui diversas espécies de bambus herbáceos (Olyreae) e lignificados (Arundinarie e Bambuseae). Bambus lignificados geralmente apresentam dificuldades de delimitação e identificação, devido principalmente ao ciclo monocárpico e longa amplitude temporal entre florações; enquanto que bambus herbáceos possuem ciclo pluricárpico, porém frequentemente são encontrados em estágio vegetativo. Foi estudada a anatomia da lâmina foliar de 16 espécies de Bambusoideae (Olyreae e Bambuseae), simpátricas e nativas do Brasil, visando levantar caracteres úteis para sua identificação. Todos os táxons estudados compartilham: epiderme uniestratificada; células buliformes na face adaxial; mesofilo com células invaginantes, células em roseta e células fusoides; e feixes vasculares colaterais. Bambus herbáceos compartilham: papilas dispersas na face abaxial e grupos de células buliformes organizadas paralelamente; enquanto que bambos lignificados compartilham: papilas organizadas em colunas centrais e grupos de células buliformes em forma de leque. Ainda dentre os bambus lignificados, fibras intercostais e nervura central com apenas um feixe vascular (nervura central simples) caracterizam a subtribo Arthrostylidiinae; enquanto que nervura central com mais de um feixe vascular (nervura central complexa) e complexo estomático com duas papilas por célula subsidiária caracterizam a subtribo Chusqueinae. Há ainda caracteres anatômicos diagnósticos, tais como: forma da papila, e forma e estrutura da nervura central. Uma chave de identificação é fornecida baseada nos caracteres anatômicos relevantes à identificação das espécies estudadas.

*Palavras-chave:* Arthrostylidiinae, Bambuseae, Chusqueinae, lâmina foliar, Olyreae.

## 1. Introduction

The cosmopolitan family Poaceae comprise about 11,000 species found mainly in grasslands and forest formations (Watson and Dallwitz, 1992 onwards; GPWG II, 2012). Twelve subfamilies are recognized within Poaceae (GPWG II, 2012; Soreng et al., 2015), among them Bambusoideae, a monophyletic group that currently includes 1,482 described species (Clark et al., 2015). Three Bambusoideae tribes are recognized, two of which are found in the Neotropics: Bambuseae, which comprise the woody bamboos; and Olyreae, the herbaceous bamboos (Kelchner, 2013; Clark et al., 2015). The Atlantic Rainforest is considered an important center of bamboo diversity (Judziewicz et al., 1999), and Brazil occupies a leading position based on number of species (298) and high endemism (172) (Carvalho et al., 2016).

Bambusoideae may be distinguished from other grass subfamilies by morphological, anatomical, and ecological characters. Monocarpic perennial lifecycle, lignified culms, branching nodes, pseudopetiolate leaves, and an outer ligule are characters worth mentioning for the woody bamboos (GPWG, 2001; BPG, 2012); whereas herbaceous bamboos are pluricarpic, usually unbranched, with quite weak culms and an inner ligule (Judziewicz et al., 1999). Together with, the strongly asymmetrically invaginated arm cells as seen in cross section are highly important for the recognition of Bambusoideae species (GPWG, 2001), and also represents one of the main synapomorphies for this group (Zhang and Clark, 2000; BPG, 2012).

In general, the Poaceae taxonomy is mainly based on reproductive characters, such as the shape and structure of spikelets and inflorescence types (Longhi-Wagner, 2012). This is true more particularly for the herbaceous species, which generally bloom many times in their life cycle. In contrast, the woody bamboos bloom only once during a life cycle (Janzen, 1976; Filgueiras, 1988), and sometimes even herbaceous species are found with no reproductive

structures. For this reason, searching for vegetative characters in addition to the reproductive ones is highly important to aid in species identification, and anatomical features often have provided useful findings (e.g., Brandis, 1907; Prat, 1936; Brown, 1958; Metcalfe, 1960; Calderón and Soderstrom, 1973; Renvoize, 1987; Vieira et al., 2002; Guglieri et al., 2008; Oliveira et al., 2008; Pelegrin et al., 2009; Jesus Junior et al., 2012; Viana et al., 2013a, b; Leandro et al., 2016; Aliscioni et al., 2016).

Considering that mostly bamboo plants have unique life cycles, but also the importance of the leaf blade anatomy for the taxonomy of grasses in general, we studied 16 sympatric species of native bamboos from the Atlantic Rainforest. We examined the leaf blade anatomy of three species of herbaceous bamboos and 13 species of woody bamboos in order to provide useful features for their identification.

## **2. Material and Methods**

### *2.1. Sampling area*

The study was carried out with 16 native species sampled at Parque Estadual das Fontes do Ipiranga - PEFI (23° 38' 08" S and 23° 40' 18" S - 46° 36' 48" W and 46° 38' 00" W) [Ipiranga State Park], a fragment of Atlantic Rainforest located in the State of São Paulo, Brazil. We have analysed three specimens per species, but only one voucher per specimens was included in the herbarium of the Instituto de Botânica (SP) (Table 1). The choice of taxa was based on a floristic study of the area that indicated the necessity of providing additional data in order to aid in species identification and conservation (Shirasuna and Filgueiras, 2013). *Olyra lorentensis* Mez was not included in this study due to its uncertain occurrence in the PEFI [see Shirasuna and Filgueiras (2013) for details about each species].

## 2.2. Anatomical analysis

For the woody bamboos, mature leaf blades were taken from the branches at the mid-culm, whereas for the herbaceous bamboos mature leaves were taken from the third node from the base. Fresh plant material was fixed in FAA 50 (Johansen, 1940) and later stored in 70% ethanol. Found on leaves of *Arthrostylidiinae* species, the green stripe was excluded from this work due to its anatomical peculiarities in relation to the remainder of the leaf blade (Judziewicz et al., 1999).

Samples from the middle portion of the leaf blade were embedded in polyethylene glycol 1500 solution (adapted from Richter, 1985) and cross-sectioned with a rotary microtome. Sections were cleared in sodium hypochlorite 50%, washed in distilled water, stained with Astra blue and Safranin (Bukatsh, 1972), and finally mounted on semi-permanent slides with glycerol. Also, a maceration technique was performed by the Jeffrey's method (Johansen, 1940) in order to describe the epidermal features.

Descriptions were primarily based on Ellis (1976, 1979), and optical images were obtained on a Leica DM4000B microscope using the software Leica Application Suite LASV4.0.

## 3. Results

### 3.1. Surface view

All the studied taxa share an epidermis with long-short cell alternation (Figures 1A-M). Short cells occur as silica bodies (Figures 1D, F, K - arrow) or suberized cells (cork cells - arrowhead) (Figure 1A) – sometimes as silico-suberose couples in the intercostal zone (e.g., Figure 1A). The wall sinuosity of long cells may be deep (Figures 1B, E, K), moderate (Figures 1A, F) or slight (Figures 1G, J). Papillae commonly occur on the abaxial surface: less pronounced in the herbaceous species (e.g., Figure 1D) and more pronounced in the woody



species (e.g., Figures 1C, H). In *Merostachys argyronema* Lindm. papillae are very conspicuous (Figure 1H; Table 2), and in *Merostachys neesii* Rupr. they have a concave apex (Figure 1C; Table 2). A scattered distribution of papillae is observed in the herbaceous bamboos (e.g., Figure 1D; Table 2), whereas the organization in the woody bamboos is often in a single central row (e.g., Figures 1G, I), but may be variable in some intercostal cells (1-2 rows) (e.g., Figure 1H).

Trichomes mainly occur on the abaxial surface and they may be of three types: (i) prickly hairs (short and silicified, microscopic unicellular) (Figures 1C, F); (ii) macrohairs (macroscopic unicellular) (Figures 1E); (iii) or microhairs (microscopic bicellular) (Figures 1E, L, M). The occurrence of these trichomes is variable among the studied species and only *Chusquea capituliflora* Trin var. *pubescens* McClure & L.B. Sm. has all the three types (Figures 1E, F). Prickly hairs of most of the species develop an enlarged base, usually as seen in *Chusquea capituliflora* var. *pubescens* (Figure 1F), but in *M. neesii* this base is more pronounced (Figure 1C). Macrohairs occur on the abaxial surface of *C. capituliflora* var. *pubescens* (Figure 1F) and *Chusquea meyeriana* Rupr. ex Doell (Figure 1I - scars). Bicellular microhairs often consist of cells of about the same size (e.g., Figure 1M), except for *C. capituliflora* var. *pubescens*, in which the apical cell is reduced (Figure 1L). Microhairs often occur on the abaxial surface in the woody species and *Parodiolyra micrantha* (Kunth) Davidse & Zuloaga.

Stomata are paracytic and occur on the abaxial surface of all the studied species, but also on the adaxial surface in *A. aristulata* and *Olyra humilis* Nees (Figure 1B; Table 2). Stomatal apparatus comprise triangular subsidiary cells (Figures 1B, D, H, K) or semi-circular (cupuliform) cells (Figures 1A, E, G). In species of *Chusquea* the stomatal apparatus bears two papillae per subsidiary cell as seen in *Chusquea capituliflora* var. *pubescens* (Figure 1E detail inset; Table 2).

### 3.2. Cross section

The epidermis consists of a single stratum of cells with slightly thickened outer walls (Figures 2A-Q). Epidermal cells are visually about the same size (Figures 2E, G, J), but may be larger on the adaxial side (Figures 2F, H, M) – excluding the bulliform cells. Bulliform cells occur as part of the adaxial epidermis (Figures 2D-M) and form a fan-shaped array in the woody bamboos, (e.g., Figures 2G, J, K; Table 2); whereas the herbaceous bamboos share a parallel-sided array of bulliform cells (e.g., Figures 2E, I; Table 2).

The mesophyll comprise arm cells, fusoid cells, rosette cells, fibers, and vascular bundles. Asymmetrically invaginated arm cells are parallel to the epidermis (Figures 2E-M), including the midrib portion (Figures 2A-D). Herbaceous species (Figures 2E, I) and *Chusquea bambusoides* (Figure 2H) develop arm cells with invaginations only from the abaxial side, whereas the other species develop invaginations from both sides (Figures 2F, G, J-M). The number of rosette cells between each fusoid cell is often variable (one to four) within the same sample/specimen (Figures 2E-M). Fusoid cells occur adjacent to the vascular bundles and arm cells (Figures 2E-M); and their outline may be short and wide, as seen in *A. aristulata* (Figure 2G), or long and narrow, as in *M. neesii* (Figure 2F). Intercostal fibers located adjacent to the bulliform cells (sometimes also opposite) occur just among species of Arthrostylidiinae (e.g., Figures 2D, F, G; Table 2). Collateral vascular bundles are surrounded by a double sheath (Figures 2A-M): the outer one is parenchymatic and may be interrupted by fibers from both sides, as seen in *Merostachys speciosa* Spreng. (Figure 2M) or only from the abaxial side, as in *Merostachys skvortzovii* Send. (Figure 2L); and the inner one is pericyclic (mestome) with thick-walled cells (Figures 2A-M). First and third order vascular bundles are observed in all the studied species (Figures 2F-M).

In most of the studied species the midrib is flat (e.g., Figure 2D), but it is abaxially projected in species of *Chusquea* (Figures 2A, B) and adaxially projected in *Padoriolyra*

*micrantha* (Figure 2C; Table 2). The midrib comprise one first order vascular bundle (Figures 2C, D), except among species of *Chusquea*, in which the midrib includes minor vascular bundles adjacent to the central one (Figures 2A, B; Table 2).

With regard to the margin, the leaf blade may be acute (Figure 2N-P) or obtuse (Figures 2Q); always with thick-walled epidermal cells and fibers immediately subjacent to the epidermis (e.g., Figures 2N-Q).

### 3.3. Taxonomic treatment

The main anatomical features are summarized in Table 2. These data in tabular form are available upon request from the first author.

#### **Identification key to the native Bambusoideae species from PEFI, SP, based on the leaf blade anatomical data (surface view and cross section)**

1. Papillae scattered on the abaxial surface; parallel-sided arrays of bulliform cells (Tribe Olyreae)
  2. Prickle hairs on the abaxial surface developed; midrib adaxially projected .....  
..... *Parodiolyra micrantha*
  - 2'. Prickle hairs on the abaxial surface lacking; midrib slightly convex on both surfaces
    3. Leaves amphistomatic; adaxial epidermal cells larger than abaxial epidermal cells (excluding the bulliform cells) ..... *Olyra humilis*
    - 3'. Leaves hypostomatic; adaxial epidermal cells equal to sub-equal to the abaxial epidermal cells (excluding the bulliform cells) ..... *Olyra glaberrima*
- 1' Papillae centrally organized in a single or double row on the abaxial surface; fan-shaped arrays of bulliform cells (Tribe Bambuseae)

4. Intercostal fibers developed; midrib with only one vascular bundle (simple midrib); stomata apparatus without papillae (Subtribe Arthrostylidiinae)
5. Stomata on both surfaces; papillae on the adaxial surface developed .....  
 ..... *Aulonemia aristulata*
- 5'. Stomata only on the abaxial surface; papillae on the adaxial surface lacking
6. Central vascular bundle in the midrib (major one) with outer sheath interrupted by fibers from both sides
7. First order vascular bundle with outer sheath interrupted by fibers from the abaxial side ..... *Merostachys scandens*
- 7'. First order vascular bundle with outer sheath interrupted by fibers from both sides
8. Fusoid cells long and narrow ..... *Merostachys magellanica*
- 8'. Fusoid cells short and wide
9. Adaxial epidermal cells larger than abaxial epidermal cells (excluding the bulliform cells) ..... *Merostachys riedeliana*
- 9'. Adaxial epidermal cells equal to sub-equal to the abaxial epidermal cells (excluding the bulliform cells)
10. Prickle hairs on the abaxial surface developed ..... *Merostachys burmanii*
- 10'. Prickle hairs on the abaxial surface lacking ..... *Merostachys pluriflora*
- 6'. Central vascular bundle in the midrib (major one) with outer sheath interrupted by fibers only from the abaxial side
11. First order vascular bundle with outer sheath interrupted by fibers from both sides
12. Bicellular microhairs on the adaxial surface developed; adaxial epidermal cells larger than abaxial epidermal cells (excluding the bulliform cells) .....

- ..... *Merostachys neesii*
- 12'. Bicellular microhairs on the adaxial surface lacking; adaxial epidermal cells equal to sub-equal to the abaxial epidermal cells (excluding the bulliform cells)
- ..... *Merostachys skvortzovii*
- 11'. First order vascular bundle with outer sheath interrupted by fibers only from the abaxial side
13. Fusoid cells short and wide; bicellular microhairs on the adaxial surface developed ..... *Merostachys argyronema*
13. Fusoid cells long and narrow; bicellular microhairs on the adaxial surface lacking ..... *Merostachys speciosa*
- 4'. Intercostal fibers lacking; midrib with more than one vascular bundle (complex midrib); stomatal apparatus bearing two papillae per subsidiary cell (Subtribe Chusqueinae)
14. Midrib with two vascular bundles subjacent to the adaxial epidermis and opposite to the central one ..... *Chusquea meyeriana*
- 14'. Midrib with one vascular bundle subjacent to the adaxial epidermis and opposite to the central one
15. Prickle hairs on the adaxial surface developed; macrohairs on the abaxial surface developed; bicellular microhairs developed ..... *Chusquea capituliflora* var. *pubescens*
- 15'. Prickle hairs on the adaxial surface lacking, macrohairs on the abaxial surface lacking; bicellular microhairs lacking ..... *Chusquea bambusoides*

#### 4. Discussion

Our anatomical study demonstrates that papillae scattered on the abaxial surface and parallel-sided arrays of bulliform cells are exclusive features among the herbaceous bamboos sampled; whereas centrally organized papillae and fan-shaped arrays of bulliform cells are exclusive features among the woody bamboos sampled.

Within the herbaceous bamboos sampled, the midrib outline and amphistomatic leaves may distinguish *Parodiolyra* Soderstr. & Zuloaga from *Olyra* L. Although this may be true, it is not clear if these features are consistent among all Brazilian species of *Olyra* (20) and *Parodiolyra* (four) (Oliveira and Filgueiras, 2016a, b). Comparatively, within the woody bamboos sampled, intercostal fibers and a midrib with only one vascular bundle (simple midrib) characterize the subtribe Arthrotyliidiinae; whereas a stomata apparatus bearing two papillae per subsidiary cell and a midrib with more than one vascular bundle (complex midrib) characterize the subtribe Chusqueinae. The presence of two papillae per subsidiary cell herein supports the assumption of this feature as a synapomorphy for *Chusquea* (Fisher et al., 2009, 2014), although there are not enough studies on micromorphology and anatomy to clarify its value. Currently, the set of features herein observed for Arthrotyliidiinae and Chusqueinae is common among all species known within each subtribe and extremely applicable for recognizing these groups (BPG, 2012; Clark et al., 2015).

The comparative anatomical analysis herein performed demonstrates that the variation in the distribution of papillae is useful for delimiting tribes. There are some reports showing the importance of this feature in bamboo systematics (e.g., Soderstrom and Ellis, 1987; Paisooksantivatana and Pohl, 1992; Yang et al., 2008; Gomes and Neves, 2009; Mota, 2013), but also for other closely related groups (e.g., Pelegrin et al., 2009). Our study is not able to define the value of this feature to the systematics of Olyreae and Bambuseae, therefore a detailed work to evaluate both distribution and type of papillae within different groups would be informative.

Our study also indicates that some features may be considered diagnostic at the species level. Among them, the stomata on the adaxial surface in *Aulonemia aristulata* must be mentioned, since their occurrence is considered as rare for *Aulonemia* (Arthrotyliidiinae) (Viana et al., 2013a), but usually typical for species within the subtribe Guaduineae

(Soderstrom and Ellis, 1987). Adaxial stomata were also recently observed in other species within *Aulonemia* (Viana, 2010; Viana et al., 2011), and thus it reinforces the anatomical affinity between the subtribes Arthrostylidiinae and Guadineae (Bambuseae) (Soderstrom and Ellis, 1987; Zhang and Clark, 2000; Ruiz-Sánchez et al., 2008), as well as the necessity of a broad anatomical study in order to elucidate the systematic value of this feature for Bambuseae.

It is important to highlight that the size and shape of bulliform cells may be influenced by environmental factors (Shields, 1951), but the structural variation herein observed deserves more attention in order to verify its constancy among bamboo groups. Also, the fusoid cell is another feature that requires additional attention since its environmentally influenced morpho-anatomical variations (March and Clark, 2011; T. D. Leandro, unpubl. data). In the present study, we consider the structure of bulliform cells and the outline of fusoid cells as relevant features for delimiting species given that all specimens were sampled under the same environmental conditions.

## **5. Conclusion**

Although most of the information herein provided is not a novelty for Bambusoideae, our results reinforce the importance of leaf blade anatomy studies for grass systematics, specially when we consider the great number of questions that are still unclear. The inclusion of anatomical data as a routine on bamboo studies may be really useful for identifying diagnostic features and additional synapomorphies, in which certainly will aid in species circumscription.

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# TABLES

**Table 1.** Specimens used in this study, with classification and voucher information provided.

Taxa and classification	Voucher information
TRIBE OLYREAE	
Subtribe Olyrineae	
<i>Olyra glaberrima</i> Raddi	A. Custodio Filho 124 (SP 160961)
<i>O. humilis</i> Nees	R. T. Shirasuna 2617 (SP 415204)
<i>Parodiolyra micrantha</i> (Kunth) Davidse & Zuloaga	R. T. Shirasuna 2863 (SP 420323)
TRIBE BAMBUSEAE	
Subtribe Chusqueinae	
<i>Chusquea bambusoides</i> (Raddi) Hack.	R. T. Shirasuna & A. Costa 1809 (SP 409031)
<i>C. capituliflora</i> Trin var. <i>pubescens</i> McClure & L.B. Sm.	R. T. Shirasuna 2760 (SP 415735)
<i>C. meyeriana</i> Rupr. ex Döll	R. T. Shirasuna 2697 (SP 415247)
Subtribe Arthrostylidiinae	
<i>Aulonemia aristulata</i> (Döll) McClure	R. T. Shirasuna 2860 (SP 420320)
<i>Merostachys argyronema</i> Lindm.	R. T. Shirasuna 2868 (SP 420326)
<i>M. burmanii</i> Send.	J. F. Toledo s/n° (SP 238492)
<i>M. magellanica</i> Send.	M. T. Grombone s/n° (SP 412132)
<i>M. neesii</i> Rupr.	R. T. Shirasuna 2864 (SP 430324)
<i>M. pluriflora</i> Munro ex. E.G. Camus	R. T. Shirasuna 1811 (SP 409023)
<i>M. riedeliana</i> Rupr. ex Döll	R. T. Shirasuna 2872 (SP 426233)
<i>M. scandens</i> Send.	R. T. Shirasuna 2993 (SP 441817)
<i>M. skvortzovii</i> Send.	T. Sendulsky 1318 (SP 166796)
<i>M. speciosa</i> Spreng.	R. T. Shirasuna 2798 (SP 417980)







# FIGURES

**Figure 1.** Surface view of leaf blades of Bambusoideae species. Adaxial surface (B, F, K) and abaxial surface (A, C-E, G-J). (A) *Chusquea bambusoides* with cupuliform subsidiary cells (\*) and silico-suberose couples; (B) *Olyra humilis* with triangular subsidiary cells (\*); (C) *Merostachys neesii* showing papillae with concave apex (white circle); (D) *Olyra glaberrima* with silica body (black arrow) and papillae scattered; (E) *Chusquea capituliflora* var. *pubescens* with macrohairs (Ma), microhairs (Mi), and a detail showing the stomata apparatus bearing two papillae per subsidiary cell (white arrows); (F) *Chusquea capituliflora* var. *pubescens* with prickle (Pr); (G) *Aulonemia aristulata* with microhair (Mi); (H) *Merostachys argyronema* with papillae organized in a central row (or double row in some long cells); (I) *Chusquea meyeriana* showing the epidermis with macrohair scars (Ma); (J) *Merostachys riedeliana* with silica body (black arrow); (K) *Parodiolyra micrantha* showing silica body (black arrow); (L) *Chusquea capituliflora* var. *pubescens* detail of a bicellular trichome (microhair) with reduced apical cell; (M) *Aulonemia aristulata* detail of a bicellular trichome (microhair) with cells about the same size. (Ma) macrohair; (Mi) microhair; (Pr) prickles. Black arrows: silica bodies; white arrows: papillae; arrowhead: suberized cells; asterisks: stomata; white circle: papillae with concave apex.

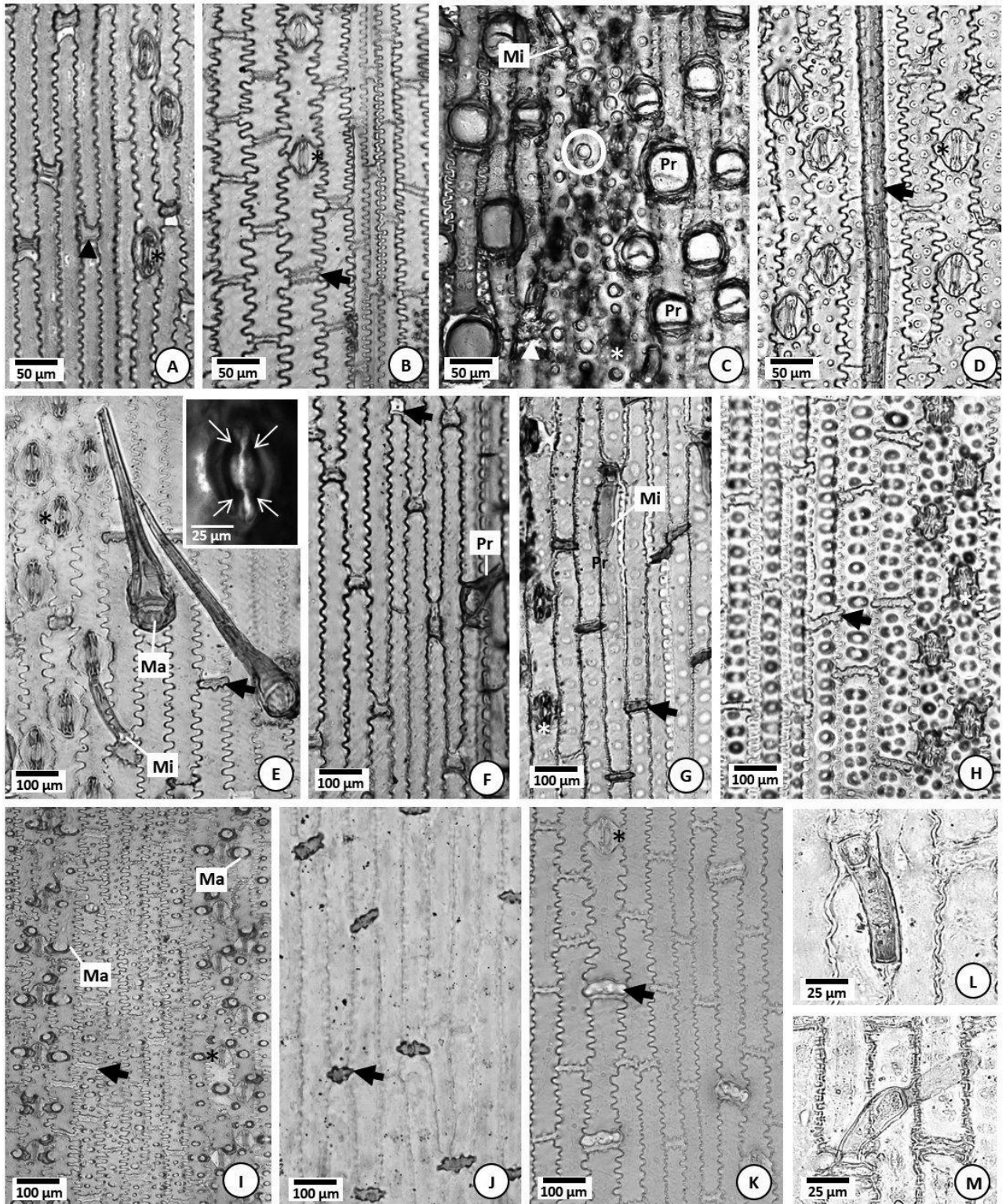


FIG 1.

**Figure 2.** Cross section of leaf blades of Bambusoideae species. Midrib (A-D), mesophyll (E-M) and margin (N-Q). (A) *Chusquea capituliflora* var. *pubescens* showing complex midrib with one vascular bundle opposite to the central one; (B) *Chusquea meyeriana* showing complex midrib with two vascular bundles opposite to the central one; (C) *Parodiolyra micrantha* showing midrib with only one vascular bundle (simple midrib); (D) *Aulonemia aristulata* with intercostal fibers (If); (E) *Olyra humilis* showing arm cells with invaginations only from the abaxial side; (F) *Merostachys neesii* with intercostal fibers (If), bulliform cells (Bc) and arm cells (Ac); (G) *Aulonemia aristulata* showing arm cells with invaginations from both sides; (H) *Chusquea bambusoides* showing rosette cells (Rc); (I) *Parodiolyra micrantha* with papillae (black circle); (J) *Chusquea capituliflora* var. *pubescens* showing prickly hair (Pr); (K) *Merostachys burmanii* with silica body (arrowhead); (L) *Merostachys skvortzovii* showing vascular bundles with outer sheath interrupted by fibers from the abaxial side; (M) *Merostachys speciosa* showing vascular bundles surrounded by a double sheath; (N) *Aulonemia aristulata* with acute margin and thick-walled epidermal cells; (O) *Chusquea capituliflora* var. *pubescens* with acute margin and a few fibers; (P) *Chusquea bambusoides* with thick-walled epidermal cells; (Q) *Chusquea meyeriana* with obtuse margin and many fibers. (Ac) arm cells; (Bc) bulliform cells; (Fi) fibers; (If) intercostal fibers; (Pr) prickly hair; (Rc) rosette cells. Arrows: fusoid cells; arrowheads: silica bodies; black circles: papillae.

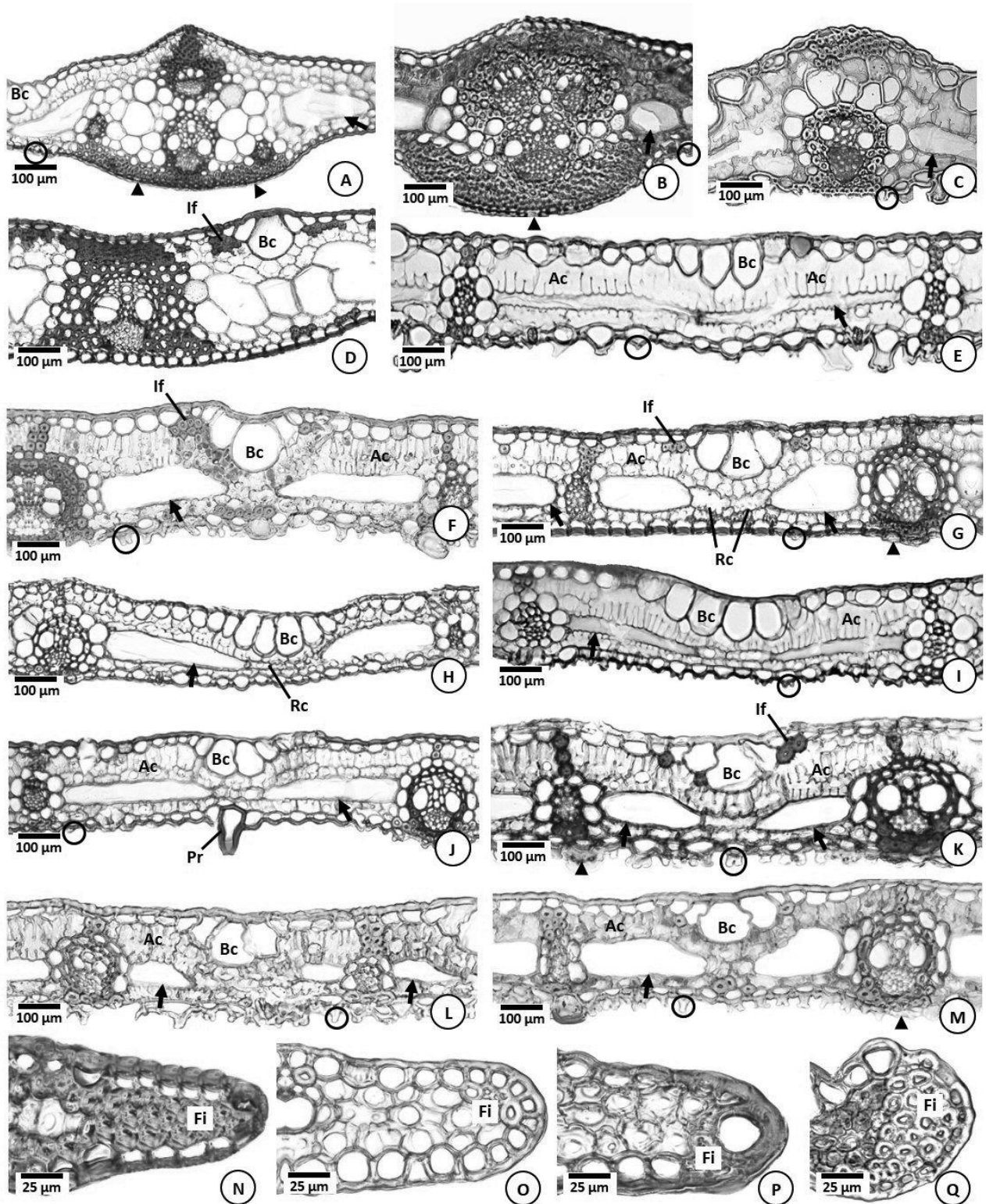


FIG 2.

## CAPÍTULO II

**The contribution of foliar micromorphology and anatomy to the  
circumscription of species within the *Chusquea ramosissima* Clade**

**(Poaceae, Bambusoideae, Chusqueinae)**

*T. D. Leandro, V. L. Scatena e L. G. Clark*

ENVIADO PARA O PERIÓDICO PLANT SYSTEMATICS AND EVOLUTION

## Abstract

*Chusquea* is a diverse but monophyletic genus of Neotropical woody bamboos from primarily montane forests that comprises four well-supported lineages: subg. *Magnifoliae*, subg. *Platonia*, subg. *Rettbergia* and the *Euchusquea* clade (comprising subg. *Swallenochloa* and subg. *Chusquea*). The *Euchusquea* clade encompasses about 75% of the described species diversity within *Chusquea* and includes five minor clades with at least moderate support from molecular data. However, the relationships among and within the minor clades inferred from molecular data are mostly not congruent with those inferred from morphological evidence, consequently limiting our ability to understand species relationships. In this study we generated foliar micromorphological and anatomical data for one of the minor clades (referred to as the *Chusquea ramosissima* Clade) including three sampled species *Chusquea ramosissima*, *C. tenella*, and *C. longispiculata*, and for the morphologically closely related species *C. attenuata*, *C. tenuiglumis* and *Chusquea* sp. nov., in order to test the value of these types of characters for defining species and to seek potential synapomorphies for this clade. Our results demonstrate that epidermal features, mainly with regard to the stomatal apparatus, proved to be more valuable in distinguishing species than anatomical characters. The type of trichomes, type and arrangement of silica bodies, type of arm cells and the number of their layers in the mesophyll, and midrib structure may be useful to lesser degree. The inclusion of *C. tenuiglumis* within the *Chusquea ramosissima* Clade and the *C. ramosissima* informal group is supported based on morpho-anatomical similarities. Support for the recognition of a new species in this group from Bolivia is also provided by micromorphological characters. An identification key based on leaf blade features is provided for the six studied species.

**Keywords** Bambuseae, Monocotyledons, Poales, woody bamboos



## Introduction

Bambuseae comprise one of three lineages within Bambusoideae and include at least 812 species in 66 genera (Clark et al. 2015). This tribe is mostly diversified in tropical forests, both lowland and montane, and humid habitats (Judziewicz et al. 1999; Clark et al. 2015). Two major lineages are currently recognized for Bambuseae based on molecular data (Sungkaew et al. 2009; Kelchner and BPG 2013): Paleotropical woody bamboos (four subtribes, 407 species), and Neotropical woody bamboos (three subtribes, 405 species) (BPG 2012; Clark et al. 2015). The phylogenetic relationship of the three subtribes within the Neotropical woody bamboos [(Arthrostylidiinae + Guaduinae) + Chusqueinae] has good support (Kelchner and BPG 2013; Wysocki et al. 2015), although no defining characters have been identified (Clark et al. 2015). Sister to each other, Arthrostylidiinae and Guaduinae are each well supported as monophyletic also based on morphological and molecular data (Ruiz-Sanchez 2011; Tyrrell et al. 2012; Kelchner and BPG 2013), while the monophyly of Chusqueinae, which includes only the genus *Chusquea* Kunth, is currently based primarily on molecular evidence although all members share the presence of two papillae per subsidiary cell and one-flowered spikelets with four glumes and no rachilla extension (Fisher et al. 2009; Kelchner and BPG 2013; Fisher et al. 2014).

Bamboos grow vegetatively for long periods and then their life cycle usually ends after a single gregarious flowering episode (Seifriz 1950; Janzen 1976; Judziewicz et al. 1999). For this reason, vegetative morphological data from their rhizomes, culms and leaves remains extremely important for bamboo studies. Anatomical and micromorphological surveys in addition to morphological ones have established the importance of these types of features in providing the basis for a more natural classification within the grass family (e.g., Guglieri et al. 2008; Oliveira et al. 2008; Gomes and Neves 2009; Pelegrin et al. 2009; Aliscioni, et al. 2016; Leandro et al. 2016a,b). Although diagnosable macro-morphological characters of *Chusquea*

and its subgenera are well defined (Fisher et al. 2009; 2014), there is a lack of knowledge with respect to the value of leaf blade anatomical and micromorphological features across the genus, even considering the amount of data available in the literature (Clark 1986; Clark et al. 1989; Clark 1990; Guerreiro et al. 2013).

*Chusquea* is the most diverse genus of Neotropical woody bamboos, comprising more or less 175 described species primarily from montane forests (Judziewicz et al. 1999; Fisher et al. 2014). Considering the classification previously provided by taxonomic work [see Clark (1989) and Fisher et al. (2009; 2014) for a detailed taxonomic history of the genus], plastid sequence data supports the existence of four clades within *Chusquea* (Fisher et al. 2014) (Fig. 1): (i) subg. *Magnifoliae* and (ii) subg. *Platonia*; (iii) subg. *Rettbergia*; and (iv) the major *Euchusquea* clade, comprising subg. *Swallenochloa* and subg. *Chusquea* and including about 75% of the species diversity of the genus. Fisher et al. (2014) also identified five minor clades (I-V) within the *Euchusquea* clade, but inferring their relationships with confidence remains a challenge because the morphologically-based infrageneric classification is largely incongruent with the molecular topology. In this study, our focus was on the minor but relatively well supported clade referred to as the *Chusquea ramosissima* Clade (Clade II in Fisher et al. 2014; Fig. 1), a group that includes several species distributed in Paraguay, Uruguay, Argentina and Brazil.

Well supported as monophyletic, the *Chusquea ramosissima* Clade includes three sampled species (Fisher et al. 2014): *Chusquea ramosissima* Lindman, *C. tenella* Nees, and *C. longispiculata* L.G. Clark. Morphologically, all three species are classified within subg. *Chusquea* based on their extravaginal or infravaginal branching and the more or less constellate arrangement of subsidiary buds with respect to the central bud (Clark 2004; Fisher et al. 2009, 2014). [Note that the statement in the caption of Fig. 3 in Fisher et al. 2014 that *C. longispiculata* and several other species were not placed in a subgenus is erroneous.] In Fisher et al. (2009), *C. ramosissima* and *C. tenella* were included within the *C. ramosissima* informal group, within

subg. *Chusquea*, along with *C. tenuiglumis* Döll, based on culm leaves with pseudopetiolate, deciduous blades that usually remain green and racemose or weakly paniculate synflorescences (Fisher et al. 2014). Also, *C. longispiculata*, along with *C. anelythra* Nees, *C. anelythroides* Döll, *C. attenuata* (Döll) L.G. Clark and *C. meyeriana* Rupr. ex Döll, was classified as a member of the *C. meyeriana* informal group within subg. *Chusquea* based on the presence of spatheate bracts subtending the synflorescences, reflexed lower synflorescence branches and reduced glumes I and II (Clark 2004; Fisher et al. 2009). Previous work identified *C. ramosissima*, *C. tenella*, *C. tenuiglumis*, *C. anelythra*, *C. anelythroides* and others as included within subg. *Rettbergia* (Judziewicz et al. 1999), but Fisher et al. (2009, 2014) and Mota (unpublished data) based on molecular and morphological evidence excluded these species from subg. *Rettbergia* and placed them within subg. *Chusquea*.

Considering its complex taxonomic history and the incongruence between the chloroplast phylogeny *versus* the morphology-based classification of *Chusquea*, the main objective of the current work was to test the value of leaf blade micromorphology and anatomy across species within the *Chusquea ramosissima* Clade of Fisher et al. (2014), and some of the species assigned to the two informal morphological groups that comprise this clade. To that end, *Chusquea ramosissima*, *C. tenella*, *C. longispiculata*, *C. attenuata* and *C. tenuiglumis* were studied. We also included a putatively undescribed species of *Chusquea* from Bolivia due to its strong vegetative similarity to *C. ramosissima*.

## **Materials and Methods**

### **Taxon sampling**

The following three species comprising the *Chusquea ramosissima* Clade (Fisher et al. 2014) were studied: *C. ramosissima* and *C. tenella* of the *C. ramosissima* informal group and *C.*

*longispiculata* of the *C. meyeriana* informal group. For comparison, we also included two morphologically related species assigned to these informal groups (Fisher et al. 2009): *Chusquea attenuata* of the *C. meyeriana* informal group and *C. tenuiglumis* of the *C. ramosissima* informal group; and a putative new species similar to *C. ramosissima* referred to as *Chusquea* sp. nov. The species were studied from herbarium material deposited as vouchers at the Ada Hayden Herbarium of Iowa State University (ISC) and the Missouri Botanical Garden (MO) for those specimens of *Chusquea* sp. nov. (Table 1). While we were able to sample all species assigned to the *C. ramosissima* informal group, we sampled only the two species in the *C. meyeriana* informal group that have been well characterized morphologically. Circumscriptions for *C. meyeriana*, *C. anelythra* and *C. anelythroides* are currently being worked out but we did not sample these species for this study in order to avoid creating additional confusion.

### **Micromorphology (scanning electron microscopy)**

Two pieces of approximately 0.5 cm<sup>2</sup> of the middle portion of the leaf blade were removed from herbarium specimens. Pieces representing both surfaces were attached to a cylindrical sample holder (stub) and coated with a thin sample of gold (Denton vacuum Desk III). At least two specimens per species were treated with xylene to eliminate the epicuticular wax in order to provide a better view of micromorphological features (Dávila and Clark 1990). Observation and images of the surface view were obtained with the aid of a scanning electron microscope (JEOL JSM-5800LV) in the Microscopy and Nano-Imaging Facility (MNIF) at Iowa State University (ISU).

## **Anatomy (light microscopy)**

Fully expanded leaf blades were taken from herbarium specimens and the middle portion was excised and then immersed in polyethylene glycol 1.500 solution and kept in an incubator at 60°C for fifteen days (adapted from Richter 1985). Cross sections were made using a *Spencer* 820 rotary microtome, and then they were cleared in 50% sodium hypochlorite, rinsed in distilled water, and then stained in Epoxy Tissue Stain (Spurlock et al. 1966). Semi-permanent slides were mounted in 50% glycerin and analyzed under a light microscope (Olympus BX-40).

Leaf clearings were performed in order to describe the structures in surface view. For this purpose, pieces of approximately 0.5 cm<sup>2</sup> from the middle portion of the leaf blade were removed. These pieces were hydrated through a graded series of ethyl alcohol (50, 25 and 10%) and then soaked in 1:1/dH<sub>2</sub>O until the samples were translucent. Samples were rinsed with dH<sub>2</sub>O, dehydrated through a graded series of ethyl alcohol (25, 50, and 70%), and then stained with Safranin and Fast Green (Johansen 1940). Stained samples were treated with ethyl alcohol (95 and 100%), xylene, and then xylene and Permount (1:1). Permanent slides were mounted with Permount.

## **Images and terminology**

For both sections and clearings, photomicrographs were obtained with the aid of a Zeiss AXIO Observer microscope in the MNIF at ISU using ZEN 2.0 blue software, and applying different light contrast regimes. The terminology for the leaf blade in cross-section and in surface view primarily followed Ellis (1976; 1979).

## Results

The following data in tabular form are available upon request from the first author. A summary of the most important micromorphological and anatomical features for each species is provided in Table 2.

### *Epidermal surfaces (SEM and clearings)*

Data for epidermal cells as seen with scanning electron microscopy (Figs. 2a-i, 3a-i) and with light microscopy of clearings (Fig. 4a-j, l, n) are merged in order to provide a better understanding.

The epidermis consists of alternating long and short cells (Figs. 2a-i, 3a-i) with epicuticular wax (e.g., Fig. 2g). In general, long cells are tabular-shaped with thin, undulating anticlinal walls (Figs. 2a-i, 3a-i), except in *C. attenuata*, which exhibits highly thick anticlinal walls (Fig. 4f). Intercostal long cells on both leaf blade surfaces are usually densely covered by dome-shaped papillae that are often organized in one central row (Figs. 2a-c, g, 3a, g, 4i), but scattered papillae may be observed on the abaxial long cells of *C. longispiculata* (Fig. 2e-f), *C. ramosissima* (Fig. 2i) and *C. tenella* (Fig. 3b-c). Papillae are usually absent or poorly developed in the interstomatal band of the intercostal zone in *C. longispiculata*, *C. ramosissima*, and *C. tenuiglumis* (Figs. 2e, h, 3e), but are well developed in *C. attenuata*, *C. tenella*, and *Chusquea* sp. nov. (Fig. 2b, 3b, h). Intraspecific variation in the density of papillae is observed, in which papillae may be well-developed or poorly developed.

Short cells may be developed as silica bodies or cork cells (Fig. 4d, i). Silica bodies occur in the costal zone on both surfaces and they are associated with tabular short cells (e.g., Fig. 4g, h), but also occur scattered on the adaxial surface in the intercostal zone (e.g., Figs. 3g, 4c). Cork cells are tall and narrow and occur as silico-suberose pairs mostly in the intercostal zone (Fig. 4d, i). In the costal zone, silica bodies are vertically oriented (e.g., Fig. 4a) or

horizontally oriented (e.g., Fig. 4b), but in the interstomatal band of the intercostal zone, silica bodies are always vertically oriented (e.g., Fig. 3b, 4i). With regard to shape, silica bodies are mainly saddle-shaped (e.g., Fig. 4a, g), but dumbbell-shaped (Fig. 4b, h) and equidimensional-shaped (not shown) bodies can be observed in the same specimen/sample. The vertically oriented pattern in the costal zone is observed just in *C. attenuata* and *C. longispiculata* (Fig. 4a), and in these two species, the silica bodies are exclusively saddle-shaped.

Stomata are always observed on the abaxial epidermis (Figs. 2b-c, e-f, h-i, 3b-c, e-f, h-i), but they occur scattered on the adaxial epidermis in *C. longispiculata*, *C. tenuiglumis* (Figs. 3d, 4l), and *Chusquea* sp. nov. (Fig. 4n). On the abaxial epidermis, the number of rows of stomata can be variable (three to six) among species (e.g., Figs. 2h, 3e), and usually there is only one interstomatal long cell separating the stomata (e.g., Fig. 3f). The stomatal apparatus is paracytic (e.g. Fig. 4j) with subsidiary cells that are low dome-shaped (Fig. 4c) or low triangular-shaped (Fig. 4j) and bearing two papillae, each one branched (Figs. 3c, i, 4f) or not (Fig. 2f, 3c, i). Branched papillae completely overarch the guard cells and form a chamber above the pore in *C. ramosissima* (Fig. 2i) and *C. tenuiglumis* (Fig. 3f), whereas in *C. attenuata* the branches do not overarch the pore (Fig. 2c). In *C. tenella* and *Chusquea* sp. nov., the simple papillae arch over and meet across the guard cells, but do not form a chamber (Fig. 3c, i). Papillae are not branched in *C. longispiculata*, but the stomatal apparatus is often overarched by papillae from adjacent intercostal long cells (Fig. 2f).

Trichomes can be of five types: (i) silicified and pointed unicellular with the base forming an integral part of the epidermis (prickle hair); (ii) silicified unicellular with a cell that emerges straight out of the epidermis (microhair); (iii) non-silicified unicellular (macrohair); (iv). non-silicified bicellular with a basal and a distal cell (microhair); (v). non-silicified bicellular with many specialized epidermal cells adjacent to the hair base (microhair). Prickle hairs occur on both surfaces in *C. attenuata* (Fig. 2a-b), *C. tenella* (Fig. 3a-b), and *Chusquea* sp. nov. (Fig. 3h). Non-silicified macrohairs occur on both surfaces of *Chusquea* sp. nov. (Fig.

3g-h) and may be observed on the abaxial surface of *C. ramosissima*, *C. attenuata* (Fig. 2b), and *C. tenella* (Fig. 3b). Silicified unicellular microhairs occur often on the abaxial surface in *C. tenella*, *C. attenuata* (Fig. 2c), *Chusquea* sp. nov. (Fig. 3g), and *C. longispiculata* (Fig. 4e), whereas bicellular microhairs occur on the adaxial surface of all the studied species (e.g., Figs. 2h, 3b, e, h).

#### *Cross section*

The outline of the leaf blade as seen in cross section is gently undulated (Fig. 4k-n). The epidermis consists of a single layer of cells with slightly thickened outer periclinal walls in most of the species (Fig. 4l-n), but strongly thickened ones in *C. longispiculata* (Fig. 4k, q). Epidermal cells are all about the same size except for the presence of fan-shaped arrays of bulliform cells in between the bundles as part of the adaxial epidermis (Fig. 4k-n). The number of bulliform cells in each array is variable among samples within the same species.

The mesophyll comprises asymmetrically invaginated arm cells, fusoid cells, rosette cells, and vascular tissue (Fig. 4k-n). Arm cells are organized in one or two layers in *C. longispiculata* (e.g., Fig. 4k, q), and in two layers in the remainder of species (e.g., Fig. 4l-n). Invaginations of the arm cells are mostly from the abaxial side (Fig. 4l-n, r), but invaginations from both sides are observed in *C. longispiculata* (Fig. 4k, q), *C. attenuata*, and *C. ramosissima*. Just one layer of arm cells is observed adjacent to the abaxial surface, in which the invaginations often occur just from the adaxial side (Fig. 4k, r). Surrounded by arm cells and between vascular bundles, the fusoid cells are highly variable in shape and size among and within species/specimens (Fig. 4k-n). These cells occur along the entire lamina, except at the margins. Rosette cells occur between fusoid cells (Fig. 4k-n), with the number of cells extremely variable in each group along the leaf blade.



With regard to the vascular tissue, xylem and phloem are collateral and surrounded by a double sheath, the inner mestome sheath and the outer parenchymatic sheath (Fig. 4k-n). Vascular bundles comprise two types: (i) first order with distinguishable metaxylem and phloem (Fig. 4k-n); and (ii) third order, small vascular bundles with usually a few lignified tracheary elements and a small patch of phloem (Fig. 4k-n). Girders occur in both types of vascular bundles, but only the parenchymatic sheath from the first order vascular bundles is abaxially interrupted by this arrangement of fibers (Fig. 4k-n). The midrib can be of two types: (i) composed of two opposing vascular bundles sharing the same inner sheath (Fig. 4o); or (ii) composed of three vascular bundles, two opposite to the central one, but all three sharing the same inner sheath (Fig. 4p).

Leaf blade margins are mainly acute, but one acute and one obtuse margin (dimorphic) may be observed within the same leaf blade of *C. attenuata* and *C. ramosissima*. Also, the amount of sclerenchyma cells in this region is variable among species and within the same sample.

## **Discussion**

### *Micromorphological and anatomical data*

Long cells with papillae; fan-shaped arrays of bulliform cells; a mesophyll with arm cells, fusoid cells, rosette cells, collateral vascular bundles surrounded by a double sheath; and a C<sub>3</sub> photosynthetic pathway are features shared by all the studied species, and also are general for Bambusoideae (Metcalf 1960; Hattersley 1987; Judziewicz et al. 1999; Sanchez-Ken et al. 2001; Oliveira et al. 2008; Gomes and Neves 2009; Viana 2010; Mota 2013; Leandro et al. 2016b). This study reveals many anatomical similarities among the sampled species while also demonstrating that micromorphological features prove to be more systematically informative

even considering notable intraspecific variation observed in papillae development. Presence or absence of trichomes and their type, type and arrangement of silica bodies, type of arm cells and the number of their layers in the mesophyll, and the midrib structure may be useful at the species level.

### *Implications for systematics*

Anatomically, the occurrence of intercostal fibers, presence or absence of papillae on the subsidiary cells and midrib structure seem to be the primary differences between the subtribes Chusqueinae and Arthrostylidiinae (Soderstrom and Ellis 1987; Leandro et al. 2016b). Intercostal fibers and a simple midrib are diagnostic features for Arthrostylidiinae, whereas species within Chusqueinae may be recognized by the presence of stomatal apparatus bearing two papillae per subsidiary cell, lack of intercostal fibers, and a complex midrib (Fisher et al. 2009; 2014; Clark et al. 2015). Comparatively, Guaduinae may be distinguished from these two subtribes by the upper epidermis having abundant stomata and often well-developed papillae (Judziewicz et al. 1999; Ruiz-Sanchez et al. 2008; BPG 2012; Clark et al. 2015). Guaduinae also lack intercostal fibers and possess a complex midrib, and both the Arthrostylidiinae and Guaduinae lack any papillae on the subsidiary cells (Clark et al. 2015).

The stomatal apparatus in Bambusoideae (and Poaceae) is composed of a pair of guard cells surrounded by two subsidiary cells (Metcalf 1960; Ellis 1979; Judziewicz et al. 1999). Subsidiary cells bearing two papillae is a feature shared by all the studied species, and is putatively a synapomorphic character for *Chusquea* (Fisher et al. 2009, 2014; Clark et al. 2015). Although the presence of branched papillae has been previously reported for other species of *Chusquea* (Clark 1986, Clark 1989), the presence of two branched papillae that completely overarch the guard cells herein observed in *Chusquea ramosissima* and *C. tenuiglumis* is highly informative to identify these two species.

Another interesting feature is the midrib. Species herein studied share a complex midrib vasculature as expected for *Chusquea* (BPG 2012; Clark et al. 2015), although not composed solely of true vascular bundles. The complex midrib in species placed within subg. *Rettbergia* (currently 15 described species) often comprise at least two vascular bundles, each one usually with their own bundle sheaths (except for *C. pulchella* L.G. Clark and *C. sellowii* Rupr.) (Mota 2013); whereas a midrib composed of one central vascular bundle that shares its own bundle sheaths with adjacent vascular bundles is a common feature among the species herein studied, which are all placed within subg. *Chusquea* (Fisher et al. 2009). In the latter case, a midrib with a few elements of xylem and phloem embedded in fibers is observed adjacent to the central vascular bundle in the midrib—and sometimes these are difficult to distinguish. Hence, considering the differences in midrib development observed thus far between these subgenera, midrib vasculature in *Chusquea* deserves a broad study in order to verify its value for systematics across the genus.

With regard to morphology, the sympatric *C. ramosissima* and *C. tenella*, and also *C. tenuiglumis* exhibit culm leaves with pseudopetiolate, deciduous blades that usually remain green and racemose or weakly paniculate synflorescences (Fisher et al. 2014). Micromorphologically, these three species exhibit branched papillae, but they may be easily distinguished by a set of features including type of papillae on the stomatal apparatus (simple or branched), type of invagination of the arm cells (abaxial or from both sides), and midrib structure (comprising two or three vascular bundles). The stomatal apparatus is also useful to distinguish *C. ramosissima* and *Chusquea* sp. nov., species closely related by sharing a similar culm leaf structure, branch complement, foliage leaf blade size, and horizontally oriented silica bodies—although the much wider leaf blades of *Chusquea* sp. nov. suggested that it might deserve recognition as a distinct species. The results of this study support this, and we are in the process of describing it as a new species.

The *Chusquea meyeriana* informal group (Fisher et al. 2009), herein represented by *C. longispiculata* and *C. attenuata*, can be recognized morphologically by the spathe bracts often subtending the synflorescences, reflexed lower inflorescence branches, and reduced glumes I and II (Fisher et al. 2014). The species exhibit many micromorphological and anatomical similarities, such as silica bodies vertically oriented, subsidiary cells low-dome shaped, and arm cells with invaginations from both sides. The latter is also one of the features observed in *C. meyeriana* (Leandro et al. 2016b), and *C. ramosissima*, within the *Chusquea meyeriana* and *C. ramosissima* informal groups, respectively (Fisher et al. 2009). The orientation of the silica bodies was the only consistent difference herein observed between these informal groups, in which species in the *Chusquea ramosissima* informal group exhibit horizontally oriented silica bodies and in the *Chusquea meyeriana* informal group vertically oriented.

As a final conclusion, considering our results and in parallel with the anatomical data presented by Mota (2013) for subg. *Rettbergia*, the inclusion of *C. ramosissima* and similar species within the *Euchusquea* clade as proposed by Fisher et al. (2014) is confirmed. The presence of branched papillae on the subsidiary cells and horizontally oriented silica bodies support the inclusion of *C. tenuiglumis* as member of the *Chusquea ramosissima* Clade (Fisher et al. 2014), whereas vertically oriented silica bodies support the recognition of the *Chusquea meyeriana* informal group (Fisher et al. 2009). We are not yet able to define an additional feature or a set of features that support the monophyly of the *Chusquea ramosissima* Clade. Lastly, we corroborate the molecular evidence that supported *C. ramosissima* as sister to *C. tenella* (Fisher et al. 2014)—although *C. tenuiglumis* (not sampled in their work) exhibits more morpho-anatomical similarities to *C. ramosissima*.

## Taxonomic treatment

### Identification key based on micromorphological and anatomical data of species of *Chusquea* herein studied

- 1a. Each subsidiary cell bearing two branched papillae (e.g., Fig. 2c, i) ..... 2
- 1b. Each subsidiary cell bearing two simple papillae (e.g., Fig. 3c) ..... 4
- 2a. Papillae with branches that completely overarch the guard cells forming a chamber above the pore (e.g., Fig. 2i) ..... 3
- 2b. Papillae with branches that do not overarch the guard cells (Fig. 2c) ..... **4. *C. attenuata***
- 3a. Arm cells with invaginations from the abaxial side only (e.g., Fig. 4r); stomata on both surfaces of the leaf blade (Fig. 4l) ..... **5. *C. tenuiglumis***
- 3b. Arm cells with invaginations from both sides (e.g., Fig. 4q); stomata on only the abaxial surface of the leaf blade ..... **3. *C. ramosissima***
- 4a. Midrib composed of two opposing vascular bundles (e.g., Fig. 4o) ..... 5
- 4b. Midrib composed of three vascular bundles, two smaller ones opposing the major one (e.g., Fig. 4p) ..... **1. *C. tenella***
- 5a. Costal silica bodies horizontally oriented (e.g., Fig. 4b); prickles and macrohairs developed; arm cells with invaginations from the abaxial side only (e.g., Fig. 4r) ..... **6. *Chusquea* sp. nov.**
- 5b. Costal silica bodies vertically oriented (Fig. 4a); prickles and macrohairs lacking; arm cells with invaginations from both sides (Fig. 4q) ..... **2. *C. longispiculata***

### General features for all species of the *Chusquea ramosissima* Clade herein studied

**ADAXIAL SURFACE:** LONG CELLS tabular with undulate anticlinal walls. PAPPILLAE often present on the long cells (usually absent or poorly developed in *C. longispiculata* and *C.*

*tenuiglumis*); often present on the bulliform cells (except in *C. longispiculata* and *C. tenuiglumis*). SHORT CELLS over the veins and usually scattered between the veins: SILICA BODIES saddle-shaped, dumbbell-shaped or equidimensional; vertically oriented or horizontally oriented. CORK CELLS usually present; occurring in silico-suberose pairs. PRICKLES often present (absent in *C. longispiculata* and *C. tenuiglumis*). MICROHAIRS often present; unicellular and silicified or bicellular and non-silicified. MACROHAIRS rarely present (observed in *Chusquea* sp. nov.); unicellular. STOMATAL APPARATUS usually absent (but often observed in *C. tenuiglumis* and rarely in *C. longispiculata*); with subsidiary cells low dome-shaped or low triangular-shaped; two papillae per subsidiary cell, branched or simple.

**ABAXIAL SURFACE:** LONG CELLS tabular with undulate anticlinal walls. PAPILLAE often present on the long cells (except over the veins in *C. longispiculata*); often present on the bulliform cells (except in *C. longispiculata* and *C. tenuiglumis*). SHORT CELLS mainly over the veins: SILICA BODIES saddle-shaped, dumbbell-shaped or equidimensional; vertically oriented or horizontally oriented. CORK CELLS usually present; occurring in silico-suberose pairs. PRICKLES often present (absent in *C. longispiculata* and *C. tenuiglumis*). MICROHAIRS often present; unicellular and silicified or bicellular and non-silicified. MACROHAIRS usually observed in *C. attenuata*, *C. tenella*, and *Chusquea* sp. nov.; unicellular. STOMATAL APPARATUS present; with subsidiary cells low dome-shaped or low triangular-shaped; two papillae per subsidiary cell, branched or simple.

**CROSS SECTION:** BULLIFORM CELLS adaxially present; organized in fan-shaped arrays. MESOPHYLL with one or two layers of asymmetrically invaginated arm cells beneath the epidermis; adaxial arm cells with invaginations from both sides or just from the abaxial side; abaxial arm cells mostly with invaginations from the adaxial side. FUSOID CELLS always present. VASCULAR BUNDLES collateral; first and third orders. MIDRIB complex

(comprising more than one vascular bundle). GIRDERS always present adjacent to the vascular bundles. MARGINS mainly acute; sometimes acute and obtuse (dimorphic).

*Description of each species including only differences from the general condition*

**1. *Chusquea attenuata*** PAPILLAE well developed on both surfaces. SILICA BODIES mainly saddle-shaped; vertically oriented. PRICKLES on both surfaces. MICROHAIRS on the abaxial surface; mostly bicellular and non-silicified; less frequently unicellular and silicified. MACROHAIRS sometimes observed on the abaxial surface; unicellular. STOMATAL APPARATUS with subsidiary cells low dome-shaped; two simple papillae per subsidiary cell. MESOPHYLL comprising two adaxial layers of arm cells; arm cells with invaginations from both sides. MIDRIB comprising three vascular bundles; two smaller ones opposite to the major one, but all three sharing the same bundle sheath. MARGINS acute and obtuse (dimorphic).

**2. *Chusquea longispiculata*** PAPILLAE usually well developed on the abaxial surface, but commonly absent over the veins. SILICA BODIES mainly saddle-shaped; vertically oriented. PRICKLES absent. MICROHAIRS on both surfaces; mostly bicellular and non-silicified; less frequently unicellular and silicified. MACROHAIRS absent. STOMATAL APPARATUS with subsidiary cells low dome-shaped; two simple papillae per subsidiary cell; usually covered by papillae from adjacent intercostal long cells. MESOPHYLL comprising one or two adaxial layers of arm cells; arm cells with invaginations from both sides. MIDRIB comprising two opposing vascular bundles sharing the same bundle sheath. MARGINS acute.

**3. *Chusquea ramosissima*** PAPILLAE usually well developed on both surfaces, but sometimes poorly developed or absent. SILICA BODIES dumbbell-shaped; horizontally oriented. PRICKLES absent. MICROHAIRS on both surfaces; bicellular and non-silicified. MACROHAIRS rarely observed on the abaxial surface. STOMATAL APPARATUS with low triangular-shaped subsidiary cells; two branched papillae per subsidiary cell. MESOPHYLL

comprising two adaxial layers of arm cells; arm cells with invaginations from both sides. MIDRIB comprising two opposing vascular bundles sharing the same bundle sheath. MARGINS acute and obtuse (dimorphic).

**4. *Chusquea tenella*** PAPILLAE well developed on both surfaces. SILICA BODIES saddle-shaped and dumbbell-shaped; horizontally oriented. PRICKLES on both surfaces. MICROHAIRS on the abaxial surface; mostly bicellular and non-silicified; less frequently unicellular and silicified. MACROHAIRS sometimes observed on the abaxial surface; unicellular. STOMATAL APPARATUS with subsidiary cells low triangular-shaped; two branched papillae per subsidiary cell. MESOPHYLL comprising two adaxial layers of arm cells; arm cells with invaginations from the abaxial side. MIDRIB comprising three vascular bundles; two smaller ones opposing the major one, but all three sharing the same bundle sheath. MARGINS acute.

**5. *Chusquea tenuiglumis*** PAPILLAE absent from the long cells. SILICA BODIES saddle-shaped and dumbbell-shaped; horizontally oriented. PRICKLES absent. MICROHAIRS rarely observed on the abaxial surface; bicellular and non-silicified. MACROHAIRS absent. STOMATAL APPARATUS with subsidiary cells low triangular-shaped; two branched papillae per subsidiary cell. MESOPHYLL comprising two adaxial layers of arm cells; arm cells with invaginations from the abaxial side. MIDRIB comprising two opposing vascular bundles sharing the same bundle sheath. MARGINS acute.

**6. *Chusquea* sp. nov.** PAPILLAE well developed on both surfaces. SILICA BODIES mostly dumbbell-shaped, and also equidimensional-shaped; horizontally oriented. PRICKLES on both surfaces. MICROHAIRS on both surfaces; bicellular and non-silicified. MACROHAIRS on both surfaces; unicellular. STOMATAL APPARATUS with subsidiary cells low dome-shaped; two simple papillae per subsidiary cell. MESOPHYLL comprising two adaxial layers of arm cells; arm cells with invaginations from the abaxial side. MIDRIB comprising two opposing vascular bundles sharing the same bundle sheath. MARGINS acute.



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# TABLES

**Table 1** List of *Chusquea* species, vouchers and their use in this study based on Fisher et al. (2009). LM

= light microscopy; SEM = scanning electron microscopy.

Taxa	Voucher number	Analysis performed	
		SEM	LM
<b><i>Chusquea ramosissima</i> informal group</b>			
<i>Chusquea ramosissima</i>	Anderson 1188		X
<i>C. ramosissima</i>	Hatschbach 40533	X	X
<i>C. ramosissima</i>	Foster 76-35	X	
<i>C. ramosissima</i>	Hatschbach 42746	X	X
<i>C. ramosissima</i>	Clark and Windisch 849		X
<i>C. ramosissima</i>	Soderstrom and Sucre 1986	X	
<i>C. ramosissima</i>	Klein 8307	X	
<i>C. ramosissima</i>	Klein and Klein 11041	X	
<i>Chusquea tenella</i>	Klein and Bresolin 9477		X
<i>C. tenella</i>	Klein and Bresolin 7766	X	X
<i>C. tenella</i>	Clark and Windisch 725		X
<i>C. tenella</i>	Swallen 9004	X	
<i>C. tenella</i>	Palacios-Cuezzo 474	X	
<i>C. tenella</i> var. <i>latifolia</i>	Rambo 47076	X	
<i>Chusquea tenuiglumis</i>	Clark and Morel 706	X	X
<i>C. tenuiglumis</i>	Clark and Windisch 864	X	X
<i>C. tenuiglumis</i>	Soderstrom and Sucre 1918		X
<i>Chusquea</i> sp. nov.	Fuentes et al. 9662	X	X
<i>Chusquea</i> sp. nov.	Layola et al. 6	X	X
<i>Chusquea</i> sp. nov.	Uzquiano et al. 10	X	X
<b><i>Chusquea meyeriana</i> informal group</b>			
<i>Chusquea attenuata</i>	Campos et al. sn (CFSC 13.176)		X
<i>C. attenuata</i>	Clark and Morel 765		X
<i>C. attenuata</i>	Clark and Morel 714	X	X
<i>C. attenuata</i>	Irwin et al. 29242	X	
<i>C. attenuata</i>	Kuhlmann 2740	X	
<i>Chusquea longispiculata</i>	Kuhlmann 3141	X	X
<i>C. longispiculata</i>	Clark and Windisch 645	X	X
<i>C. longispiculata</i>	Clark and Windisch 727	X	X

**Table 2** Summary of the main foliar micromorphological and anatomical features of the species herein studied. **1.** *Chusquea ramosissima*; **2.** *C. tenella*; **3.** *C. tenuiglumis*; **4.** *Chusquea* sp. nov. **5.** *C. longispiculata*; **6.** *C. attenuata*. The classification used in this table was based on Fisher et al. (2009).

+ Presence; - Absence

TAXA	FEATURES	Subg. <i>Chusquea</i>					
		<i>C. ramosissima</i> group				<i>C. meyeriana</i> group	
		1	2	3	4	5	6
<b>Surface view</b>							
<i>Epidermis</i>							
	Silica bodies horizontally oriented	+	+	+	+	-	-
	Silica bodies vertically oriented	-	-	-	-	+	+
	Subsidiary cells bearing two branched papillae	+	-	+	-	-	+
	Subsidiary cells bearing two simple papillae	-	+	-	+	+	-
	Subsidiary cells low dome-shaped	-	-	-	+	+	+
	Subsidiary cells low triangular-shaped	+	+	+	-	-	-
<b>Cross section</b>							
<i>Mesophyll</i>							
	Arm cells with invaginations from the abaxial side	-	+	+	+	-	-
	Arm cells with invaginations from both sides	+	-	-	-	+	+
<i>Midrib</i>							
	Composed of two vascular bundles	+	-	+	+	+	-
	Composed of three vascular bundles	-	+	-	-	-	+
<i>Margin</i>							
	Acute	+	+	+	+	+	+
	Obtuse	+	-	-	-	-	+



# FIGURES

Fig 1. Summary cladogram of the subtribes of Neotropical woody bamboos, the genus *Chusquea* showing its infrageneric classification, and the *Chusquea ramosissima* Clade (II) (inset). Cm *Chusquea meyeriana* informal group, Cr *Chusquea ramosissima* informal group. Redrawn from Fisher et al. (2014).

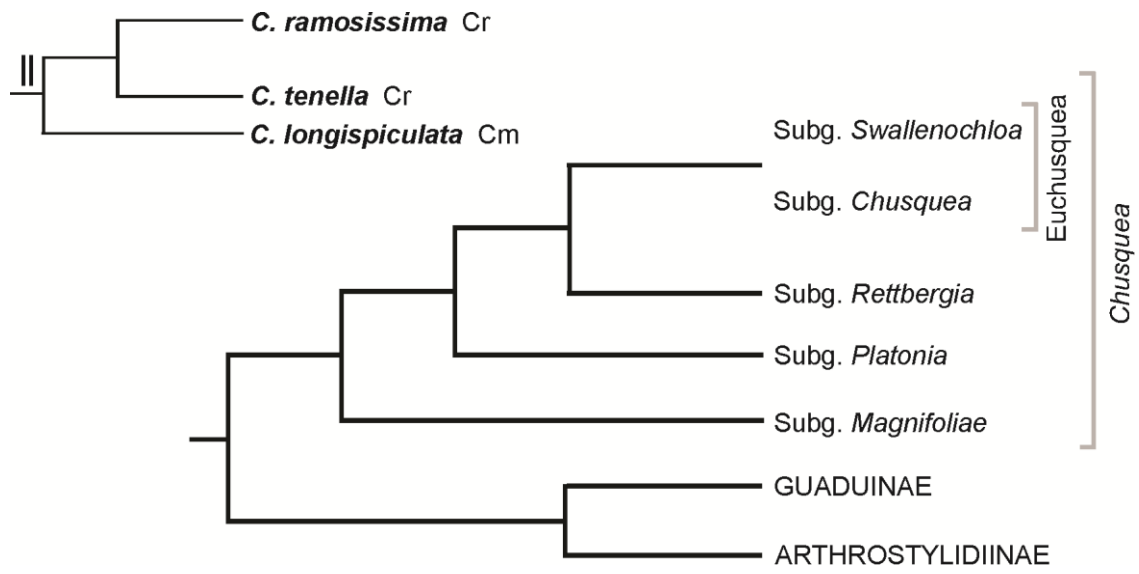


FIG. 1

Fig 2. Micromorphology from SEM of *Chusquea* species. Adaxial surface (**a, d, g**), abaxial surface (**b-c, e-f, h-i**). **a.** *C. attenuata* (Clark and Morel 714); **b.** *C. attenuata* (Irwin et al. 29242); **c.** *C. attenuata* (Kuhlmann 2740); **d.** *C. longispiculata* (Clark and Windisch 645); **e.** *C. longispiculata* (Clark and Windisch 645); **f.** *C. longispiculata* (Clark and Windisch 645); **g.** *C. ramosissima* (Hatschbach 40533); **h.** *C. ramosissima* (Hatschbach 42746); **i.** *C. ramosissima* (Soderstrom and Sucre 1986). *bc* bulliform cells, *ilc* intercostal long cell, *ma* macrohair, *mi* microhair, *pa* papilla, *pr* prickle, *sb* silica body, *smi* silicified unicellular microhair, *st* stomatal apparatus. Scale bars: 60  $\mu\text{m}$  (**a-b, d-e, g-h**), 13  $\mu\text{m}$  (**c, f, i**).

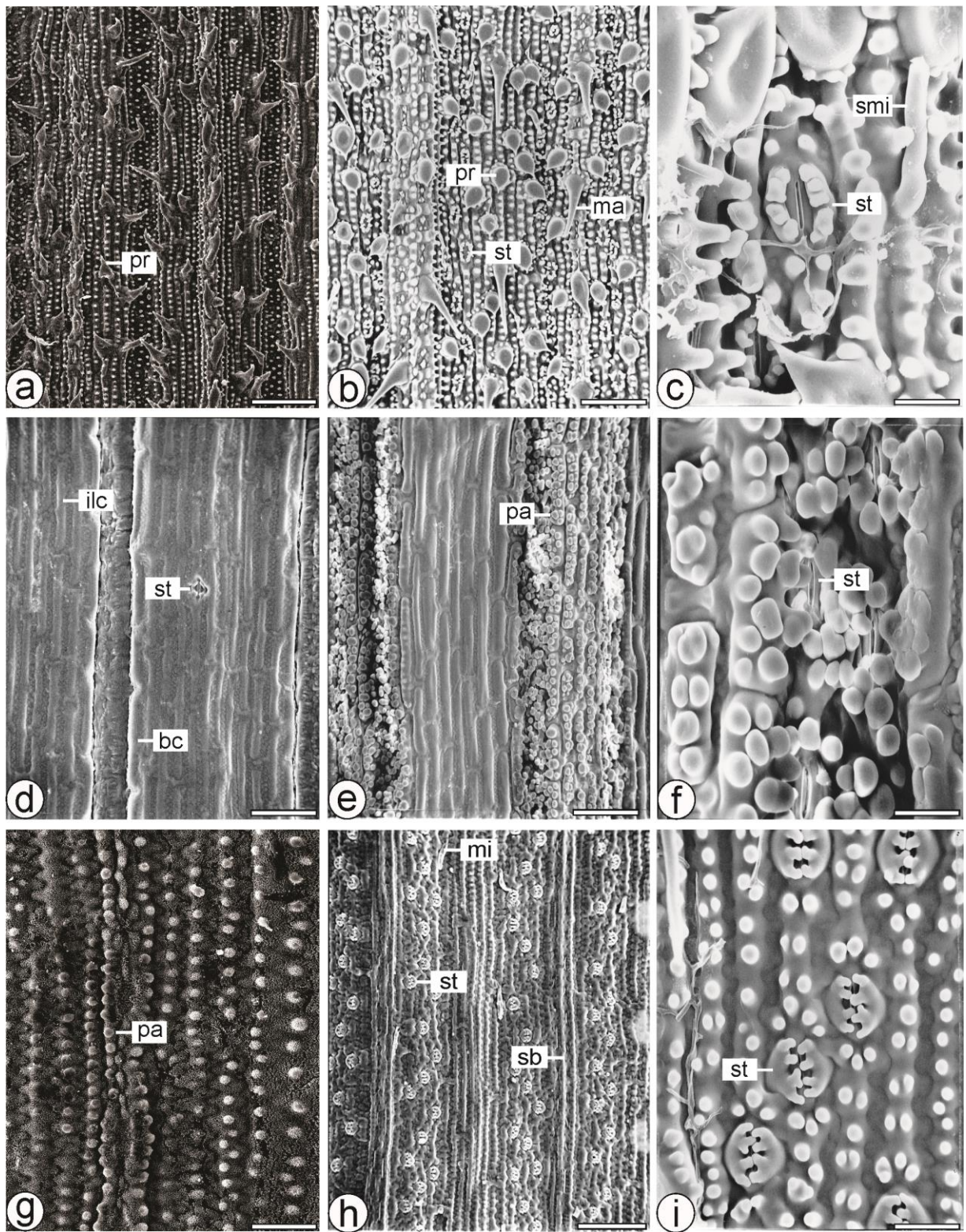


FIG. 2

Fig 3. Micromorphology from SEM of *Chusquea* species in surface view. Adaxial surface (**a**, **d**, **g**), abaxial (**b-c**, **e-f**, **h-i**). **a**. *C. tenella* (Klein and Bresolin 7766, inset Swallen 9004); **b**. *C. tenella* (Swallen 9004); **c**. *C. tenella* (Palacios-Cuezzo 474, inset Swallen 9004); **d**. *C. tenuiglumis* (Clark and Windisch 864). **e**. *C. tenuiglumis* (Clark and Morel 706, inset Hatschbach 42746); **f**. *C. tenuiglumis* (Clark and Morel 706); **g**. *Chusquea* sp. nov. (Fuentes et al. 9662); **h**. *Chusquea* sp. nov. (Layola et al. 6, inset Uzquiano et al. 10); **i**. *Chusquea* sp. nov. (Fuentes et al. 9662). *ilc* intercostal long cell, *ma* macrohair, *mi* microhair, *pr* prickle, *sb* silica body, *smi* silicified unicellular microhair, *st* stomatal apparatus. Scale bars: 60  $\mu\text{m}$  (**a-b**, **d-e**, **g-h**), 13  $\mu\text{m}$  (**c**, **f**, **i**), 6.5  $\mu\text{m}$  (insets).



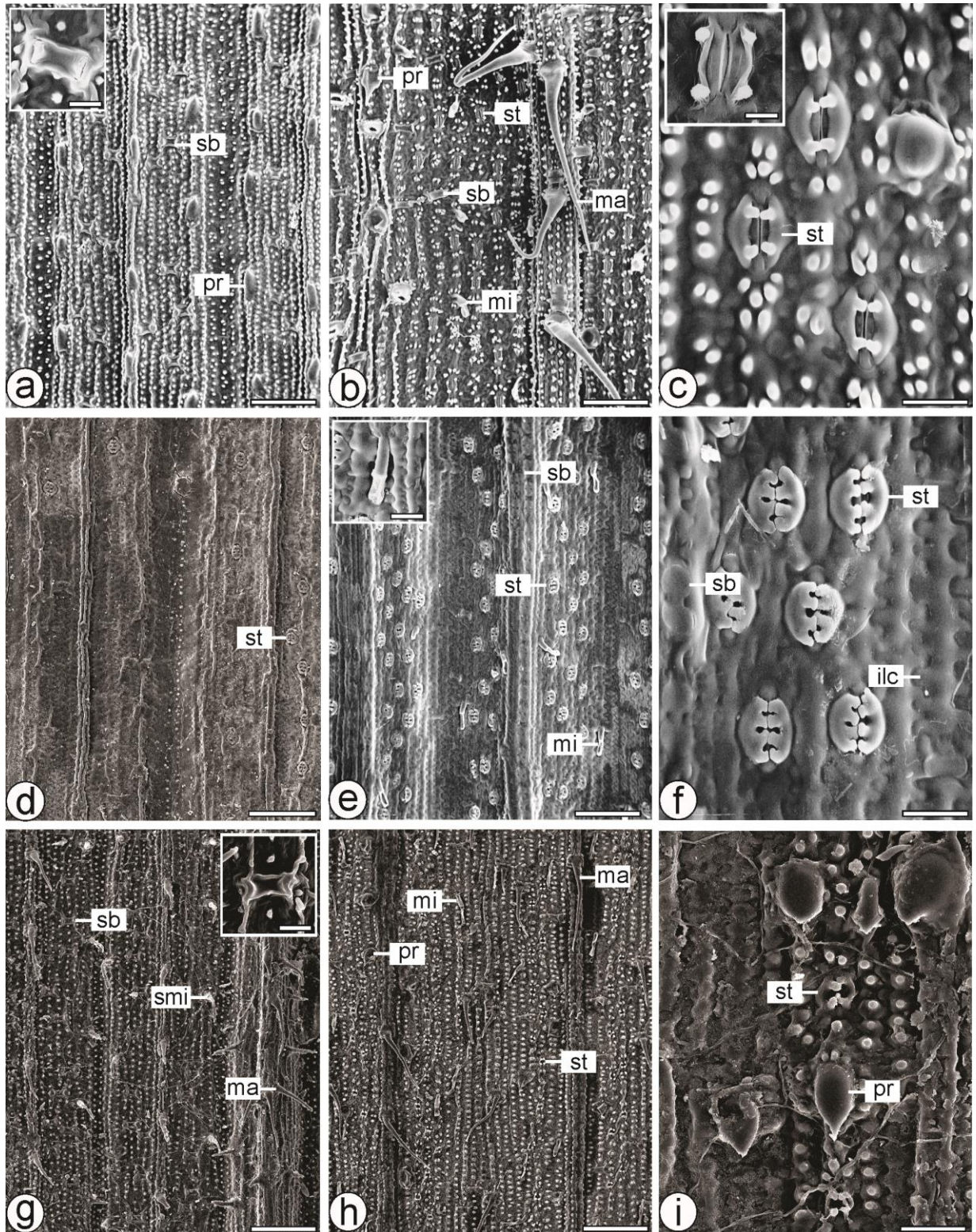


FIG. 3

Fig. 4. Micromorphology and anatomy from light microscopy of *Chusquea* species. Surface view (**a-j**), Cross section (**k-r**). **a.** *C. longispiculata* (Clark and Windisch 727); **b.** *C. ramosissima* (Clark and Windisch 849); **c.** *Chusquea* sp. nov. (Uzquiano et al. 10); **d.** *Chusquea* sp. nov. (Layola et al. 6); **e.** *C. longispiculata* (Clark and Morel 716) showing a silicified unicellular microhair; **f.** *C. attenuata* (Clark and Morel 765); **g.** *Chusquea* sp. nov. (Layola et al. 6); **h.** *C. ramosissima* (Clark and Windisch 849); **i.** *Chusquea* sp. nov. (Layola et al. 6); **j.** *C. ramosissima* (Clark and Windisch 849); **k.** *C. longispiculata* (Kuhlmann 3141); **l.** *C. tenuiglumis* (Clark and Windisch 864); **m.** *C. tenella* (Klein and Bresolin 9477); **n.** *Chusquea* sp. nov. (Uzquiano et al. 10); **o.** *C. tenuiglumis* (Clark and Windisch 864); **p.** *C. tenella* (Clark and Windisch 725); **q.** *C. longispiculata* (Kuhlmann 3141); **r.** *C. tenuiglumis* (Clark and Windisch 864). *ac* arm cell; *bc* bulliform cells, *cc* cork cell, *csc* costal short cell, *ds* dumbbell-shaped silica body, *fc* fusoid cell, *gc* guard cells, *hsb* horizontally oriented silica body, *ilc* intercostal long cell, *ma* macrohair, *mi* microhair, *pa* papilla, *rc* rosette cell, *sb* silica body, *ss* saddle-shaped silica body, *st* stoma, *vsb* vertically oriented silica body. Scale bars: 5  $\mu\text{m}$  (**d-j**), 10  $\mu\text{m}$  (**c**), 15  $\mu\text{m}$  (**q-r**), 20  $\mu\text{m}$  (**a-b**), 30  $\mu\text{m}$  (**k-p**).



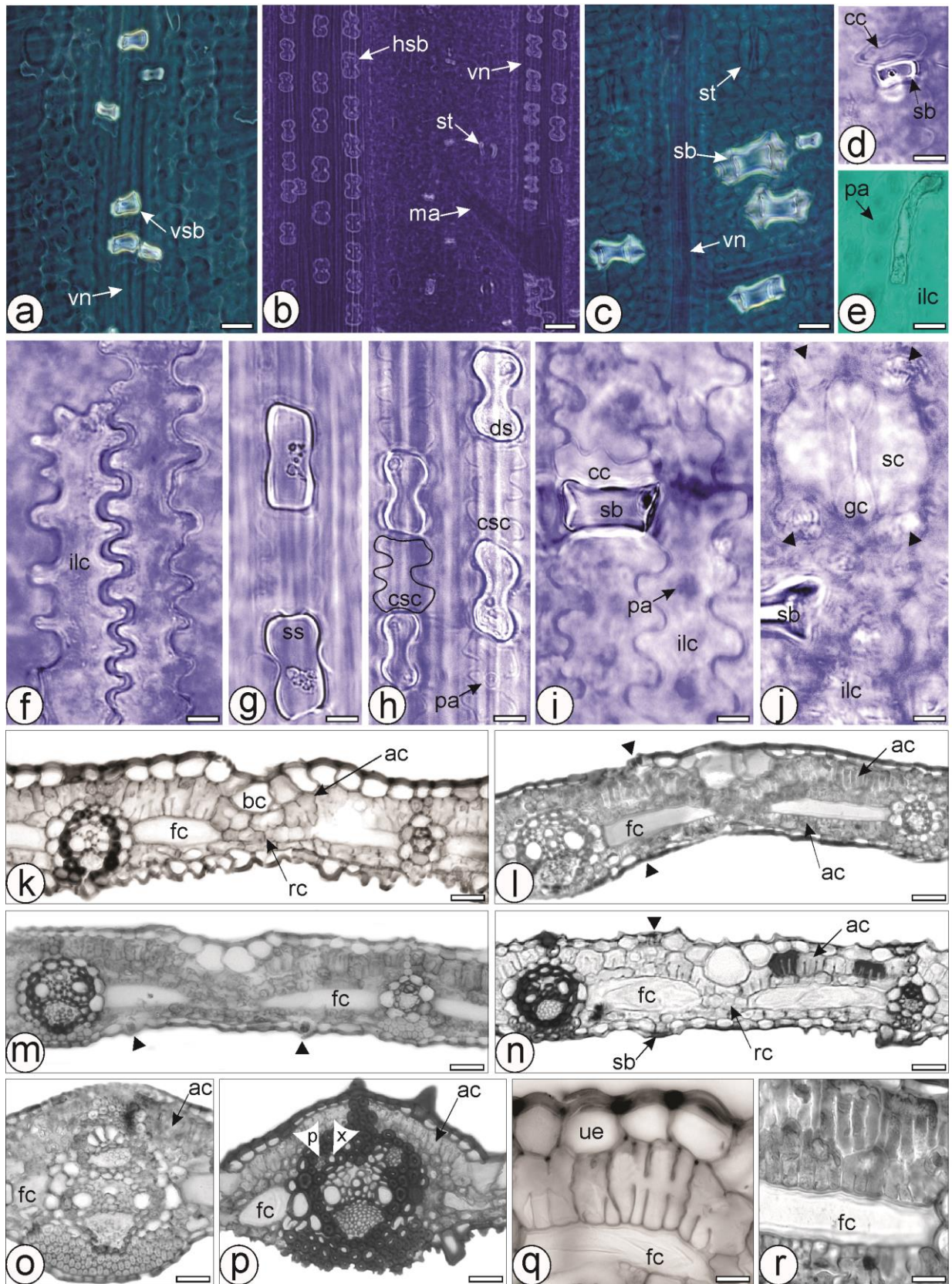


FIG. 4

## CAPÍTULO III

**Fusoid cells in the grass family Poaceae (Poales):  
a developmental study reveals homologies and suggests insights into their  
functional role in young leaves**

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SERÁ ENVIADO PARA O PERIÓDICO ANNALS OF BOTANY

*Background and Aims* In mature grass leaf blades as seen in cross section, oblong cell-like structures have been interpreted most recently as intercellular gas spaces delimited by successive collapsed fusoid cells. These cells have been reported in at least seven of 12 subfamilies of Poaceae and are considered a synapomorphy for the family; however, no developmental work has been performed to verify their meristematic origin or to assess possible homologies within the Graminid clade (= Flagellariaceae + [(Joinvilleaceae + Ecteiocoleaceae) + Poaceae]) or among subfamilies of Poaceae. A developmental study was therefore carried out, including 15 species in three families (Flagellariaceae, Joinvilleaceae, and Poaceae), representing the earlier diverging and derived branches within the Graminid clade and Poaceae.

*Methods* Light microscopy (LM) was combined with transmission electron microscopy (TEM) to study the development of leaves taken from the shoot apex of young plants. Also, mature leaf blades were taken from living or dried plants and the mid-portion studied.

*Key Results* Our developmental results show that, in mature leaf blades, a fusoid cell as seen in cross section is typically a cavity resulting from the collapse of several fusoid cells. Each cavity is delimited by successive collapsed fusoid cells arranged perpendicular to the veins. Fusoid cells in all studied Poaceae members originate from the ground meristem as do the colourless cells in *Joinvillea ascendens* (Joinvilleaceae). Both types of mesophyll cells have a strongly similar ontogeny, distinguished by the collapse of the fusoid cells in Poaceae, which is not observed in the colourless cells in *Joinvillea ascendens*.

*Conclusions* The same topography and meristematic origin suggest that fusoid cells in Poaceae and colourless cells in Joinvilleaceae are homologous and that fusoid cells possibly evolved from colourless cells on the stem of the [(Joinvilleaceae + Ecteiocoleaceae) + Poaceae] Clade. Within the Poaceae, the meristematic origin of fusoid cells is the same in the early-diverging lineages, the BOP clade and the Panicoideae (Poaceae), and thus they are homologous within the family. In Poaceae, the presence of the fusoid cells in early-diverging

lineages, Streptogyneae, Zizaniinae, a few Pooideae and Paspaleae, and generally in the Bambusoideae; and their lack in Ehrharteae, Phyllorachideae, Oryzinae and most Pooideae and Panicoideae clearly suggest multiple losses within the BOP and PACMAD clades.

**Key words:** BOP Clade, early-diverging grass lineages, foliar anatomy, Graminid Clade, grasses, mesophyll cells, ontogeny, Panicoideae.

## INTRODUCTION

Poales is a diverse group that currently comprises 14 families with morphological, anatomical, embryological and molecular similarities (APG IV, 2016; Stevens, 2001 onwards). There are six clades within this order, of which the {Flagellariaceae + [(Joinvilleaceae + Ecteiocoleaceae) + Poaceae]} is recognized as the Graminid clade (Linder and Rudall, 2005); these four families share primarily morphological and embryological features (Stevens, 2001 onwards). The graminid family Poaceae comprises nearly 12,000 species in 12 subfamilies widespread mainly in grasslands and forests all over the world (GPWG II, 2012; Soreng *et al.*, 2015), diversity for which anatomical studies have often provided useful features that aid in species identification and delimitation (e.g., Calderón and Soderstrom, 1973; Zuloaga *et al.*, 1992, 1993; Guglieri *et al.*, 2008; Oliveira *et al.*, 2008; Pelegrin *et al.*, 2009; Aliscioni *et al.*, 2003, 2016; Leandro *et al.* 2016a), and also oftentimes indicate potential synapomorphies for clades (GPWG, 2001; Judziewicz *et al.*, 1999; Clark *et al.*, 2015; Leandro *et al.*, 2016b).

Although knowledge of grass anatomy is constantly growing and there are many contributions in the literature, anatomical work on grasses has concentrated mainly on transverse leaf blade analyses. Strongly asymmetrically invaginated arm cells, intercostal fibers, Kranz anatomy, and bundle spacing are examples of highly systematically informative leaf blade features (e.g., Judziewicz *et al.*, 1999; GPWG 2001, Ueno *et al.* 2006, Viana *et al.*, 2011; GPWG II 2012, Clark *et al.*, 2015; Leandro *et al.* 2016b); however, the systematic value of fusoid cells, an important anatomical feature for Poaceae, is still controversial and conflicting findings regarding their taxonomic distribution and functional role(s) in mature leaves have been published (GPWG 2001; Clark, 1991; Vieira *et al.*, 2002; March and Clark, 2011; Vega *et al.*, 2016; Wang *et al.*, 2016).

The term “fusoid cells” was originally assigned by Metcalfe (1956) who studied the leaf blade anatomy of many bamboo species (see the terminological history in Table 1). Even

today, agrostologists assume that the fusoid cells are typical of Bambusoideae species (BOP clade—formerly the BEP clade), even though fusoid cells are also observed in other subfamilies across the family (GPWG, 2001): (i) the early-diverging lineages (Anomochlooideae, Pharoideae, and Puelioideae) (see Ellis, 1987 as Bambusoideae); (ii) the BOP clade (Bambusoideae and also in a few members of Oryzoideae and Pooideae) (see Tateoka, 1963; Barkworth *et al.*, 2007; Leandro *et al.*, 2016a); (iii) and the PACMAD clade (in a few members of Panicoideae) (see Watson *et al.*, 1985; Clayton and Renvoize, 1986; Killeen and Clark, 1986; Zuloaga *et al.*, 1992). Given their wide occurrence, the presence of fusoid cells has been utilized as an important character in taxonomic descriptions and also in trying to establish phylogenetic relationships within the family. However, previous work has shown that these cells are environmentally influenced and thus their occurrence may be facultative within the same species or even within the same sample or individual (e.g., Metcalfe, 1956; Wu 1962; Pearson *et al.*, 1994; March and Clark, 2011). In bamboos, plants living in sunny habitats often lack these cells, whereas those growing shade always have them. In contrast, Oryzeae (Oryzoideae) comprise aquatic species primarily living in open habitats and also exhibit fusoid cells (Tateoka, 1963; Leandro *et al.*, 2016a).

Fusoid cells are recognized as a prominent feature of early-diverging lineages and Bambusoideae, but the role played by these cells in mature leaves has never been satisfactorily explained. Clark (1991) proposed that fusoid cells act as reservoirs for CO<sub>2</sub> from photorespiration, hypothesis later rejected by March and Clark (2011) based on evidence of less rates of photorespiration in shaded environments. Also, in their work, March and Clark (2011) proposed that cavities formed by the collapse of fusoid cells may be related to intra- or intercellular reflectance, which contribute to the absorption of light in shaded environments. Vega *et al.* (2016) proposed that the I-shaped fusoid cell as seen in longitudinal section may play a structural role, allowing the mature leaf blade architecture. Vieira *et al.* (2002) and Wang *et al.* (2016), in turn, suggested their relation with storage, transportation, and water

balance. Wang *et al.* (2016) also reinforced the role of fusoid cells as CO<sub>2</sub> reservoirs and their relation with photorespiration based on greenhouse experiments under high temperatures.

In mature leaf blades, the fusoid cells are large, mainly cigar-shaped with thin cell walls, and apparently with no content (GPWG, 2001; Kellogg, 2015). This set of features makes these cells visually similar to cavities or air spaces in the mesophyll, consequently generating doubts about whether they are cells or intercellular spaces (Karelstschicoff, 1868; Brandis, 1907). Their cellular nature was confirmed in studies of leaf blade development in *Streptochaeta*, in which the fusoid cells were called “enlarged cells” (Page, 1947) (Table 1). A recent study of the development of fusoid cells in two species of *Guadua* (Bambusoideae) show that these cells differentiate, enlarge, and then collapse to form intercellular gas spaces as seen in cross section (Vega *et al.* 2016). Although these two developmental works address aspects of fusoid cell ontogeny, the origin of the fusoid cells has not been yet completely clarified nor has their occurrence or homology across the grass family.

Since there are no leaf developmental studies addressing the occurrence of fusoid cells and their relationships among families within the Graminid Clade, there is no consensus with regard to many subjects: (i) a retention of the plesiomorphic condition (GPWG, 2001)—considering their occurrence in early-diverging lineages and the presence of colourless cells similar to fusoid cells in the mesophyll of Joinvilleaceae members; (ii) losses within the BOP clade—considering the absence of fusoid cells in many Oryzoideae and in most Pooideae members (GPWG, 2001); (iii) whether their origin is the same across the entire family—they are called “fusoid-like cells” in the Panicoideae (see Killeen and Clark, 1986). Hence, given the importance of fusoid cells for grass systematics and phylogeny, the aims of this work were to: (i) study leaf blade development within Poaceae mainly looking at the development of fusoid cells in order to verify their meristematic origin, putative homologies and evolutionary pattern; (ii) to determine whether the mesophyll cells similar to fusoid cells observed in Joinvilleaceae have the same origin as in the Poaceae. To that end, we studied the

development of 16 species within Poaceae, Joinvilleaceae, and Flagellariaceae (Graminid clade) in order to clarify their relationship with regard to this feature.

## MATERIALS AND METHODS

### *Sampling*

For the developmental work the following taxa were studied: (i) *Joinvillea ascendens* (Joinvilleaceae); (ii) and several Poaceae members in six subfamilies: the early-diverging lineages (Anomochlooideae and Pharoideae), the BOP clade (Oryzoideae, Bambusoideae, and Pooideae), and the PACMAD clade (Panicoideae). Since we are looking at the leaf blade development in Joinvilleaceae in order to compare it with Poaceae, we also included *Flagellaria indica* (Flagellariaceae)—placed within the Graminid clade and sister to [(Joinvilleaceae + Ecteiocoleaceae) + Poaceae] (Stevens, 2001 onwards; Linder and Rudall, 2005). To define homologies properly we also included selected species across the grass family in which fusoid cells are lacking. Living plants were sampled in their natural habitat or from the R. W. Pohl Conservatory at Iowa State University, U.S.A.

We were not able to collect living plants to represent the subfamily Puelioideae (from Tropical Africa), but we included anatomical results from mature dried leaves of *Guaduella oblonga* (Puelioideae) and also from other Poaceae species in order to provide a broad approach.

All species included in this work, sampling method, purpose of study, and vouchers are provided in Table 2.

### *Developmental study (young leaves)*

Light microscopy (LM). Leaves were taken from the shoot apex of young plants, fixed in FAA<sub>50</sub> for 48 hours (Johansen, 1940), and then stored in 70% ethanol. Each sample was



dehydrated through a graded n-butyl series and embedded in glycol methacrylate (*Leica* Histo-resin Embedding Kit) following conventional methods. Cross sections were obtained with a rotary microtome (*Leica* RM 2145 or *Spencer* 820) and stained with Periodic Acid Schiff (PAS) plus Toluidine Blue (Feder and O'Brien, 1968). Permanent slides were mounted with Entellan (Merck, Germany) or Permount (Fisher, U.S.A).

Transmission electron microscopy (TEM). Small leaf blade pieces (1 mm diameter) from the shoot apex of young plants were fixed with 2% paraformaldehyde / 2% glutaraldehyde in a 0.1 M cacodylate buffer at 4°C for 48 hours (Horner, 2012). The pieces were buffer washed three times for a total of 1 hour, dehydrated through an ethanol series, and embedded in Spurr's resin (Spurr Low-Viscosity Embedding Kit). Ultra-thin sections were cut with diamond knife, then placed on copper grids and post-stained with lead and uranium.

#### *Anatomical study (mature leaf blades)*

Mature leaf blades were taken from living or dried plants and sampled from the middle portion of the blade. Each sample was fixed in FAA<sub>50</sub> for 48 hours and stored in 70% ethanol. Due to the amount of sclerified and silicified tissues, sections were made by using two distinct methods: (i) leaf blade pieces (0.5 cm<sup>2</sup>) were embedded in polyethylene glycol 1.500 solution and kept in an incubator at 60°C for fifteen days, dried, and then sectioned (adapted from Richter, 1985); (ii) samples were treated with 30% hydrofluoric acid for 72 hours, dehydrated through an graded n-ethanol series, embedded in paraffin, and then sectioned. For both methods, sections were made using rotary microtome *Leica* RM 2145 or *Spencer* 820.

Also, pieces of approximately 0.5 cm<sup>2</sup> from the middle portion of the leaf blade of *Chusquea attenuata* were removed for analyzing the fusoid cells through the depth of the leaf blade. These pieces were hydrated through a graded series of ethyl alcohol (50, 25 and 10%) and then soaked in 1:1/dH<sub>2</sub>O until the samples were translucent. Samples were rinsed with

dH<sub>2</sub>O, dehydrated through a graded series of ethyl alcohol (25, 50, and 70%), and then stained with Safranin and Fast Green (Johansen 1940). Stained samples were treated with ethyl alcohol (95 and 100%), xylene, and then xylene and Permount (1:1). Permanent slides were mounted with Permount.

#### *Description, observation, images, and tridimensional reconstruction*

Descriptions and illustrations were mainly concentrated on the development of fusoid cells and the terminology primarily followed Ellis (1976; 1979). In order to better present the results, the progression of leaf development was divided in four stages. For LM analyses, photomicrographs were obtained with Zeiss AXIO Observer microscope through the ZEN 2.0 blue software or with *Leica* DM4000B microscope through the *Leica* Application Suite LASV4.0. TEM images were captured with a JEOL 1200 or FEI Tecnai™ Spirit transmission electron microscope.

In order to build a paradermal scan with tridimensional reconstruction showing the fusoid cells through the depth of the leaf blade, each 5- $\mu$ m thick section of a leaf blade clearing of *Chusquea attenuata* was photographed along the Z-axis to create a stack of eight to ten 0.5- $\mu$ m thick optical sections. Z-stacks from each section were aligned and modeled also using ZEN 2.0 blue.

The analyses herein described were performed in the Departamento de Botânica at UNESP-Rio Claro, Brazil, in the Centro de Microscopia Eletrônica (CME) at UNESP-Botucatu, Brazil, and in the Microscopy and Nano-Imaging Facility (MNIF) at Iowa State University, U.S.A.

## RESULTS

The following descriptions include four stages of leaf development with emphasis on the fusoid cells (Figs. 1A-F, 2A-J, 3A-I, 4A-X). Images for the development of leaves with no

fusoid cells are provided as Supplementary Data Fig. S1A-O, as well as the video showing a tridimensional reconstruction of mature fusoid cells (Supplementary Data – Video).

*Stage 1 - Meristems and initial differentiation of cells into dermal and vascular tissues (LM)*

The initial stage of leaf blade development is the same in all studied species and occurs from the base toward the apex (acropetally) and from the center toward the margins (centrifugally) (Fig. 1A-F, Supplementary Data Fig. S1A-D). The differentiation of tissues may occur simultaneously, but often the protoderm is the first meristem to become differentiated, followed by procambium and ground meristem (Fig. 1A-F, Supplementary Data Fig. S1A-D).

Protodermal cells start anticlinal divisions to form a single stratum of epidermal cells (Fig. 1A and B). Simultaneously, procambial cells start anticlinal, periclinal, and tangential divisions to give rise to vascular elements (Fig. 1A-C). Considering the centrifugal differentiation of tissues, the first major procambial strand will form the midvein (Fig. 1A), and then additional veins are formed by adjacent and also major procambial strands (Fig 1B-F). Minor procambial strands are formed between pre-existing major ones as a result of leaf blade enlargement. Mesophyll precursor cells from the ground meristem are mainly isodiametric and still undifferentiated at this point in time (Fig. 1A-F, Supplementary Data Fig. S1A and B)—although some ground meristem cells start their differentiation into mesophyll cells, in which cells below the epidermis and adjacent to vascular bundles start to divide mainly in the anticlinal and periclinal planes (Fig. 1D and E, Supplementary Data Fig. S1B).

*Stage 2 - Ongoing differentiation of dermal and vascular tissues, and initial differentiation of ground meristem cells (LM)*

At this stage, the epidermis is completely differentiated (except the bulliform cells)

and the development of some trichomes may be observed (Fig. 2B). The midvein and lateral vascular bundles are quite distinguishable, although the maturation of the conducting elements is still in progress (Fig. 2A-D).

According to their position in the mesophyll, cells from the ground meristem will give rise to bundle sheath extensions, sclerenchyma, and parenchyma cells. Girders are formed above and below the vascular bundles, whereas sclerenchyma is mainly formed at the margins, but also appears as intercostal fibers adjacent to the bulliform cells in *Aulonemia aristulata* (Poaceae) (Fig. 4G) and strands adjacent to the adaxial or abaxial epidermis in between fusoid cells (e.g., Fig. 4F-G). Parenchymatous cells are formed primarily between vascular bundles and can be of six types according to the anatomy path of each species: (i) peg cells, (ii) colourless cells, (iii) arm cells, (iv) rosette cells, (v) fusoid cells, and (vi) irregular-shaped cells.

Parenchymatous cells differentiate mainly into peg cells in *Flagellaria indica* (Flagellariaceae) (Supplementary Data Fig. S1I); irregular-shaped cells in Panicoideae members (Poaceae) (e.g., Supplementary Data Fig. S1E); rosette cells in Oryzoideae (Poaceae) (Fig. 4D and E); and into arm cells in Bambusoideae (Poaceae) (Fig. 4F-H). These cell types visually exhibit contents and will form the chlorenchyma tissue (Fig. 2A-J). Irregular-shaped cells become arranged in a variable number of layers parallel to the epidermis, but in *Ruguloa pilosa* (Fig. 2A) and *Setaria scabrifolia* (Supplementary Data Fig. S1D and E) (both Panicoideae, Poaceae) they become radiate and surround the vascular bundles. In Bambusoideae (Poaceae), arm cells become arranged into two layers of cells below the upper epidermis and in just one layer above the lower epidermis (Fig. 2A), whereas in Oryzoideae (Poaceae) the number of layers of rosette cells is usually variable (not shown).

During the development of the chlorenchyma cells, the cell adjacent to the vascular bundle sheath that occupies the central portion of the mesophyll starts to become distinguishable from the other parenchymatous cells and will form a colourless cell in

*Joinvillea ascendens* (Supplementary Data Fig. S1J-O) and a fusoid cell in the members of Poaceae that produce them (Figs. 1F, 2A and B). This occurs in the same mesophyll region that differentiates the peg cells in *Flagellaria indica* (Supplementary Data Fig. S1I) and irregular-shaped cells in Poaceae members with no development of fusoid cells (Supplementary Data Fig. S1E-H).

*Stage 3 - Final differentiation of tissues and the ongoing development of fusoid cells (LM and TEM)*

In *Joinvillea ascendens*, a single colourless cell divides to form three to four daughter cells; it is possible to observe the complete formation of their cells walls with LM (Supplementary Data Fig. S1J-M). They then gradually increase in size along with the differentiation of the leaf blade (Supplementary Data Fig. S1M-N). Fusoid cell differentiation in members of Poaceae occurs in much the same way, however, with LM we often observe the increase in size of the single fusoid cell (Fig. 2A, B inset, E, G-H), with some content but no apparent cell divisions [note that to better illustrate this, we included many images clearly showing cell divisions with LM—e.g., Fig. 2A, C-J]. With TEM, however, we are able to easily observe division of the mother fusoid cell to form daughter cells (as in *Joinvillea ascendens*—not shown), the vacuoles increasing in size and high nuclear activity with initial formation of cell walls (Fig. 3A-F). A number of amyloplasts with starch grains are also often observed (Fig. 3G-I).

At this point in time, peg cells, irregular-shaped cells, and arm cells are almost all completely differentiated and the rosette cells (abaxially positioned) in members of Bambusoideae (Poaceae) become topographically distinct from the arm cells in differentiation (Fig. 2B). Except for the fusoid cells, mesophyll differentiation often occurs simultaneously, but there are temporal differences in reaching complete maturity depending on the developmental characteristics of each species. Also, along with the leaf enlargement, there is

degradation of the middle lamella between fusoid and chlorenchyma cells and the fusoid cells are stretched and flattened (Fig. 2E).

#### *Stage 4 - Final development of fusoid cells and mature leaves (LM)*

Figure 4A-X shows many images of mature leaf blades of Poaceae members with fusoid cells, organized according to the most recent phylogenetic classification by Soreng *et al.* (2015).

Peg cells in *Flagellaria indica* (Supplementary Data Fig. S1I), irregular-shaped cells in Pooideae and Panicoideae (Supplementary Data Fig. S1E-G), rosette cells in Oryzoideae and Bambusoideae (Fig. 4D-G), arm cells in Bambusoideae (Fig. 4F-G), and the colourless cells between vascular bundles (usually 3-4) in *Joinvillea ascendens* are completely differentiated (Supplementary Data Fig. S1N). Yet, along with the breakdown of the middle lamella during leaf enlargement (Fig 2F and J), fusoid cells gradually collapse perpendicular to the long axis of the leaf blade, leaving an intracellular space between adjacent successive fusoid cells (Fig. 4K-R). In mature leaf blades, the fusoid cell in cross section is a usually cigar-shaped cavity (Fig. 4A-J), and due to the collapse, an I-shaped cell in longitudinal view (Fig. 4K-R). However, longitudinal and paradermal sections reveal that not all fusoid cells collapse (Fig. 4M-N, Q, R, W), since their cell walls still have a contact zone with the bundle sheath and other parenchymatous mesophyll cells (Fig 4K-X, Supplementary Data – Video).

## DISCUSSION

### *Fusoid cell origin and development*

Mesophyll with large colourless cells has been reported in Joinvilleaceae (Bayer and Appel, 1998), a family which currently comprises only two known species (*Joinvillea ascendens* and *J. elegans*). In this family, colourless cells curiously occur in the same mesophyll region as fusoid cells in the Poaceae, adjacent to and between vascular bundles.

Also, we herein observed that the origin of a fusoid cell is from the ground meristem and thus is the same as a colourless cell in *Joinvillea ascendens*—even though there is a developmental variation worth taking into account. In *Joinvillea ascendens*, a single colourless cell divides to form daughter cells and, along with the leaf maturation, mother and daughter cells do not collapse and thus remain intact until their complete development; whereas in Poaceae, the development of a fusoid cell occurs mostly in the same way as a colourless cell, but mother and daughter cells during their development often collapse perpendicularly to the long axis of the leaf blade and thus leave a wide cavity in the mesophyll. Hence, the development of colourless and fusoid cells is quite similar and, considering their same topography and ground meristem origin, they are clearly homologous—as well as being homologous to the mesophyll cells adjacent to the vascular bundles in those species with no fusoid cells.

It is noteworthy that our results show that the origin of a fusoid cell is always from the ground meristem and occurs at least seven times across Poaceae: early-diverging lineages (Anomochlooideae, Pharoideae, and Puelioideae), within the BOP clade (Oryzoideae, Bambusoideae, and Pooideae), and in the Panicoideae (PACMAD clade). Within Poaceae, the meristematic origin of the fusoid cells in Paniceae (Panicoideae) has been speculated as being distinct of those from early-diverging lineages and BOP clade (GPWG, 2001 as BEP clade). According to this work, these cells appear to be derived from laterally extended bundle sheaths and thus were referred to as fusoid-like cells. The homologies herein observed demonstrate that there is no reason to attribute distinctive terminology for these cells in Panicoideae.

According to Sugimoto *et al.* (2011), transdifferentiation encompasses the process by which cells transform into another cell type outside of their already established differentiation paths. In contrast, a putative fusoid cell is clearly distinguishable from any other mesophyll cell even at earlier stages and throughout development until its complete differentiation. The most recent work on development of fusoid cells in *Guadua chacoensis* and *Guadua trini*

reported evidence of transdifferentiation of chlorenchyma cells into fusoid cells (Vega *et al.*, 2016), however, our the developmental work herein reported shows no evidence of transdifferentiation for the 13 species we sampled from the grass family.

Vega *et al.*, (2016) also described a conspicuous cell wall invagination during the differentiation into fusoid cells, which likewise was not herein observed. Since the cell wall invagination reported in that work are also evidenced in adjacent bundle sheath cells (see Fig. 1. B-C in Vega *et al.*, 2016), which do not have any lobes in their mature age, it is very likely that the formed invaginations are a technical artifact caused by fixative methods. We had similar results in materials pre-fixed for less than 48 hours.

What appears to be a mature fusoid cell in cross section is herein interpreted as a cavity delimited by successive collapsed fusoid cells arranged perpendicularly to the veins, with the caveat that sometimes fusoid cells do not collapse. A similar interpretation was given by Vega *et al.*, (2016) referring to these apparent “cells” as intercellular gas spaces delimited by successive collapsed fusoid cells. We consider “cavity” to be the appropriate term because many Poaceae species have intercellular gas spaces in the mesophyll (not necessarily only as seen in cross section and not in this configuration), and thus it distinguishes more clearly between the types of intercellular spaces in order to avoid possible misinterpretation.

A single cavity seen in cross section of a mature leaf blade is the result of the development and collapse of several fusoid cells (mother and daughter cells). During leaf development, we are not often able to perceive fusoid cell division with LM, and thus the successive I-beam shaped collapsed fusoid cells delimiting the cavities have always been interpreted as each resulting from the collapse of a single fusoid cell. Light microscopy techniques were able to clearly show fusoid cell divisions just in *Streptochaeta spicata* (Anomochlooideae) and *Pharus latifolius* (Pharoideae), early-diverging lineages that exhibited large fusoid cells. The gradual collapse of these cells observed in all studied species strongly suggests that they exhibit programmed cell death (PCD), as previously reported for



two *Guadua* species (Bambusoideae) (Vega *et al.*, 2016). PCD will be discussed in detail in a subsequent section.

#### *Phylogenetic implications of fusoid cells for the Graminid clade*

The homology herein observed between the colourless cells in Joinvilleaceae and the fusoid cells in Poaceae is helpful in understanding the relationships within the Graminid Clade. Considering that Joinvilleaceae diverged from the other graminid lineages at 76.7 Ma (Late Cretaceous), followed by Poaceae at ~56 Ma (Palaeocene), and Ecdeiocoleaceae at ~36.7 Ma (Late Eocene) (Bouchenak-Khelladi *et al.*, 2014), it is plausible that the fusoid cells in Poaceae evolved from colourless cells of Joinvilleaceae along the stem of the [(Joinvilleaceae + Ecdeiocoleaceae) + Poaceae] clade. Within Flagellariaceae there is no evidence of differentiated colourless or fusoid cells in the mesophyll, consistent with its position as sister to the [(Joinvilleaceae + Ecdeiocoleaceae) + Poaceae] clade. And of course, Ecdeiocoleaceae do not produce developed leaves. Further anatomical, developmental, and molecular studies are needed to better document the origin of the fusoid cells in the Graminid clade.

The first attempt to produce a phylogenetic hypothesis of relationships within Poaceae was based exclusively on morphological data, in which Bambusoideae (including herbaceous tribes such as Anomochloaeae, Phareae, Streptochaetae) was interpreted as monophyletic based on the presence of arm cells and fusoid cells (Kellogg and Campbell, 1987). Clark *et al.* (1995), based on a family-wide analysis of plastid *ndhF* sequence data, resolved the three tribes mentioned above as early-diverging lineages within the family with a more narrowly circumscribed Bambusoideae placed in the BOP clade. They therefore concluded that the presence of fusoid cells, a character previously useful for delimiting Bambusoideae, was probably a synapomorphy for Poaceae. A broader phylogenetic analysis of the Poaceae, incorporating both molecular sequence and morphological data, was elaborated by the Grass

Phylogeny Working Group (GPWG, 2001) and clearly showed the fusoid cells as plesiomorphic within the family—but also typically found in Bambusoideae species and *Streptogyna*, then of uncertain placement in the BOP clade. There is no doubt with regard to the plesiomorphic condition of fusoid cells within Poaceae, however, the evolution of this character within the family is still unclear.

As previously mentioned, fusoid cells occur in early-diverging lineages (Anomochlooideae, Pharoideae, Puelioideae), some BOP clade members (Bambusoideae, Oryzoideae, and Pooideae), and in a few Panicoideae species. In the BOP clade, Oryzoideae is sister to Bambusoideae + Pooideae; and within Oryzoideae, the tribe Streptogyneae is sister to the remainder of the subfamily: Ehrharteae, Oryzeae (including the subtribes Oryzinae and Zizaniinae), and Phyllorachideae (Soreng *et al.*, 2015). Within Oryzoideae, fusoid cells are known only in the early-diverging *Streptogyna* (Streptogyneae) (Soderstrom *et al.*, 1987) and in some species within the tribe Oryzeae subtribe Zizaniinae (Tateoka, 1963; Kellogg, 2015; Soreng *et al.*, 2015; Leandro *et al.*, 2016a); within Pooideae, fusoid cells are reported only in *Brachyelytrum* (Barkworth *et al.*, 2007) [herein not observed in *Brachyelytrum erectum*]. Hence, the presence of the fusoid cells in early-diverging lineages, Streptogyneae, Zizaniinae, a few Pooideae and generally in the Bambusoideae, and their lack in Ehrharteae, Phyllorachideae, Oryzinae and most Pooideae clearly suggest multiple losses within the BOP clade (Kellogg, 2015).

The same developmental origin of the fusoid cells herein confirmed between Panicoideae species and other Poaceae members is also relevant to phylogenetic inferences within the family. Fusoid cells have been reported in a few species of *Rugoloa*, *Dallwatsonia* (Killeen and Clark, 1986; Zuloaga *et al.*, 2012—both as *Panicum*), *Homolepis* (Watson and Dallwitz, 1992 onwards), and *Canastra* (Morrone *et al.*, 2001). These genera are all classified within the tribe Paspaleae, which along with Paniceae and other tribes with no fusoid cells reported, comprise the subfamily Panicoideae (Soreng *et al.*, 2015). Thus, fusoid cells were

probably lost in the early evolution of the PACMAD clade but regained in the Paspaleae. A broader sampling within Panicoideae as well as a formal character optimization of the presence of fusoid cells using the most recent molecular phylogeny of the family are needed to verify the evolution of this character within Poaceae.

*Some evolutionary and functional insights on fusoid cells based on TEM data*

As previously mentioned, there are many possible functional roles for fusoid cells in mature leaves, in summary: structural (Vega *et al.* 2016), light-scattering (March and Clark 2011), photorespiration-related (Clark 2001; Wang *et al.* 2016), and water dynamics (Vieira *et al.* 2002; Wang *et al.* 2016). It is noteworthy that these functions may not necessarily be mutually exclusive and thus may occur simultaneously in mature leaves. Hence, in this section, we made a correlation of these findings with environmental characteristics and our developmental results from young leaf blades.

Poales has evolved mainly in open habitats and the first transitions to shade habitats have been dated to the Late Cretaceous for the early-diverging graminid lineages (Bouchenak-Khelladi *et al.*, 2014). The early-diverging lineages of Poaceae comprise plants growing in shaded forests and thus it is plausible that these lineages also evolved in the Late Cretaceous, since the origin and diversification of the BOP + PACMAD Clades dated to the Palaeocene and occurred mainly in dry and sunny environments (Bouchenak-Khelladi *et al.*, 2014). The exceptions are Bambusoideae, *Brachyelytrum* (Pooideae), Streptogyneae, Phyllorachideae and some Panicoideae, which are associated with wet and shaded environments. The presence of fusoid cells in most of these taxa suggests the correlation of fusoid cells with shaded environments; in contrast, the presence of these cells in other Oryzoideae, which comprise plants growing in open and often wet environments, may suggest the influence of other environmental factors. This background should be the first step to understand the role played by the fusoid cells.

Our developmental results show fusoid cells with no chloroplasts, but with several amyloplasts. Plants living in shaded environments often have wide leaves, presumably to maximize light absorption, which optimizes their survival under low levels of light. In this kind of environment, field experiments have clearly demonstrated the suppression of photorespiration, with stomatal closure and consequent increase in plant growth (Long *et al.*, 2006; Peterhansel and Maurino, 2011). The combination of enhanced CO<sub>2</sub> supply, wide leaves, and soil moisture promote the photosynthesis process, which produces carbohydrates (glucose) in the chlorenchyma cells. The glucose, in turn, may be metabolized in the photorespiration process or converted into starch and stored. Thus, considering the low rates of photorespiration in shaded environments, along with our results that show no evidence of starch storage in the differentiating chlorenchyma cells, one may argue that, at early stages of development, the fusoid cells play a critical role in the synthesis and storage of starch granules. The evidence of plasmodesmata between chlorenchyma cells and fusoid cells supports the possibility of glucose transport from photosynthetic cells to fusoid cells. Further ultrastructural and functional studies should assist in the interpretation of the relationship of CO<sub>2</sub> supply to the presence of fusoid cells, particularly in aquatic species of Poaceae.

As previously discussed, during the leaf development, a single fusoid cell typically undergoes divisions, with the daughter fusoid cells enclosed within the cell wall of the mother fusoid cell, and then all cells die, collapsing and leaving a space between successive fusoid cells. Collapse and cell death are often observed in mature leaves and thus it is conceivable that the ultimate fate of fusoid cells is determined by the PCD process. At early stages of development, our TEM analysis reveals vacuoles increasing in size, high nuclear activity with formation of cell walls, and reminiscent of membranes resultant from lyse of organelles. At some point, however, we are not able to distinguish the tonoplast and thus we believe it ruptures releasing hydrolases that gradually degrade the cytoplasm, thus resulting in self-degradation but without affecting the integrity of at least the mother fusoid cell wall (van

Doorn and Woltering, 2005; 2010). It is therefore tempting to speculate that PCD represents the end point of later fusoid cell development and that fusoid cell death may be needed to allow the hydrolases to break down the starch granules to make nutrients available to the developing leaves and the plant as a whole. The direct role of the PCD process and starch accumulation in the fusoid cells needs further study; however, observations on grass reproductive structures that show dynamic changes of starch synthesis, storage, degradation, and nutrient transport in developing cells could support this idea (Zhou *et al.*, 2009; Li *et al.*, 2010).

### *Conclusions*

During the leaf differentiation, a single fusoid cell originated from the ground meristem divides to form several fusoid cells. In mature leaf blades, a fusoid cell seen in cross section is actually a cavity as a result of the collapse of these several fusoid cells. Each cavity is delimited by successive collapsed fusoid cells arranged perpendicularly to the veins and along the entire lamina. Considering the same topography and origin, fusoid cells in Poaceae and colourless cells in Joinvilleaceae are homologous and it is plausible that fusoid cells evolved from colourless cells in the base of the [(Joinvilleaceae + Ecdeiocoleaceae) Poaceae] Clade. Also, the origin of fusoid cells in Panicoideae is the same from early-diverging lineages and BOP Clade (Poaceae), and thus they are homologous within the family. Further developmental work, above all a functional study, would be optimal to verify analogies since this foundation is necessary to understand the evolution of mesophyll cells within the Graminid Clade. We are hypothesizing that the role played by the fusoid cells are related to synthesis and storage of starch granules in early stages of development and it is PCD process-dependent. Looking at the pattern of occurrence of fusoid cells within Poaceae and its correlation with ancestral and environmental reconstructions is a great source of information in trying to find out their evolutionary and functional significance.

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# TABLES

TABLE 1. Brief terminological history of the fusoid cells.

References	Taxa	Anatomical term
Karelstschicoff (1868)	<i>Dendrocalamus strictus</i>	<b>Faltenzellen</b> “bended cells” (described as many thin-wall cells with different planes overarched).
Brandis (1907)	Bambusoideae	<b>apparent cavities</b> (although described as cavities, they are recognized as cells).
Page (1947)	<i>Streptochaeta spicata</i>	<b>enlarged mesophyll cell</b> (with regard to the big cells in the mesophyll as seen in cross, longitudinal, and paradermal sections).
Jacques-Felix (1954)	Bambusoideae	<b>lacune</b> ou <b>parenchyme différencié</b> (with regard to the anastomosing network of canals that branch and then reunite as before).
Metcalf (1956)	Bambusoideae	<b>fusoid cells</b> (to define the fusiform-shaped cells as seen in cross section).
Metcalf (1960)	Bambusoideae	<b>fusoid cells</b>
Wu (1960)	Bambuseae	<b>translucent fusoid cells</b> (according to Metcalf (1956) who defined these as translucent cells).
Aber (1965)		<b>colourless central mesophyll cells</b> (also according to Metcalf (1956) who defined these as translucent cells).
Renvoize (1985)	Bambusoideae	<b>fusoid cells</b>
Killeen and Clark (1986)	<i>Panicum</i> sect. <i>Laxa</i>	<b>fusoid-like cells</b> similar to cavities which apparently are derived from laterally extended bundle sheaths.
Renvoize (1987)	Bambuseae	<b>fusoid cells</b>
Clark (1991)	Bambusoideae	<b>fusoid cells</b> or <b>spaces formed by their collapse</b> (with regard to the spaces as seen in longitudinal section).
Zuloaga <i>et al.</i> (1992)	<i>Panicum</i> subg. <i>Phanophyrum</i> sect. <i>Laxa</i>	<b>translucent fusoid cells, fusoid cells, and fusoid cavities</b> (used in the same work to refer to the same cells).
GPWG (2001)	Poaceae	<b>fusoid cells</b> for early-diverging lineages and Bambusoideae, and <b>fusoid-like cells</b> for Panicoideae.
Vega <i>et al.</i> (2016)	<i>Guadua</i> species	<b>Intercellular gas spaces</b> (with regard to fusoid cell collapse and the space left between adjacent fusoid cells)

TABLE 2. List of studied species, occurrence of fusoid cells, vouchers and types of microscopy and analysis. AN = anatomical work; DV = developmental work; LM = light microscopy; TEM = transmission electron microscopy; (+) presence; (-) absence

TAXA	FUSOID CELLS	VOUCHER	ANALYSIS PERFORMED		
			LM		TEM
			DV	AN	
<b>FLAGELLARIACEAE</b>					
<i>Flagellaria indica</i> L.	-	Clark & Zhang 1305 (ISC)	X	X	
<b>JOINVILLEACEAE</b>					
<i>Joinvillea ascendens</i> Gaudich. ex Brongn. & Gris	-	Clark (ISC)	X	X	X
<b>POACEAE</b>					
<b>Anomochloideae</b>					
<i>Streptochaeta spicata</i> Schrad. ex Nees	+	Clark & Lewis 1642 (ISC)	X	X	X
<b>Pharoidae</b>					
<i>Pharus latifolius</i> L.	+	Klahs (ISC)	X	X	X
<b>Puelioideae</b>					
<i>Guaduella oblonga</i> Hutchinson ex W. D. Clayton	+	Baldwin 6704 (ISC)		X	X
<b>Oryzoideae</b>					
<i>Ehrharta erecta</i> Lam.	-	Clark (ISC)	X	X	
<i>Luziola spruceana</i> Benth. ex Döll	+	Moreira 249 (CGMS)		X	
<i>Oryza latifolia</i> Desv.	+	Guglieri-Caporal 3172 (CGMS)		X	
<b>Bambusoideae</b>					
<i>Raddia brasiliensis</i> Bertol.	+	Clark & Attigala 1713 (ISC)	X	X	

<i>Parodiolyra micrantha</i> (Kunth) Davidse & Zuloaga	+	RT Shirasuna 2863 (SP)		X	
<i>Aulonemia aristulata</i> (Döll) McClure	+	RT Shirasuna 2860 (SP)	X	X	X
<i>Chusquea attenuata</i> (Döll) L.G. Clark	+	Campos et al. s/n (ISC)	X		
<i>Otatea rzedowskiorum</i> Ruiz-Sanchez	+	Clark (ISC)	X	X	
<b>Pooideae</b>					
<i>Brachyelytrum erectum</i> (Schreb.) P. Beauv.	-	Clark & Dixon 1669 (ISC)	X	X	
<i>Diarrhena obovata</i> (Gleason) Brandenburg	-	Clark & Dixon 1667 (ISC)	X	X	
<i>Glyceria</i> sp.	+	Clark & Dixon 1671 (ISC)	X	X	
<b>Panicoideae</b>					
<i>Centotheca lappacea</i> (L.) Desv.	-	Sanchez-Ken s/n (ISC)	X	X	
<i>Rugoloa pilosa</i> (Sw.) Zuloaga	+	Leandro 161 (CGMS)	X	X	
<i>Setaria scabrifolia</i> (Nees) Kunth	-	Leandro 160 (CGMS)	X	X	

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**FIGURES**

**AND**

**SUPPLEMENTARY DOCUMENTS**

**FIG. 1.** Cross sections of leaf blades (LM) at early stages of development with emphasis on the development of fusoid cells. (A), (B), (D) and (E) *Aulonemia aristulata*; (C) and (F) *Rugoloa pilosa*. Red arrows show cell divisions, blue shadows highlight procambial strands (pr), and pink shadows highlight the differentiation of ground meristem (gm) into fusoid cells. (A) Early procambial strand of the midvein, protoderm (pt) and ground meristem. (B) Midvein and initial development of minor procambial strands. (C) Minor procambial strands are formed between major pre-existing ones. (D) Major and minor procambial strands and initial differentiation of ground meristem cells into fusoid cells. (E) Ongoing differentiation of procambial strands into vascular bundles and ground meristem cells into fusoid cells. (F) Subsequent differentiation of protoderm and procambium, as well as ground meristem cells with fusoid cells quite distinguishable. Scale bars: 15  $\mu\text{m}$ .

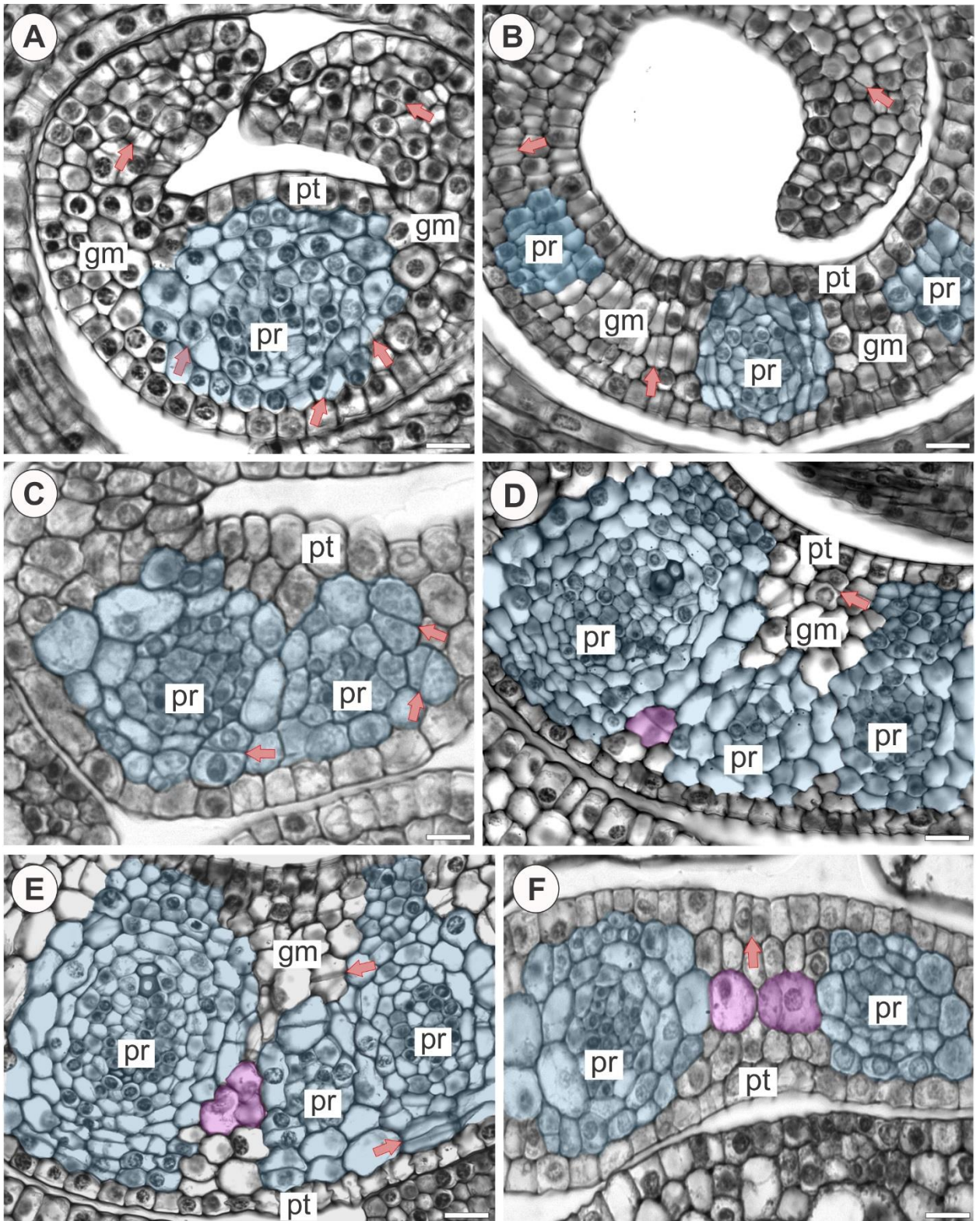


FIG. 1.

**FIG. 2.** Cross sections of leaf blades (LM) at early stages of development with emphasis on the development of fusoid cells. (A) *Rugoloa pilosa*; (B) *Aulonemia aristulata*; (C), (D), (E) and (H) *Otatea rzedowskiorum*; (F), (G), (I) and (J) *Pharus latifolius*. Red arrows show cell divisions, and pink shadows highlight the differentiation of ground meristem into fusoid cells. Asterisks = cavities. (A) Adaxial (ad) and abaxial (ab) epidermis and vascular bundles (vb) differentiated (except the bulliform cells), and ongoing differentiation of ground meristem cells with fusoid cells quite distinguishable. (B) Several fusoid cells adjacent to the vascular bundles and inset showing a young fusoid cell with content. (C) Mother fusoid cell giving rise to a daughter fusoid cell. (D) Subsequent division from mother and daughter fusoid cells. (E) Vascular bundle between one (left) and three (right) fusoid cells showing nuclei. (F) Degradation of the middle lamella between fusoid and chlorenchyma cells. (G) and (H) Fusoid cell divisions accompanying vascular bundle maturation (note the fully differentiated vascular tissue and enlarged bundle sheath cells in G). (I) Degradation of the middle lamella following bundle maturation. (J) Fusoid cell stretched and flattened as a result of the degradation of the middle lamella and leaf blade enlargement. Scale bars: 10  $\mu\text{m}$  (C, D, E, H); 15  $\mu\text{m}$  (A); 30  $\mu\text{m}$  (I), 40  $\mu\text{m}$  (F, G, J), 50  $\mu\text{m}$  (B, inset).



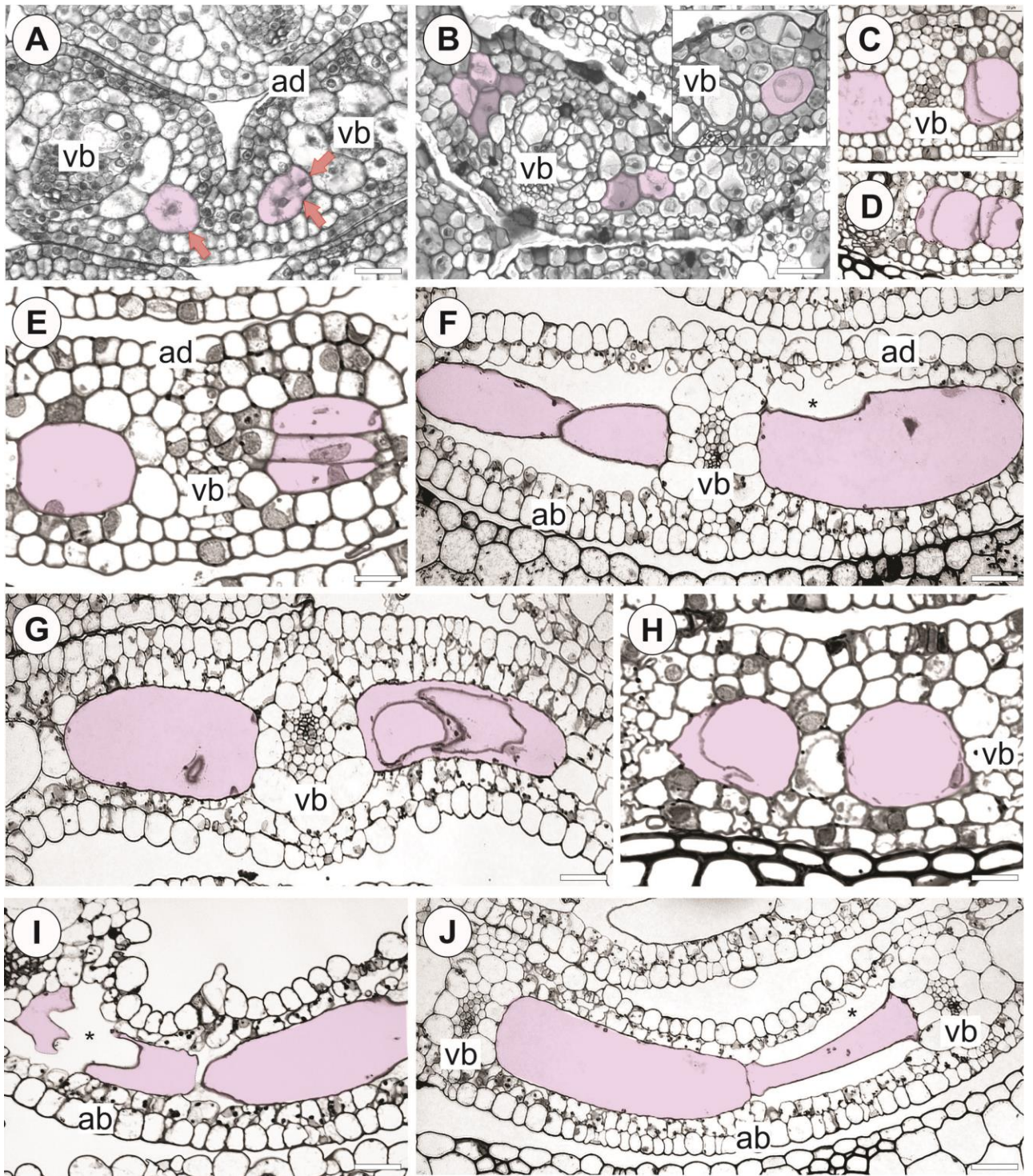


FIG. 2.

**FIG. 3.** Cross sections of leaf blades (TEM) at early stages of development with emphasis on the development of fusoid cells (fc). (A), (B), (D) and (F) *Aulonemia aristulata*; (C) and (E) *Rugoloa pilosa*; (G) and (H) *Streptochaeta spicata*; (I) *Pharus latifolius*. ad = adaxial epidermis; ab = abaxial epidermis; cc = chlorenchyma cells. White arrows show amyloplasts with starch grains. (A) Mother and daughter fusoid cells showing high cytoplasmic activity, large vacuoles, and formation of cell walls. (B) A fusoid cell and its derivative cell, with initial formation of cell wall. (C) Fusoid cells (apparently two) with large vacuole. (D) Several fusoid cells; the central one with its nucleus and the others with no organelles and large vacuole. (E) Several fusoid cells with high activity showing three nucleoli. (F) Large vacuoles with cell membranes from dead organelles. (G), (H) and (I) Fusoid cells with starch granules and initial degradation of the middle lamella. Scale bars: 1  $\mu\text{m}$  (F, G, H); 3  $\mu\text{m}$  (B, E, I); 4  $\mu\text{m}$  (A, C, D).



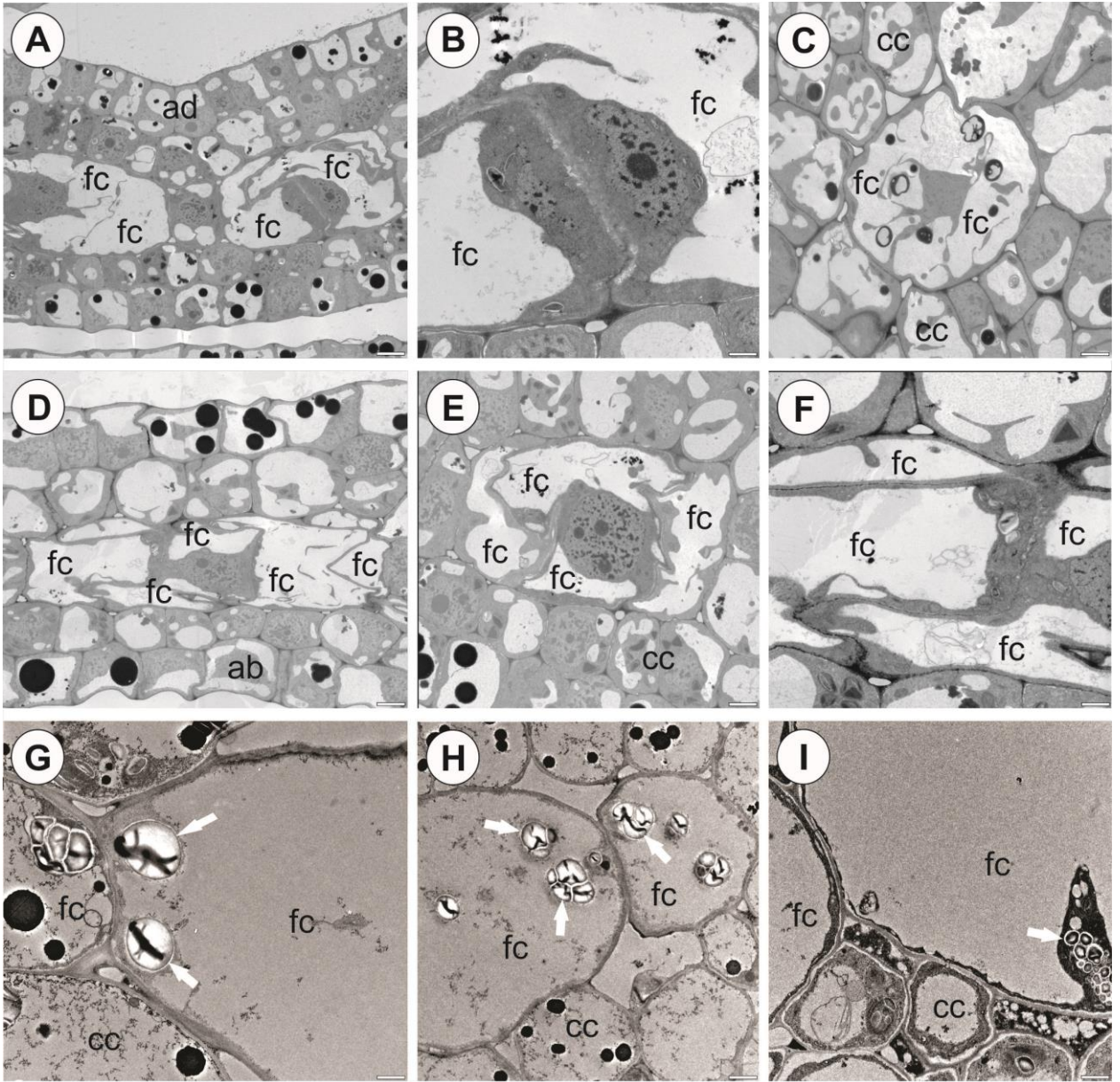


FIG. 3.

**FIG. 4.** Mature leaf blades (LM) with emphasis on fusoid cells. Cross sections (A-J) show cavities (asterisks) as a result of the collapse of several fusoid cells (black arrows). Longitudinal (K-R) and paradermal (S-X) sections show that each cavity is delimited by successive collapsed (rarely non-collapsed) fusoid cells arranged perpendicular to the veins and along the entire lamina. (A), (K) and (S) *Streptochaeta spicata*. (B), (L), (M), (N) and (T) *Pharus latifolius*. (C) and (U) *Guaduella oblonga*. (D) *Luziola spruceana*. (E) and (O) *Oryza latifolia*. (F) *Parodiolyra micrantha*. (G), (Q) and (W) *Aulonemia aristulata*. (H) and (V) *Otatea rzedowskiorum*. (I) *Glyceria* sp. (J), (R) and (X) *Rugoloa pilosa*. (P) *Raddia brasiliensis*. Scale bars: 12  $\mu\text{m}$  (W); 25  $\mu\text{m}$  (P, R, X); 30  $\mu\text{m}$  (M, N); 40  $\mu\text{m}$  (K, L, O, Q, S, V); 50  $\mu\text{m}$  (A, B, C, E, F, G, H); 60  $\mu\text{m}$  (D); 90  $\mu\text{m}$  (I, U); 100  $\mu\text{m}$  (J); 150  $\mu\text{m}$  (T).



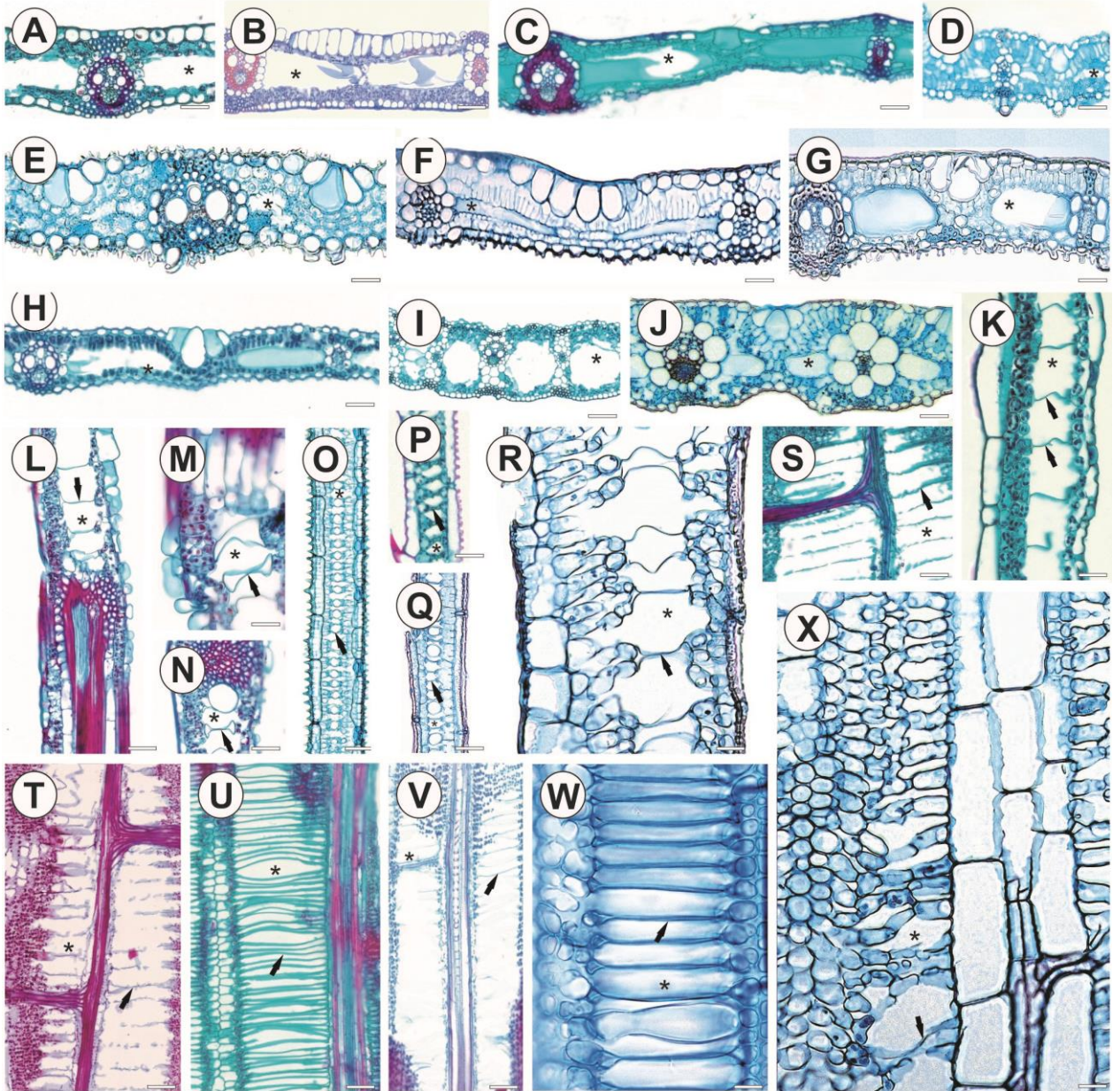


FIG. 4.

## SUPPLEMENTARY DOCUMENTS

**FIG. S1.** Cross sections of leaf blades with no fusoid cells at early stages of development (A-D and J-M) and mature (E-I, N and O) on LM. (A), (B), (C), (D), (E) and (F) *Setaria scabrifolia*. (G) *Brachyelytrum erectum*. (H) *Centotheca lappacea*. (I) *Flagellaria indica*. (J-O) *Joinvillea ascendens*. ad = adaxial epidermis; ab = abaxial epidermis; cc = chlorenchyma cells; vb = vascular bundle. (A) Early procambial strand (pr) of the midvein, protodermis (pt) and ground meristem (gm). (B) Midvein and initial development of minor procambial strands. (C) Ongoing development of procambial strands. (D) Major and minor procambial strands and initial differentiation of ground meristem cells into chlorenchyma cells (cc). (E), (F), (G), (H), (I) Major and minor vascular bundles completely differentiated, as well as chlorenchyma cells. (J), (K), (L) and (M) Subsequent developmental stages of several colourless cells (pink arrows) in *Joinvillea ascendens*. (N) Colourless cells completely developed showing their cell walls. (O) Colourless cells always non-collapsed. Scale bars: 13  $\mu\text{m}$  (A, B, C, D); 20  $\mu\text{m}$  (K, L, M); 25  $\mu\text{m}$  (J); 40  $\mu\text{m}$  (F, H, N, O); 50  $\mu\text{m}$  (E, I); 100  $\mu\text{m}$  (G).



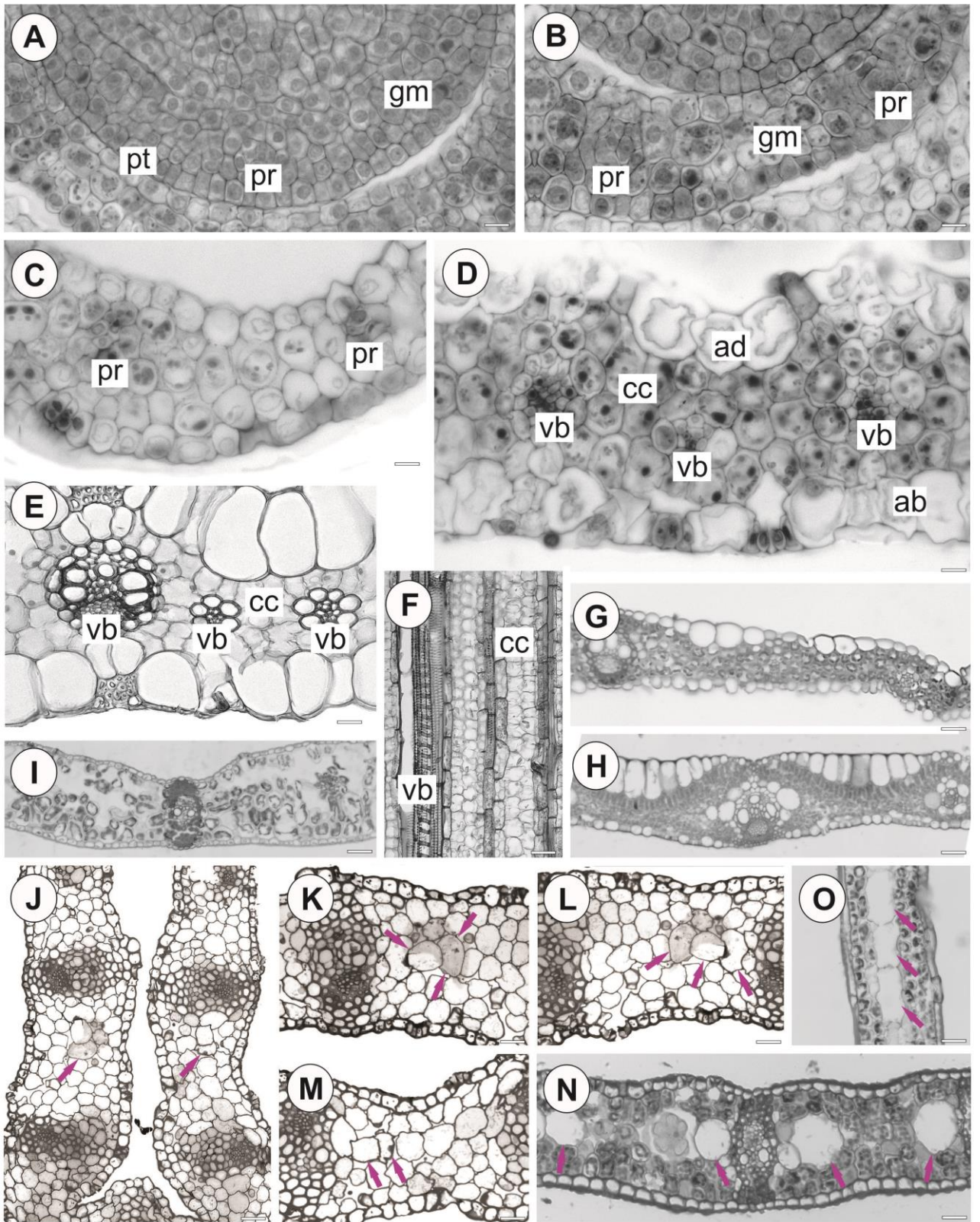


FIG. S1.

**VIDEO.** Paradermal scan with tridimensional reconstruction showing the fusoid cells through the depth of the leaf blade of *Chusquea attenuata*, in which each 5- $\mu\text{m}$  thick section was photographed along the Z-axis to create a stack of eight to ten 0.5- $\mu\text{m}$  thick optical sections. Z-stacks from each section were aligned and modeled also using ZEN 2.0 blue. The video starts by showing the upper epidermis (paradermal view) and goes through the leaf blade toward the lower epidermis. Fusoid cells with collapsed cell walls become quite distinguishable at 00:00:08.



## CAPÍTULO IV

**An update on comparative leaf blade anatomy in the systematics of**

**Poaceae (Poales): the past thirty years since Ellis**

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SERÁ ENVIADO PARA O PERIÓDICO

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Grass anatomical studies have been increasingly growing since the review of comparative leaf blade anatomy in the systematics of Poaceae undertaken by Ellis, in 1987. At that point, the number of subfamilies was stabilized at five major groups, some of them showing a unique combination of features but also similarities between closely related taxa. After thirty years, the grass family Poaceae has passed through a number of taxonomic changes, and descriptions of new genera and species were carried out; in addition, new structural concepts with reference to the terminology of the mesophyll cells were introduced. The standardization of anatomical descriptions and reevaluation of anatomical features in defining and delimiting major or minor groups thus becomes necessary. In this review we provide a brief history of grass subfamilial classification since 1987, general leaf blade anatomical descriptions of the 12 currently recognized grass subfamilies, as well as, when possible, a structural definition and reevaluation of the relevance of anatomical features in grass systematics. Leaf blade anatomical features remain systematically informative, however, toward a broad overview, we recommend more detailed studies prioritizing the subfamilies Puelioideae, Bambusoideae (particularly Temperate and Palearctic woody bamboos), Aristidoideae, Panicoideae, Micrairoideae, and Arundinoideae.

## Introduction

At the International Symposium on Grass Systematics and Evolution held at the Smithsonian Institution, in Washington, D.C, in July 1986, agrostologists presented scientific conclusions that summarized the state-of-the-art, challenges and perspectives in research on grasses. As a result, the book “Grass Systematics and Evolution” was organized by Thomas R. Soderstrom and collaborators (1987a), which included many studies arranged into sections such as: structural and biochemical diversity, evolution, systematics of major groups, and application of taxonomic methods. Within the “Structural Diversity” section, Roger P. Ellis, an agrostologist from the Botanical Research Institute, in Pretoria, South Africa, provided a review of comparative leaf blade anatomy in the systematics of Poaceae taking into account twenty-five years of published papers, which were brilliantly discussed therein (Ellis, 1987). In his work, Ellis has emphasized the usefulness of leaf blade anatomical features in defining subfamilies of Poaceae, stabilized at five major groups at that point (Pooideae, Bambusoideae, Arundinoideae, Chloridoideae, and Panicoideae).

Herein we review structural studies that include relevant data with reference to anatomical and taxonomic value since Ellis’s work (1987), toward characterizing a unique combination of leaf blade anatomical features useful in defining each one of the 12 subfamilies currently recognized for Poaceae (Soreng et al. 2015). Furthermore, when possible, we provide comments on particular genera or species, showing that, in some cases, anatomical data are also useful in delimiting minor groups or in diagnosing species. This review also provides a discussion and general illustrations of the most important anatomical features for Poaceae, which are considered diagnostic attributes, putative, or true synapomorphies.



*Brief History of Subfamilial Classifications with Emphasis on the Three Major Collaborative Works: GPWG (2001), GPWG II (2012), and Soreng et al. (2015)*

Phenetically, a previous classification system has been proposed based on morphology of spikelets, in which two major groups were later recognized as subfamilies by Hitchcock (1950): (i) spikelets exhibiting two anthechia, the basal one often staminate or neutral, with disarticulation below the glumes (Panicoideae); and (ii) spikelets exhibiting one or many anthechia, all bisexual or at least the basal one bisexual, with articulation above the glumes and often between the florets (Festucoideae). After that, several classification systems were proposed recognizing different amount of subfamilies: Andropogonoideae, Anomochlooideae, Eragrostoideae, Festucoideae, Micrairoideae, Olyroideae, Oryzoideae, and Panicoideae (Pilger 1956); Argentinean grasses into Bambusoideae, Oryzoideae, Phragmitoideae, Festucoideae, Eragrostoideae, and Panicoideae (Parodi 1961); North American grasses into Bambusoideae, Oryzoideae, Arundinoideae, Festucoideae, Eragrostoideae, and Panicoideae (Stebbins and Crampton 1961); Brazilian grasses into Bambusoideae, Oryzoideae, Arundinoideae, Pooideae, Chloridoideae, and Panicoideae (Valls 1980); and finally Bambusoideae, Arundinoideae, Pooideae, Chloridoideae, Centothecoideae, and Panicoideae (Clayton and Renvoize 1986). For a detailed discussion of these circumscriptions, see Longhi-Wagner (2012).

In the 15 years following the first grass symposium in 1986, many studies were undertaken to address grass relationships based on phenetic and phylogenetic analyses (Kellogg and Campbell 1987; Hamby and Zimmer 1988; Esen and Hilu 1989; Doebley et al. 1990; Hilu and Johnson 1991; Watson and Dallwitz 1992a,b onwards; Davis and Soreng 1993; Nadot et al. 1994; Verboom et al. 1994; Barker et al. 1995; Clark et al. 1995; Clark and Judziewicz 1996; Duvall and Morton 1996; Liang and Hilu 1996; Soreng and Davis 1998; Barker et al. 1999; Hilu et al. 1999; Hsiao et al. 1999; Clark et al. 2000; Mathews et al. 2000; Zhang 2000). These papers were the basis for the first broad work on grass systematics and classification published

in the early 2000s, undertaken by a large collaborative group including agrostologists mainly from the U.S.A., which was referred to as the Grass Phylogeny Working Group (GPWG 2001).

The GPWG (2001) work included a broad study with 62 sampled grass species and four outgroups. Its primary result was the reevaluation of subfamilial classification based on morphological, biochemical, anatomical, embryological, and molecular evidence, in which 12 subfamilies were recognized and classified as follows: (i) early-diverging lineages comprising Anomochlooideae, Pharoideae, and Puelioideae; (ii) the BEP clade comprising Bambusoideae, Ehrhartoideae, and Pooideae; and (iii) the PACCAD clade comprising Aristidoideae, Danthonioideae, Arundinoideae, Chloridoideae, Centothecoideae, and Panicoideae. This work was widely accepted by agrostologists until the emergence of a new classification by the Grass Phylogeny Working Group (GPWG II 2012).

This second collaborative work expands the sampling from 62 to 531 taxa, with emphasis on  $C_4$  grass species. Carried out by agrostologists mostly from U.S.A and Argentina, the GPWG II (2012) also recognized Anomochlooideae, Pharoideae, and Puelioideae as early-diverging lineages, but also the BEP and PACMAD clades with strong support, totaling 12 subfamilies. Note that the PACMAD clade, previously referred to as the PACCAD clade in the GPWG (2001), was therein recognized also comprising six subfamilies, but taking into account the inclusion of Centothecoideae within Panicoideae (Sánchez-Ken and Clark 2010; Morrone et al. 2012), and the reinstatement and emendation of Micrairoideae as a valid subfamily (Sánchez-Ken et al. 2007).

Lastly, Soreng and collaborators (2015) published the most recent work on the phylogenetic classification of Poaceae including a large dataset of 448 species based on the plastid *matK* and *ndhF* DNA markers. Their work also includes a detailed taxonomic classification resulting into six supertribes, 51 tribes, and 80 subtribes. The number of subfamilies was stabilized at 12, but the subfamilial name Oryzoideae was proposed over Ehrhartoideae.

### *Leaf Blade Anatomical Data in the Grass Systematics*

Since the classification of Poaceae is traditionally based on morphological data from vegetative and reproductive structures (Longhi-Wagner 2012), the absence of fertile materials or incongruences between morphological and molecular evidence can create a challenge for grass systematics. Among vegetative structures, anatomical data from the leaf blade has been demonstrated to be highly informative in defining major groups but also in showing intermediate characters, indicating, thus, priorities of study within the family (Ellis 1987).

After thirty years of advances in the grass systematics, many new taxa have been published. Along with the phylogenetically-based work of the GPWG (2001), the early 2000s was also marked by new structural concepts with reference to the mesophyll cells, introduced in botanical conferences in the U.S.A. Hence, the standardization of anatomical descriptions, and above all, the reevaluation of anatomical features that define and delimit major and minor groups, have become highly necessary.

#### **Leaf Blade Anatomical Features Revisited**

In this section, the most important leaf blade anatomical features in mature leaves as seen in cross sections and epidermal views are structurally defined and discussed with reference to their character states, occurrence, and usefulness in delimiting taxa. Some images are provided in Fig. 1—these are merely illustrative and not necessarily related to a specific taxon. Features that are better observed using scanning electron microscopy (SEM) (e.g., papillae and the stomatal apparatus) are also discussed and illustrated.

##### *Plicate mesophyll parenchyma: arm and rosette cells*

*Arm cells.* Thin-walled photosynthetic mesophyll cells that exhibit cell wall

invaginations (lobes) extending to different depths from one side (often abaxial) or both sides of the leaf blade (Fig. 1A-E, H). Complementary images in: Judziewicz et al. (1999); Viana (2010); Viana et al. (2011); Jesus Junior et al. (2012); Lizarazu (2012); Mota (2013); Leandro et al. (2016b); Second Chapter; Third Chapter.

These cells were originally reported in bamboos (Bambusoideae) and in species that are currently regarded as basal grasses (e.g., Metcalfe 1956, 1960; Ellis 1987; Soderstrom and Ellis 1987; Judziewicz et al. 1999; GPWG 2001; BPG 2012; Clark et al. 2015). Arm cells occur closely appressed and vertically oriented, forming rows above and below cavities (when present) and parallel to the epidermis (Fig. 1A-C). Below the upper epidermis one or two layers (or more) of arm cells with vertically oriented lobes occur (Fig. 1A), although sometimes is observed just one layer in Olyreae species (Zuloaga et al. 1993) (e.g. Fig. 1B-D) and some of the temperate bamboos (Arundinarieae) (LG Clark personal observation); and usually just one layer above the lower epidermis (Fig. 1B-D, G and H).

Within Bambusoideae, arm cells exhibiting asymmetrically strongly invaginated arm-like lobes from both sides (Fig. 1C) or just from the abaxial side (Fig. 1B) of the leaf blade is one of the set of anatomical features considered a synapomorphy for the group (GPWG 2001). Arm cells weakly lobed can be observed in early-diverging lineages and Oryzoideae (Judziewicz et al. 1999; Leandro et al. 2016b), although few species of the Bambusoideae (Bambuseae and Olyreae) apparently have reverted to the putative ancestral state (Zhang 1996). These two types of lobed arm cells along with the number of layers arranged below the upper epidermis might be potentially taxonomically informative for delimiting species (e.g., Leandro et al. 2016b).

*Rosette cells.* Thin-walled photosynthetic mesophyll cells that exhibit weak cell wall invaginations (lobes) all around the cell as seen in cross section (Fig. 1D). Complementary images in: Viana (2010); Viana et al. (2011); Lizarazu (2012); Mota (2013); Leandro et al. (2016a,b); Second Chapter.

Formerly lumped as arm cells, rosette cells are closely appressed and vertically oriented like an arm cell, but distinguish themselves by their lobes all around the cell as a whole not really asymmetric. Although has been interpreted as a putative synapomorphy for the bistigmatic clade (Puelioideae + [BOP + PACMAD]), these cells occur mainly in Oryzoideae and Bambusoideae members and their presence in few pooids and in the PACMAD clade may be the result of an ancestral retention (LG Clark personal observation).

*Midrib vasculature.* With reference to the number of vascular bundles that comprise the midvein (Fig. 1A and E). Complementary images in: Tateoka (1963); Viana (2010); Viana et al. (2011, 2013a,b); Jesus Junior et al. (2012); Lizarazu (2012); Mota (2013); Leandro et al. (2016a,b); Second Chapter.

Most grass species exhibit an abaxially projecting midrib comprising several vascular bundles equidistantly arranged and constituting an arc (keel) (Metcalf 1960; Soderstrom and Ellis 1987). Although there are many variations in the pattern of distribution of vascular elements (see Metcalfe 1960 for more details), the number of vascular bundles comprising the midrib may be taxonomically informative. Therefore, with regard to the number of vascular bundles, the midrib vasculature can be classified into two types: (i) simple: comprising only one central vascular bundle (Fig. 1A); and (ii) complex: comprising two or more vascular bundles, the additional ones usually arranged opposite to (superposing) the central one (Fig. 1E).

The complex midrib is an unusual feature in the grass family (Calderón and Soderstrom 1973; Soderstrom and Ellis 1987), however, when present, it has proved to be taxonomically relevant. Within Oryzoideae, the midrib vasculature is useful for delimiting Oryzeae and Ehrharteae, which exhibit complex and simple midribs, respectively (Tateoka 1963). Also, it seems that, for oryzoid species, the arrangement of vascular bundles in the midrib is highly informative for distinguishing species, mainly with respect to closely related taxa (e.g., Tateoka

1963; Leandro et al. 2016a). Within Bambusoideae, many woody but few herbaceous bamboo species exhibit a complex midrib, although a simple vasculature is the expected condition in herbaceous species (Judziewicz et al. 1999). A simple midrib is also observed in the subtribe Arthrostylidiinae (Bambuseae) species (e.g., see Viana et al. (2011); Leandro et al. 2016b), thus being useful in delimiting and distinguishing this subtribe from Chusqueinae and Guaduinae, Neotropical woody bamboos that exhibit complex midrib. In Chusqueinae, the midrib vasculature can be of three types as known to date (Clark 1986; Mota 2013; Second Chapter): (i) comprising two opposing vascular bundles, with several adjacent ones; (ii) comprising two opposing vascular bundles sharing the same bundle sheath; and (iii) comprising three vascular bundles, two smaller ones opposite (adaxial) to the major one, but all three sharing the same bundle sheath. Although all classified as complex midrib, these differences have been shown to be highly informative in distinguishing *Chusquea* species (Mota 2013; Second Chapter).

It seems that this feature is taxonomically important just for Oryzoideae and Bambusoideae, and thus we strongly recommend its inclusion in anatomical descriptions of taxa within these two subfamilies.

*Fusoid cells.* Thin-walled non-photosynthetic mesophyll cells that collapse and die as a result of their development (Fig. 1F). Complementary images in: Vega et al. (2016); Third Chapter.

Recent developmental work has shown that, in mature leaves, intact fusoid cells are often only observed in longitudinal and paradermal sections, since cross sections actually usually show a cavity resulting from the collapse of several fusoid cells (Third Chapter) (Fig. 1A-E, G and H). Each cavity is delimited by successive collapsed I-shaped fusoid cells, arranged perpendicularly to the veins and along the entire lamina (Vega et al. 2016; Third Chapter) (Fig. 1F). During leaf differentiation, the activity of marginal meristems promotes the enlargement of the leaf blade and, consequently, the elongation and collapse of the fusoid cells (Third Chapter). Therefore, as a result, the cavity is usually cigar-shaped (Fig. 1A, C and G)

and the collapsed fusoid cell is itself I-shaped in longitudinal view (GPWG 2001; Kellogg 2015; Vega et al. 2016; Third Chapter) (Fig. 1F).

Fusoid cells have been reported in the early-diverging lineages (Ellis 1987 as Bambusoideae; GPWG 2001), Oryzoideae (Tateoka 1963; Leandro et al. 2016a), Bambusoideae (Judziewicz et al. 1999; GPWG 2001), and few Pooideae (Barkworth *et al.*, 2007) and Panicoideae (Watson et al. 1985; Clayton and Renvoize 1986; Killeen and Clark 1986; Zuloaga et al. 1992; GPWG 2001). This feature has been considered plesiomorphic for Poaceae and either retention of a plesiomorphy or a reversal after the loss of fusoid cells at the base of the BOP clade (GPWG 2001 as BEP clade). Traditionally, the presence or absence of fusoid cells has been considered useful in delimiting and defining taxa within Bambusoideae and Oryzoideae (e.g., Metcalfe 1960; Tateoka 1963; Watson et al. 1985; Clayton and Renvoize 1986; Killeen and Clark 1986; Zuloaga et al. 1992; Guala 1995; Oliveira and Longhi-Wagner 2007; Oliveira et al. 2008; Leandro et al. 2016a,b), however, there are some peculiarities worth mentioning.

Anatomical work by March and Clark (2011) demonstrated sun-shade variations in bamboo leaves, in which, within the same species, fusoid cells were observed in plants from shaded environments but not in those from sunny environments. Also, the authors reported differences found between exterior and interior leaves within one individual of *Phyllostachys aurea* Carrière ex Rivière & C. Rivière (Bambusoideae), suggesting that the development of fusoid cells is environmentally influenced by light availability (March and Clark 2011; Leandro et al. 2016a; Third Chapter). Since fusoid cells in bamboos occur primarily in plants living in shaded environments, in contrast, the presence of fusoid cells in some Oryzoideae (Tateoka 1963; Leandro et al. 2016a), a group that comprises plants living in open and wet environments, suggests a developmental influence by other environmental factors (Third Chapter).

Lastly, we infer that the presence of fusoid cells, as an anatomical feature, is most informative in establishing phylogenetic relationships rather than in defining and delimiting

species. Further experimental and molecular work, an ongoing project of our lab, is needed to understand the functional role(s) of these cells in Poaceae.

*Intercostal fibers.* Sclerenchymatous cells observed always in the intercostal zone, adjacent to the bulliform cells or opposite them adjacent to the abaxial epidermis (Fig. 1G and H). Complementary images in: Silva Filho (2006); Viana (2010); Viana et al. (2011); Leandro et al. (2016b).

This feature has been reported as a diagnostic attribute of Arthrostylidiinae (Bambusoideae), (Soderstrom and Ellis 1987), and more recently considered taxonomically consistent in defining and delimiting this subtribe (Tyrrell et al. 2012; Clark et al. 2015; Leandro et al. 2016b). Lack of intercostal fibers (near or opposite to the bulliform cells) is rarely reported in this group; when absent, considered taxonomically informative (Viana 2010). The presence of intercostal sclerenchyma in the Arthrostylidiinae appears to be an anatomical synapomorphy for this subtribe, but this needs to be tested formally in a phylogenetic framework. In any case, the presence and distribution of this feature should be included in anatomical descriptions of woody bamboos.

*Refractive papillae.* In general, a tiny outgrowth developed on leaf blade epidermal cells. Complementary images in: Gomes and Neves (2009); Viana (2010).

The occurrence of papillae in grass species is quite variable, but their presence is considered characteristic of bamboos leaves (Judziewicz et al. 1999). The term “refractive papillae” was originally assigned by Soderstrom and Ellis (1987) with no reference to a structural definition for these putative kind of papilla. Refractive papillae have been reported as a useful feature in recognizing Arthrostylidiinae (Soderstrom and Ellis 1987; Judziewicz et al. 1999; Viana 2010); however, they also occur in Guaduinae, the subtribe sister to Arthrostylidiinae that, along with Chusqueinae, comprises the Neotropical woody bamboos



(Ruiz-Sanchez et al. 2008; Tyrrell 2008). Within the Paleotropical woody bamboos (PWB), refractive papillae are also reported in Melocanninae, a subtribe strongly supported as monophyletic and sister of the remaining PWB (Sungkaew et al. 2009; Kelchner et al. 2013).

Although refractive papillae are apparently smaller than a regular papilla, further comparative developmental and experimental studies are needed to verify their putative differences and the true value of refractive papillae in defining the Arthrostylidiinae + Guaduinae (or other) clade within the Bambuseae.

*Stomatal apparatus bearing papillae on the subsidiary cells* (Fig. 1I-K). Complementary images in: Judziewicz et al. (1999); Lizarazu (2012); Mota (2013); Leandro et al. (2016b); Second Chapter. A stomatal apparatus bearing two or more papillae per subsidiary cell has been reported in the Neotropical woody bamboo genus *Chusquea* Kunth (e.g., Clark 1986; BPG 2012; Mota 2013; Clark et al. 2015; Leandro et al. 2016a,b), but also in few herbaceous bamboos (Olyreae) (Calderón and Soderstrom 1973; Judziewicz et al. 1999), *Oryza* (Oryzoideae) (Prasad et al. 2011; TD Leandro personal observation), and *Distichlis* (Chloridoideae) (Bell and Columbus 2008). Absent in other woody bamboos, this feature is regarded a putative synapomorphy of *Chusquea* (Fisher et al. 2009), proving highly informative in defining and delimiting this group (Leandro et al. 2016b; Clark et al. 2015). Furthermore, papillae from subsidiary cells can be simple (Fig. 1I) or branched (Fig. J and K), which is sufficiently distinctive to be used for diagnostic and taxonomic purposes.

Although not necessarily related to this feature, stomata in most bamboos (perhaps most of grasses) are located on the lower epidermis, with exception of the Guaduinae, a group that exhibits a large number of stomata on both surfaces of the leaf blade (Judziewicz et al. 1999). Subsidiary cell shape is another relevant descriptive characteristic, which can be, in general, triangular (panicoid type), dome-shaped, parallel-sided, or flat-topped (Ellis 1979). For instance, within Bambusoideae, the stomatal apparatus can be dome-shaped or low-triangular

shaped (Judziewicz et al. 1999).

*Silica cells.* In grass leaves, the short epidermal cell that incorporates silica within its lumen, forming a silica body; sometimes paired with cork cells; silica bodies are known as phytoliths when isolated from plant tissue (Fig. 1L). Complementary images in: Piperno and Pearsall (1998); Chaves (2012); Jesus Junior et al. (2012); Mota (2013); Rudall et al. (2014); Leandro et al. (2016a,b).

Short cells developing silica bodies is a characteristic attribute of grass leaves (GPWG 2001; Kellogg 2015). Silica bodies exhibit a large number of shapes, which are horizontally or vertically oriented on the leaf surface (see Metcalfe 1960 for more details), although some species of *Chusquea* may exhibit both horizontally and vertically oriented silica bodies within a same leaf (Second Chapter) (Fig. 1L). Apparently, silica bodies are horizontally elongated and saddle-shaped in most of the grasses (Stevens, 2001 onwards; Metcalfe 1960; Ellis 1979), however, some distinctive shapes can be defined for some groups, such as Olyreae, in which many species exhibit cross-shaped silica bodies (Judziewicz et al. 1999; Metcalfe 1960)—apparently both Parianinae + Olyrinae, but no Buergersiochloinae. Yet within Olyreae, some species also exhibit oryzoid-type silica bodies (Metcalfe 1960), which is defined by vertically elongated (also vertically oriented) dumbbell-shaped silica cells (Ellis 1979); however, the oryzoid-type has been demonstrated useful only for recognizing Oryzoideae species (e.g., Tateoka 1963; Prasad et al. 2005, 2011; Leandro et al. 2016a)—although not all the oryzoids exhibit oryzoid-type silica bodies. Within the grasses, silica bodies often occur paired with cork cells, another type of short epidermal cell (Ellis 1979).

Prychid et al. (2004) reported that attributes such as shape and orientation of silica bodies are not environmentally influenced but rather genetically defined, and thus silica bodies may be a distinctive characteristic for delimiting taxa even at lower taxonomic levels.

*Trichomes* (Fig. 1M-Q). Complementary images in: Judziewicz et al. (1999); Vieira et al. (2002); Gomes and Neves (2009); Viana (2011); Lizarazu (2012); Jesus Junior et al (2012); Guerreiro et al. (2013); Mota (2013); Vieira (2014); Leandro et al. (2016a,b), Second Chapter.

In grasses, trichomes can be of three main types (Ellis 1979): (i) macrohairs termed usually large, unicellular trichomes (Fig. 1M); (ii) microhairs usually termed bicellular trichomes, although one-celled structures also occur (Fig. 1N); and (iii) prickly hairs termed pointed, often thick-walled and silicified structures (Fig. 1O), with swollen bases arising directly from the epidermis (including hooks).

Traditionally, microhairs have been highly regarded for delimiting grass taxa (e.g., Liphshitz and Waisel 1974; Amarasinghe and Watson 1988, 1990; Stevens 2001 onwards). This kind of trichome occurs on both leaf surfaces of most of the grasses (see Metcalfe 1960 for general descriptions) and can be of three main types: (i) panicoid-type, reported in Panicoideae, Arundinoideae, Bambusoideae, and a few genera of Chloridoideae (Watson et al. 1985; Watson and Dallwitz 1994); (ii) Enneapogon-type, mainly reported in the subtribe Cotteinae (Chloridoideae) (Tateoka et al. 1959; Stewart 1964; Tivano 2011), but also in *Amphipogon* R. Br. (Johnston and Watson 1976; Watson et al. 1985); and (iii) chloridoid-type, typical of Chloridoideae and considered a synapomorphy for this group (Peterson et al. 2007, 2010; Ingram et al. 2010)—also termed salt glands by physiologists (for a detailed review of salt glands in grasses see Céccoli et al. (2015)). However, trichomes are absent in few groups, such as the early-diverging genus *Pharus* P. Browne (Pharoideae) (Judziewicz 1987; Soderstrom et al. 1987b; Judziewicz et al. 1999), or two types may occur within a same group, as observed in *Eragrostis* Wolf. (Chloridoideae) (Tateoka et al. 1959; Amarasinghe & Watson 1988). Most of the Pooideae lack microhairs, although they occur in a few Stipeae (Kellogg 2015).

Macrohairs and prickles may occur on one or both leaf surfaces, and their presence is

highly variable across the family (see Metcalfe 1960 for general descriptions). Although most grasses exhibit microhairs, macrohairs, and prickles within a same leaf blade (with exception of most the Pooideae), a detailing of these three types of trichomes may be taxonomically useful at lesser degree. For instance, *Luziola fragilis* Swallen (Oryzoideae) exhibits vertically-elongated epidermal cells surrounding unicellular trichomes, a characteristic considered unique within *Luziola* Juss., and thus being diagnostic of this species (Leandro et al. 2016a) (Fig. 1Q).

### **Subfamilial Descriptions and General Notes based on Leaf Blade Anatomy Data**

This section followed the classification provided by the recent work of Soreng et al. (2015) and includes a general leaf blade anatomical description of each subfamily of Poaceae. General descriptions primarily followed GPWG (2001), taking into account new concepts on mesophyll cell shape and additional data provided by personal observations of our group for obtaining insights into the usefulness of leaf blade anatomical features in the grass systematics. The main features in defining and delimiting major and minor groups are summarized in Tables 1 and 2. A detailed anatomical description of the family can be found in Metcalfe (1960), thus, herein, we pointed out only differences from the general condition.

*The early-diverging lineages: Anomochloideae, Pharoideae and Puelioideae*

#### **I. ANOMOCHLOIDEAE**

*Surface view:* **Papillae** absent. **Stomatal apparatus** with low dome-shaped or triangular subsidiary cells. **Microhairs** often bicellular and large-sized. **Silica bodies** vertically oriented; equidimensional or unlobed. *Cross-section:* **Bulliform cells** adaxially present. **Mesophyll** non-radiate; adaxial palisade layer absent. **Kranz anatomy** absent. **Fusoid cells** present; usually very large and well developed. **Arm cells** rarely present; when present irregular. **Rosette cells**

weakly developed. **Midrib** complex.

*Notes.* (a) There is no unique morphological synapomorphy for this subfamily (GPWG 2001).

(b) Additional information can be found in Ellis (1987) as Bambusoideae.

*Complementary literature.* Judziewicz and Soderstrom (1989).

## II. PHAROIDEAE

*Surface view:* **Papillae** absent. **Stomatal apparatus** with parallel-sided to dome-shaped subsidiary cells. **Microhairs** absent. **Silica bodies** horizontally oriented (*Pharus*); saddle-shaped. *Cross-section:* **Bulliform cells** adaxially present (?). **Mesophyll** non-radiate; adaxial palisade layer absent. **Kranz anatomy** absent. **Fusoid cells** present; usually very large and well developed. **Arm cells** present. **Rosette cells** weakly developed. **Midrib** complex.

*Note.* (a) According to GPWG (2001), bulliform cells in Pharoideae are poorly developed or absent, however, for instance, *Pharus latifolius* L. exhibits intercostal epidermal cells highly similar to bulliform cells. Further experimental and structural work are needed in order to clarify this question. (b) Additional information can be found in Ellis (1987) as Bambusoideae.

## III. PUELIOIDEAE

*Surface view:* **Papillae** often absent. **Stomatal apparatus** with dome-shaped to triangular subsidiary cells. **Microhairs** present (except in *Puelia* Franch.); usually multicellular. **Silica bodies** vertically-oriented; saddle-shaped. *Cross-section:* **Bulliform cells** adaxially present. **Mesophyll** non-radiate; adaxial palisade layer absent. **Kranz anatomy** absent. **Fusoid cells** present; usually very large and well developed. **Arm cells** present. **Rosette cells** present. **Midrib** often complex; rarely simple.

*Complementary literature.* Clark et al. (2000).

*BOP clade (Clark et al. 1995; Clark et al. 2000; as BEP): Oryzoideae, Bambusoideae, and Pooideae*

#### IV. ORYZOIDEAE

*Surface view:* **Papillae** present. **Stomatal apparatus** with dome-shaped or triangular subsidiary cells. **Microhairs** often bicellular. **Silica bodies** horizontally or vertically oriented; dumbbell-shaped (oryzoid-type) or saddle-shaped. *Cross-section:* **Bulliform cells** adaxially present. **Mesophyll** often non-radiate; adaxial palisade layer absent. **Kranz anatomy** absent; **Fusoid cells** often absent; when present poorly developed. **Arm cells** absent. **Rosette cells** present. **Midrib** simple or complex.

*Note.* (a) Fusoid cells have been reported in *Streptogyna* P. Beauv. (Soderstrom et al., 1987), sister to the rest of the subfamily, and in some Oryzeae, as following: *Chikusichloa aquatica* Koidz, *Hygroryza aristata* (Retz.) Nees, *Rhynhoryza subulata* (Nees) Baill., *Zizania aquatica* L., *Zizaniopsis miliacea* (Michx.) Doell and Aschers (Tateoka 1963); *Luziola spruceana* Benth. ex. Doell and *L. subintegra* Swallen (Leandro et al. 2016a); as well as in *Oryza glumaepatula* Steud. and *O. latifolia* Desv. (TD Leandro personal observation). (b) Additional information can be found in Ellis (1987) as Bambusoideae and Arundinoideae, for Oryzeae and Ehrharteae, respectively.

*Complementary literature.* Sánchez et al. (2003); Sánchez & Espinoza (2005); Sánchez et al. (2006); Martínez-y-Pérez et al. (2006); Sage and Sage (2009); Yost and Blinnikov (2011).

#### V. BAMBUSOIDEAE

*Surface view:* **Papillae** present, often well developed. **Stomatal apparatus** with dome-shaped, triangular, or parallel-sided subsidiary cells. **Microhairs** often bicellular; panicoid-type. **Silica bodies** often vertically oriented; usually saddle-shaped in the woody bamboos and crenate-

shaped in the herbaceous bamboos (olyroid-type). *Cross-section*: **Bulliform cells** adaxially present. **Mesophyll** non-radiate; adaxial palisade layer absent. **Kranz anatomy** absent. **Fusoid cells** often present. **Arm cells** strongly asymmetrically invaginated. **Rosette cells** present. **Midrib** simple or complex.

*Notes.* (a) Within the bamboos, the papillae are usually only abaxial, however, the Guaduiniae are often an exception in having papillae as well as stomata on both surfaces. (b) Many herbaceous bamboos exhibit distinctive cross-shaped silica bodies in the costal zone (Judziewicz et al. 1999). (c) Within woody bamboos, fusoid cells are absent in the monotypic genus *Apoclada* McClure (Guaduiniae) (Guala 1995), two species of *Filgueirasia* Guala (Arthrostylidiinae) (Guala 2003), and a few species of *Chusquea* (Clark 1986); whereas, within herbaceous species, both species of *Ekmanochloa* Hitchc., *Mniochloa pulchella* (Griseb.) Chase (Zuloaga et al. 1993), some species of *Raddia* Bertol. (Calderón and Soderstrom 1967), and *Parodiolyra ramosissima* (Trin.) Soderstr. & Zuloaga (Soderstrom and Ellis 1987) lack these cells. (d) Midrib vasculature deserves attention in anatomical descriptions of this group. (e) A brief discussion about this subfamily was provided by Ellis (1987).

*Additional literature.* Renvoize (1987a); Vieira et al. (2002); Motomura et al. (2004); Santos-Gonçalves (2005); Silva Filho (2006); Triplett et al. (2006); Oliveira et al. (2008); Gomes and Neves (2009); Lizarazu (2012); BPG (2012); Fisher et al. (2009; 2014); Jesus Junior et al. (2012); Viana (2012); Viana et al. (2011, 2013a,b); Guerreiro et al. (2013); Mota (2013); Zhang et al. (2014); Clark et al. (2015).

## VI. POOIDEAE

*Surface view*: **Papillae** usually absent. **Stomatal apparatus** with parallel-sided subsidiary cells. **Microhairs** often absent, when present unicellular or bicellular. **Silica bodies** horizontally oriented; oblong-shaped or nodular-shaped. *Cross-section*: **Bulliform cells** adaxially present. **Mesophyll** non-radiate; adaxial palisade layer absent. **Kranz anatomy** absent. **Fusoid cells**

absent. **Arm cells** absent. **Rosette cells** usually present. **Midrib** simple.

*Notes.* (a) Unicellular microhairs have been observed in a few Stipeae. Bicellular chloridoid-type have been reported for *Lygeum* Loefl. ex L. and the panicoid-type for *Nardus* L. (GPWG 2001). (b) A brief discussion about this subfamily was provided by Ellis (1987).

*Complementary literature.* Dannenhoffer and Evert (1994); Aiken and Consaul (1995); Ramesar-Fortner et al. (1995); Martre and Durand (2001); Finot et al. (2005); Gielwanowska et al. (2005); Barkworth *et al.* (2007); Namaganda and Lye (2008); Namaganda et al. (2009); Pelegrin et al. (2009); Kuzmanović et al. (2012); Ortúñez and Cano-Ruiz (2013); Dani et al. (2014); Peterson and Soreng (2016).

*PACMAD clade (Sánchez-Ken and Clark 2010); Previously Reported as PACC (Davis and Soreng 1993), PACCAD (GPWG 2001), or PACCMAD (Sanchez-Ken et al. 2007): Aristidoideae, Panicoideae, Arundinoideae, Micrairoideae, Danthonoideae, and Chloridoideae*

## VII. ARISTIDOIDEAE

*Surface view:* **Papillae** absent. **Stomatal apparatus** with dome-shaped or triangular subsidiary cells. **Microhairs** often bicellular; panicoid-type. **Silica bodies** horizontally oriented; equidimensional or dumbbell-shaped. *Cross-section:* **Bulliform cells** adaxially present. **Mesophyll** often radiate (except in *Sartidia* De Winter); adaxial palisade layer absent. **Kranz anatomy** usually present (except in *Sartidia* and *Aristida longifolia* Trin.). **Fusoid cells** absent. **Arm cells** absent. **Rosette cells** present. **Midrib** simple.

*Note.* Although C<sub>3</sub> and C<sub>4</sub> (mainly) species are found in this subfamily, Kranz anatomy is not equally developed across the genera (GPWG 2001).

*Complementary literature.* Voznesenskaya et al. (2005); Cerros-Tlatilpa and Columbus (2009).



### VIII. PANICOIDEAE

*Surface view:* **Papillae** usually present. **Stomatal apparatus** with dome-shaped or triangular subsidiary cells. **Microhairs** often bicellular (rarely absent); panicoid-type. **Silica bodies** horizontally oriented; dumbbell-shaped, cross-shaped, or nodular-shaped. *Cross-section:* **Bulliform cells** adaxially present. **Mesophyll** radiate or non-radiate; adaxial palisade layer often absent (except in *Centotheca* Desv. and *Zeugites* P. Browne). **Kranz anatomy** present or absent. **Fusoid cells** often absent. **Arm cells** absent. **Rosette cells** present. **Midrib** simple (rarely complex).

*Notes.* (a) Fusoid cells have been reported in *Homolepis* Chase, *Streptostachys* Desv., and few species of *Panicum* L. (Watson et al. 1985; Clayton and Renvoize 1986; Killeen and Clark 1986; Zuloaga et al. 1992; GPWG 2001). (b) Physiologically, there are species C<sub>3</sub>, C<sub>4</sub> and C<sub>3</sub>/C<sub>4</sub> intermediates (GPWG 2001), and thus different patterns of mesophyll arrangement have been observed. (c) A brief discussion about this subfamily was provided by Ellis (1987)—including Centotheceae (previously Arundinoideae).

*Complementary literature.* Renvoize (1987b); Long et al. (1989); Dávila and Clark (1990); Dengler et al. (1994); Ueno (1995); Dengler et al (1996); Oliveira and Longhi-Wagner (1997); Zuloaga et al. (1992; 2006); Jankovsky et al. (2001); LeBlond (2001); Brito and Rodella (2002); Aliscioni et al. (2003); Ueno et al. (2006); Alvarez et al. (2008); Machado et al. (2008); Santos (2008); Vega et al. (2008); Guglieri et al. (2008); Christin et al. (2000; 2013); Chaves (2012); Eichemberg (2012); Vieira (2013); Vieira (2014); Alisconi et al. (2016).

### IX. ARUNDINOIDEAE

*Surface view:* **Papillae** often absent (except in *Amphipogon* R. Br.). **Stomatal apparatus** low dome-shaped or triangular subsidiary cells. **Microhairs** often bicellular (rarely absent); panicoid-type (except in *Amphipogon*). **Silica bodies** horizontally oriented; square-shaped,

oblong-shaped, cross-shaped, saddle-shaped; or dumbbell-shaped. *Cross-section*: **Bulliform cells** adaxially present. **Mesophyll** often non-radiate (except in *Arundo* L. and *Amphipogon*); adaxial palisade layer absent. **Kranz anatomy** absent. **Fusoid cells** absent. **Arm cells** absent. **Rosette cells** usually present. **Midrib** simple.

*Note.* A brief discussion about this subfamily was provided by Ellis (1987).

*Complementary literature.* (Barker 1995); Monteiro et al. (2015).

## X. MICRAIROIDEAE

*Surface view*: **Papillae** sometimes present. **Stomatal apparatus** with dome-shaped subsidiary cells. **Microhairs** often bicellular; panicoid-type. **Silica bodies** horizontally oriented; sharp-angled oryzoid-type, cross to square-shaped. *Cross-section*: **Bulliform cells** adaxially present. **Mesophyll** radiate or non-radiate; adaxial palisade layer present or absent. **Kranz anatomy** usually absent (except in *Eriachne* R. Br. and *Pheidochloa* S.T. Blake). **Fusoid cells** absent. **Arm cells** absent. **Rosette cells** usually present. **Midrib** simple.

*Complementary literature.* Sánchez-Ken et al. (2007).

## XI. DANTHONIOIDEAE

*Surface view*: **Papillae** often absent (except in *Chionochloa* Zotov and *Merxmuellera* Conert). **Stomatal apparatus** with dome-shaped or parallel-sided subsidiary cells (rarely dome-shaped or slightly triangular). **Microhairs** often bicellular (rarely absent); panicoid-type. **Silica bodies** horizontally oriented; dumbbell-shaped. *Cross-section*: **Bulliform cells** adaxially present. **Mesophyll** radiate or non-radiate; adaxial palisade layer present or absent. **Kranz anatomy** absent. **Fusoid cells** absent. **Arm cells** absent. **Rosette cells** usually present. **Midrib** simple.

*Complementary literature.* Ellis (1988).

## XII. CHLORIDOIDEAE

*Surface view*: **Papillae** absent or present. **Stomatal apparatus** with dome-shaped or triangular subsidiary cells. **Microhairs** often bicellular; usually choroid-type. **Silica bodies** equidimensional; saddle-shaped, square-shaped, angular-shaped, round-shaped, dumbbell-shaped. *Cross-section*: **Bulliform cells** adaxially present. **Mesophyll** usually radiate; adaxial palisade layer absent. **Kranz anatomy** often present (except in *Ellisochloa*). **Fusoid cells** absent. **Arm cells** absent. **Rosette cells** usually present. **Midrib** simple.

*Notes*. (a) *Ellisochloa* P.M. Peterson & N.P. Barker exhibits irregular palisade-like chlorenchyma surrounding the vascular bundles (C<sub>3</sub> photosynthetic pathway). (b) A brief discussion about this subfamily were provided by Ellis (1987).

*Complementary literature*. Peterson et al. (1989); Peterson and Herrera-Arrieta (2001); Ingram (2010); Ingram et al. (2010); Peterson et al. (2011); Giraldo-Cañas et al. (2012); Jattisha and Sabu (2012); Grigore et al. (2014); Favaretto et al. (2015).

### Future Prospects for Investigating Grass Leaf Blade Anatomical Features

Since Ellis's work (1987), many studies including leaf blade anatomical descriptions have been published. Although most of them performed by anatomists, some of them are carried out by taxonomists and physiologists, or even by anatomists who do not have grass anatomy as their primary focus. Taxonomists usually carefully describe all details as seen in surface view and cross-sections, providing good sources of information in defining and delimiting taxa (e.g., Oliveira et al. 2008; Guglieri et al. 2008; Pelegrin et al. 2009; Viana 2011; Mota 2013). Physiologists, although not focused on descriptions, also provide useful information. Functional anatomy, for instance, has revealed interesting patterns of mesophyll cell shape and arrangement in rice (*Oryzoideae*) (Sage and Sage 2009). These kind of studies are becoming increasingly welcoming, however, terminological standardization is probably the big challenge

in the grass anatomy, being extremely important in providing data for a more natural classification. Therefore, for description purposes, we strongly recommend the use of Ellis's work (1976; 1979), who provided a procedure for standardizing comparative leaf anatomy in the family.

This review shows anatomical similarities among groups, but also highlights diagnostic features in defining and delimiting species within Poaceae. Among the early-diverging lineages, although including few species, Puelioideae are relatively little known and thus need further anatomical studies. Within Bambusoideae (BOP clade), Temperate woody bamboos (I-XII lineages) and Paleotropical woody bamboos (Bambusinae, Hickeliinae, Racemobambosinae, and Melocanninae) should receive the highest priority, as well as the Neotropical woody genus *Chusquea* (Chusqueinae), which shows incongruences between morphological and molecular evidence (Fisher et al. 2009; 2014). Due to the number of taxonomic rearrangements and descriptions of new taxa in the Panicoideae (PACMAD clade), we would recommend further anatomical studies in this subfamily. Aristidoideae and Arundinoideae also deserve further studies mainly with regard to Kranz anatomy.

### **Final considerations**

Grass anatomy studies has been increasingly growing since 1987. Within the grasses, leaf blade anatomy data may reveal a unique combination of features common to major and minor groups. Stomatal apparatus bearing two papillae per subsidiary cell in *Chusquea* and intercostal fibers in Arthrostylidiinae are examples of how could leaf blade anatomy be highly relevant for defining and delimiting taxonomic groups. Advances in phylogenetics and microscopy methods place anatomists, taxonomists, and physiologists in an unprecedented position to dissect and understand grass relationships and to understand the functional importance of anatomical adaptations.

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# TABLES

Table 1. General summary of the anatomical features as seen in surface view, characteristic of the subfamilies and useful in defining and delimiting taxa of Poaceae. The subfamilial classification used in this table was based on Soreng et al. (2015). Putative and true synapomorphies are shown. Same color and character state within a same column may be interpreted as synapomorphic for major groups, herein referred to as early-diverging lineages, BOP clade, and PACMAD clade. **1.** Papillae; **2.** Subsidiary cell shape (stomatal apparatus); **3.** Stomatal apparatus bearing two or more papillae per subsidiary cell. **4.** Bicellular microhairs; **5.** Silica bodies shape. (+) present; (-) absent.

SUBFAMILIES	LEAF BLADE ANATOMICAL FEATURES AS SEEN IN SURFACE VIEW				
	1	2	3	4	5
<i>Early-diverging lineages</i>					
Anomochlooideae	(-)	low-dome shaped or triangular	(-)	(+)	equidimensional or vertically-unlobed
Pharoideae	(-)	parallel-sided to dome-shaped	(-)	(-)	horizontally oriented saddle-shaped
Puelioideae	often (-)	dome-shaped to triangular	(-)	(+)	vertically-oriented saddle-shaped
<i>BOP clade</i>					
Oryzoideae	(+)	dome-shaped or triangular	rarely (+) <i>Oryza</i>	(+)	horizontally or vertically oriented dumbbell or saddle-shaped (ozyzoid-type)
Bambusoideae	(+)	dome-shaped, triangular, or parallel-sided	(+) <i>Chusqueinae</i>	(+) panicoid-type	often vertically oriented saddle-shaped, dumbbell-shaped, cross-shaped; crenate-shaped (olyroid-type)
Pooideae	usually (-)	parallel-sided	(-)	often (-)	horizontally oriented oblong-shaped or nodular-shaped

*PACMAD clade*

Aristidoideae	(-)	dome-shaped or triangular	(-)	(+) panicoid-type	equidimensional or horizontally oriented dumbbell-shaped
Panicoideae	usually (+)	dome-shaped or triangular	(-)	(+) panicoid-type	horizontally oriented dumbbell-shaped, cross-shaped, or nodular-shaped
Arundinoideae	often (-)	low dome-shaped or triangular	(-)	often (+) panicoid-type	horizontally oriented dumbbell-shaped; cross to square-shaped
Micrairoideae	sometimes (+)	dome-shaped	(-)	(+) panicoid-type	horizontally oriented; sharp-angled oryzoid-type, cross to square-shaped
Danthonoideae	often (-)	dome-shaped or parallel-sided	(-)	(+) panicoid-type	horizontally oriented dumbbell-shaped
Chloridoideae	(+) or (-)	dome-shaped or triangular	(-)	(+) chloridoid-type	equidimensional round-shaped; cross to square-shaped

Table 2. General summary of the anatomical features as seen in cross-section, characteristic of the subfamilies and useful in defining and delimiting taxa of Poaceae. The subfamilial classification used in this table was based on Soreng et al. (2015). Putative and true synapomorphies are shown. Same color and character state within a same column may be interpreted as synapomorphic for major groups, herein referred to as early-diverging lineages, BOP clade, and PACMAD clade. **1.** Bulliform cells; **2.** Mesophyll; **3.** Adaxial palisade layer; **4.** Kranz anatomy; **5.** Fusoid cells; **6.** Arm cells; **7.** Rosette cells; **8.** Complex vasculature. (+) present; (-) absent.

SUBFAMILIES	LEAF BLADE ANATOMICAL FEATURES AS SEEN IN CROSS-SECTION							
	1	2	3	4	5	6	7	8
<i>Early-diverging lineages</i>								
Anomochlooideae	adaxially	non-radiate	(-)	(-)	(+)	rarely (+)	(+)	(+)
Pharoideae	(?)	non-radiate	(-)	(-)	(+)	(+)	(+)	(+)
Puelioideae	adaxially	non-radiate	(-)	(-)	(+)	(+)	(+)	often (+)
<i>BOP clade</i>								
Oryzoideae	adaxially	often non-radiate	(-)	(-)	often (-)	(-)	(+)	often (+) (-) Ehrharteae
Bambusoideae	adaxially	non-radiate	(-)	(-)	often (+)	(+)	(+)	often (+) (-) Arthrostylidiinae
Pooideae	adaxially	non-radiate	(-)	(-)	(-)	(-)	(+)	(-)
<i>PACMAD clade</i>								
Aristidoideae	adaxially	often radiate	(-)	(+)	(-)	(-)	(+)	(-)
Panicoideae	adaxially	non-radiate or radiate	often (-)	(+) or (-)	often (-)	(-)	(+)	often (-)

Arundinoideae	adaxially	often non-radiate	(-)	(-)	(-)	(-)	(+)	(-)
Micrairoideae	adaxially	non-radiate or radiate	(+) or (-)	usually (-)	(-)	(-)	(+)	(-)
Danthonoideae	adaxially	non-radiate or radiate	(+) or (-)	(-)	(-)	(-)	(+)	(-)
Chloridoideae	adaxially	usually radiate	(-)	(+)	(-)	(-)	(+)	(-)



**FIGURE**

**Fig. 1.** Main leaf blade anatomical features of Poaceae species. (A-H) cross-sections on light microscopy, (I-Q) surface view on scanning electron microscopy. (A) *Aulonemia pumila* L.G. Clark & Lodoño (Clark 382). (B) *Raddia brasiliensis* Bertol. (Clark & Attigala 1713); (C) and (D) *Chusquea longispiculata* L.G. Clark (Kuhlmann 3141); (E) *Chusquea tenella* Nees (Clark and Windisch 725); (F), (G) and (H) *Merostachys* sp. (Clark 639); (I) *Chusquea tenella* (Klein and Bresolin 7766); (J) *Chusquea ramosissima* Lindm. (Foster 76-35); (K) *Chusquea attenuata* (Doell) (Kuhlmann 2740); (L) and (M) *Chusquea tenella* (Swallen 9004); (N) *Chusquea ramosissima* (Klein and Klein 11041); (O) *Luziola bahiensis* (Steud.) Hitchc. (Guglieri 1610); (P) *Luziola subintegra* Swallen (Carvalho 326); (Q) *Luziola fragilis* Swallen (Guglieri 2432). Arrow = collapsed fusoid cell. *ac* arm cells; *cv* cavity; *if* intercostal fibers; *ma* unicellular microhair; *mi* bicellular microhair; *rc* rosette cell; *pr* prickle; *sb* silica body; *st* stomatal apparatus; *ut* unicellular trichome. Scale bars: 4  $\mu\text{m}$  (O); 5  $\mu\text{m}$  (I, J, K, L); 6  $\mu\text{m}$  (N); 8  $\mu\text{m}$  (P, Q); 15  $\mu\text{m}$  (C, D); 20  $\mu\text{m}$  (B); 30  $\mu\text{m}$  (M); 40  $\mu\text{m}$  (A, E); 50  $\mu\text{m}$  (F, G, H).

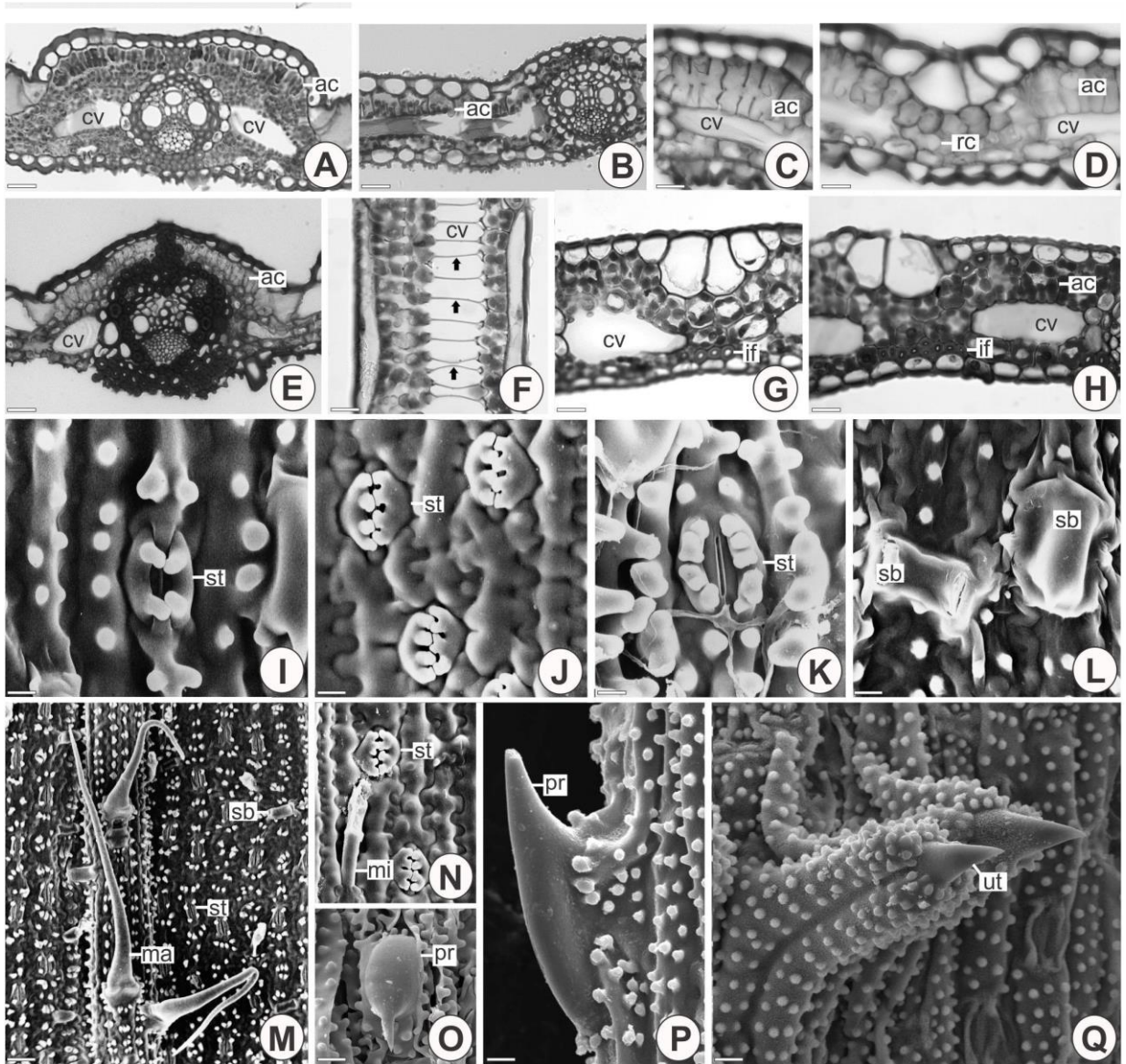


FIG. 1

## 5. CONSIDERAÇÕES FINAIS

Os dados aqui levantados por meio das espécies estudadas reforçam a importância da utilização de estruturas anatômicas da lâmina foliar na sistemática e filogenia de Poaceae.

Dentre os bambus neotropicais, estruturas anatômicas como fibras intercostais e nervura central simples são importantes para o reconhecimento de espécies de Arthrostylidiinae, assim como células subsidiárias com papilas e nervura central complexa para o reconhecimento de Chusqueinae. A presença de papilas nas células subsidiárias de complexos estomáticos foi recentemente considerada uma sinapomorfia para *Chusquea*, o que foi reforçado por ter sido observada em todas as espécies aqui estudadas pertencentes ao grupo. Por outro lado, as papilas refrativas, que são consideradas um caráter diagnóstico de Arthrostylidiinae e Guaduinae, devem ser reavaliadas quanto seu valor para a sistemática, pois, aparentemente, não apresentam distinções estruturais visíveis quando comparadas a uma papila considerada não-refrativa.

Embora não tenha sido possível levantar nenhum caráter possivelmente sinapomórfico para o clado II "*Chusquea ramosissima*", a anatomia da lâmina foliar mostrou-se útil para diferenciar espécies deste grupo e aquelas morfologicamente semelhantes. Somando-se a isso, uma nova espécie de *Chusquea* foi reconhecida baseada principalmente em características da epiderme, demonstrando a importância da superfície da lâmina foliar na sistemática do grupo.

O estudo do desenvolvimento foliar com ênfase nas células fusoides revelou sua origem proveniente do meristema fundamental, bem como homologias dentro do clado graminídeo. Dados ultraestruturais evidenciaram o armazenamento de grânulos de amido e sua posterior degradação no interior das células fusoides durante o desenvolvimento e expansão da lâmina foliar, sugerindo a função armazenadora e nutricional destas células em folhas jovens. A anatomia mostrou nova interpretação para essas células, assim como sua possível evolução a partir das células incolores, observadas em Joinvilleaceae. Considerando que as células fusoides são um caráter sinapomórfico para Poaceae e tem seu desenvolvimento influenciado por fatores

ambientais, os dados aqui apresentados contribuem para o entendimento da evolução e diversificação da família.

A revisão da aplicabilidade de estruturas anatômicas da lâmina foliar para a sistemática de Poaceae a partir dos estudos de Ellis (1987) permitiu indicar grupos com alta prioridade de estudo. Além disso, foram apresentadas descrições gerais de cada uma das 12 subfamílias e comentários sobre caracteres úteis ao reconhecimento de táxons, o que permitiu apontar possíveis sinapomorfias e também corroborar aquelas já reportadas na literatura.

O estudo da anatomia da lâmina foliar mostra-se cada vez mais importante na sistemática e filogenia de Poaceae. Com os avanços tecnológicos, dados em microscopia de luz ou em eletrônica de varredura e transmissão mostram-se extremamente informativos em busca da classificação mais natural do grupo. Estudos anatômicos ainda são necessários em representantes de Aristidoideae, Arundioideae, Bambusoideae (especialmente bambus temperados e paleotropicals), Micraioideae, Puelioideae e Panicoideae, fornecendo, portanto, um vasto campo para a formação de novos anatomistas especialistas em Poaceae.