Universidade de São Paulo

Desenvolvimento, anatomia e evolução de caracteres florais em Cyperaceae (Poales)

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DESENVOLVIMENTO, ANATOMIA E EVOLUÇÃO DE CARACTERES FLORAIS EM CYPERACEAE (POALES)

DEVELOPMENT, ANATOMY AND EVOLUTION OF FLORAL CHARACTERS IN CYPERACEAE (POALES)

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Desenvolvimento, anatomia e evolução de caracteres florais em Cyperaceae (Poales)

Development, anatomy and evolution of floral characters in Cyperaceae (Poales)

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Dedico aos pós-graduandos desse país, que mesmo com pouco incentivo, respeito e motivação, dedicam sua vida e sua saúde (mental e física) e movem a ciência no Brasil.

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CONTENTS

RESUMO	
ABSTRACT	
GENERAL INTRODUCTION	
LITERATURE CITED	
CHAPTER 1 - The effect of spatial constraints on changes in floral development of Cyperoideae spikelets (Cyperaceae)	
ABSTRACT 14 INTRODUCTION 15 MATERIAL AND METHODS 17 RESULTS 19 DISCUSSION 30 CONCLUSION 41 REFERENCES 43 APPENDIX 1 50	
CHAPTER 2 - Development and function of the stylopodium in Cyperoideae (Cyperaceae): a potential adaptative innovation	
ABSTRACT60INTRODUCTION61MATERIAL AND METHODS63RESULTS65DISCUSSION76CONCLUSION82LITERATURE CITED84	
CHAPTER 3 - Spicoid ontogeny in <i>Diplasia</i> (Cyperaceae): an approach on the developmental processes operating in Mapanioideae spicoids	
ABSTRACT93INTRODUCTION94MATERIAL AND METHODS98RESULTS99DISCUSSION105CONCLUSION116REFERENCES118	
CHAPTER 4 - Spicoid morphology of Mapanioideae (Cyperaceae): an evolutionary perspective	
ABSTRACT125INTRODUCTION126MATERIAL AND METHODS129RESULTS131DISCUSSION142CONCLUSION147	

GENERAL CONCLUSIONS	
Appendix 2	
Appendix 1	
LITERATURE CITED	

RESUMO

Cyperaceae possui ca. 5500 espécies distribuídas em 87 gêneros e divididas em duas subfamílias: Cyperoideae (76 gêneros) e Mapanioideae (11 gêneros). Inflorescências em Cyperaceae apresentam uma ampla variação em número de eixos laterais, ordens e comprimentos que resultam em uma variedade de arquiteturas, levando a muitas interpretações controversas. A família apresenta inflorescências descritas como panículas compostas com vários eixos laterais; entretanto, diferente da descrição usual de panículas, os eixos laterais não terminam em flores e sim, em estruturas reprodutivas que diferem entre as subfamílias. Em Cyperoideae, os eixos laterais terminam em espiguetas contendo várias flores. Em Mapanioideae, os eixos laterais terminam em espigas, que ao invés de conterem flores em cada bráctea, apresentam várias unidades denominadas espicoides. Os espicoides já foram previamente interpretados tanto como flores únicas, assim como inflorescências reduzidas. Considerando tais particularidades, esta tese teve como objetivo investigar os caracteres morfológicos de espiguetas e espicoides através de uma abordagem da anatomia, desenvolvimento e aspectos evolutivos. Espiguetas e espicoides de várias espécies foram analisados através de técnicas de microscopias de luz e eletrônica de varredura, testes fisiológicos e reconstruções de caracteres ancestrais. Os resultados mostraram que mudanças no início do desenvolvimento, principalmente reguladas por pressões mecânicas, parecem influenciar a variação no número e posição dos órgãos florais nas subfamílias. Além disso, caracteres florais (i.e. estilopódio) parecem ter uma notável influência no sucesso da germinação e na diversidade de gêneros de Cyperoideae. Para Mapanioideae, os resultados mostraram que processos no desenvolvimento sugerem ser consistentes na mesma tribo e o desenvolvimento dos espicoides do gênero monotípico Diplasia favoreceu sua interpretação como uma inflorescência reduzida. Finalmente, enfatiza-se o valor em entender as variações morfológicas dos espicoides em Mapanioideae como uma fonte de caracteres informativos para uma subfamília com notáveis relações filogenéticas inconclusivas. Tais resultados reforça a importância de combinar estudos anatômicos, ontogenéticos e evolutivos com o intuito de elucidar aspectos sobre diversidade e relações em uma família excepcional como Cyperaceae.

Palavras chave: Cyperaceae, Cyperoideae, Mapanioideae, ontogenia, anatomia.

ABSTRACT

Cyperaceae comprise ca. 5500 species distributed in 87 genera divided in two subfamilies: Cyperoideae (76 genera) and Mapanioideae (11 genera). Inflorescences in Cyperaceae exhibits a wide range of variation in number of lateral axes, orders and length that results in a variety of architectures, leading to many controversy and misinterpretation. The family exhibits inflorescences described as compound panicles with several lateral axes; however, different from the usual description of panicles, the lateral axes do not end in flowers, instead they hold reproductive units that differ between subfamilies. In Cyperoideae the lateral axes end in spikelets which hold several flowers. In Mapanioideae the lateral axes end in spikes, but instead of holding flowers in each bract, they hold several units known as spicoids. The spicoids have been interpreted either as a single flower or as a reduced inflorescence. Considering such particularities, this thesis aims to investigate the morphological features of spikelets and spicoids through a survey of their anatomical, developmental and evolutionary aspects. Spikelets and spicoids of several species were analyzed through scanning electron and light microscopy, added to physiological tests and ancestral character reconstruction. The results showed that changes in the beginning of development, mostly regulated by mechanical constraints, appears to influence the variation in number and position of floral organs in both subfamilies. Moreover, floral traits (i.e. stylopodium) seems to have a notable influence in the germination success and diversity of Cyperoideae genera. For Mapanioideae, our results show that developmental processes appear to be consistent within tribes and the development of the spicoid in the monotypic genus *Diplasia* favours to interpret it as a reduced inflorescence. At last, we emphasized the value of understanding the morphological variation of spicoids in Mapanioideae as a source of informative features to a subfamily with notable inconclusive phylogenetic relations. Such results reinforce the importance of combining anatomical, developmental and evolutionary studies in order to elucidate the diversity and relationships in an outstanding family such as Cyperaceae.

Keywords: Cyperaceae, Cyperoideae, Mapanioideae, ontogeny, anatomy.

General Introduction

GENERAL INTRODUCTION

Cyperaceae comprise ca. 5500 species distributed in 87 genera occurring in a wide variety of environments (Govaerts *et al.*, 2018). The family is recovered in two subfamilies, Cyperoideae and Mapanioideae (Simpson *et al.*, 2007; Muasya *et al.*, 2009, Hinchliff & Roalson, 2013; Semmouri *et al.*, 2019). Cyperoideae consist of 76 genera with a pantropical distribution among which *Carex* L. (1997 spp.), *Cyperus* L. (955 spp.) and *Rhynchospora* Vahl (361 spp.) exhibit the highest number of species (Goetghebeur, 1998; Simpson *et al.*, 2007; Muasya *et al.*, 2009; Govaerts *et al.*, 2018). Alternatively, Mapanioideae consist of 11 genera (five monotypic) with a more restricted distribution (Simpson *et al.*, 2007; Muasya *et al.*, 2009) being *Mapania* Aubl. (100 species) and *Hypolytrum* Pers. (61 species) the most species-rich genera (Simpson, 1992; Alves *et al.*, 2001; Govaerts *et al.*, 2018).

The differences between the subfamilies are not only restricted to species richness, but also concerning their particular inflorescence and floral features. The general architecture of Cyperaceae inflorescence consist of a main axis from which the ramifications of successive orders originate, thus the whole structure is known as a synflorescence. Each ramification of the synflorescence comprises a group of reproductive units, known as coflorescence (Goetghebeur, 1998; Vegetti, 2003; Guarise & Vegetti, 2008).

Cyperoideae synflorescences are interpreted as compound panicles with several lateral axes that terminate in spikelets, the smallest unit in the whole inflorescence. The spikelet is described as an open spike composed of an axis holding several bracts that may or may not subtend a flower (Eiten, 1976; Bruhl, 1995; Goetghebeur, 1998; Vegetti, 2003). Flowers in Cyperoideae exhibit a typical Bauplan of two trimerous perianth whorls, one whorl of stamens and a trimerous gynoecium (Vrijdaghs *et al.*, 2009). In

4

Mapanioideae, a synflorescence with a main axis holding several lateral axes resembles Cyperoideae architecture. However, in the former the lateral axes terminate in spikes which instead of holding flowers, it holds several reproductive units named spicoid (Kern, 1974; Simpson, 1992; Bruhl, 1995; Goetghebeur, 1998). The spicoids have been described as flowers, because of their structure composed of two bracts, two stamens and a central gynoecium (Kern, 1974; Eiten, 1976; Goetghebeur, 1998). However, for some species, the spicoids exhibit bracts between stamens and gynoecium, which raised questions whether it should be interpreted as a reduced inflorescence where each stamen is considered a unisexual male flower and the central gynoecium, a female flower (Dahlgren *et al.*, 1985; Simpson, 1992; Bruhl, 1995; Richards *et al.*, 2006; Prychid & Bruhl, 2013; Monteiro *et al.*, 2016, submitted).

Considering that spikelets (Cyperoideae) and spicoids (Mapanioideae) are the functional units of the inflorescence (Snell, 1936; Holttum, 1948; Eiten, 1976; Dahlgren *et al.*, 1985; Simpson, 1992; Vegetti, 2003; Vrijdaghs *et al.*, 2009), it is fundamental to investigate the morphological variation that undergoes in such structures in order to elucidate the possible factors influencing such changes and how consistent and informative they are for the relationships in each subfamily.

SPIKELET AND FLORAL VARIATION IN CYPEROIDEAE

Two main aspects are remarkable in Cyperoideae: 1) the wide diversity of floral morphology among and within genera and; 2) the high number of species concentrated in few genera (*Eleocharis* R.Br., *Fimbristylis* Vahl, *Rhynchospora* Vahl, *Bulbostylis* Kunth, *Carex* L., *Cyperus* L. and *Scleria* P.J.Bergius).

Variation on floral morphology in Cyperoideae

The morphological variation in the spikelets is mainly related to the arrangement of the bracts (glumes) along the axis (phyllotaxis), their fertility and floral sexuality (Dahlgren *et al.*, 1985). However, interpreting the morphology in mature flowers may hamper their understanding due to their reduced and complex nature. Therefore, it is fundamental to investigate processes occurring during the floral development, especially at the organ initiation, that may affect each floral whorl and consequently the final floral morphology (Ronse De Craene, 2016, 2018). It is well known that genetic interaction regulates the identity of floral organs, however morphological factors such as mechanical constraints of surrounding organs (i.e. bracts) are also fundamental in modulate the final morphology of the flowers (Chandler, 2014; Ronse De Craene, 2018). Such approach led to the first chapter of this thesis:

1. The effect of spatial constraints on changes in floral development of Cyperoideae spikelets (Cyperaceae) – This chapter focus on elucidating the influence of spatial constraints, mainly applied by glumes, on the further development of flower primordia in Cyperoideae species. Floral development and anatomy were investigated in species varying in phyllotaxis and floral sexuality. This study was developed in The Royal Botanic Garden, Edinburgh, Scotland (Mobilidade Internacional Santander) and it is under review in *The Botanical Journal of the Linnean Society*.

Morphological traits on the most diverse genera of Cyperoideae

It is notable that despite the diversity of genera in Cyperoideae, the highest species number are concentrated in about seven genera: *Carex* (1997 spp.), *Cyperus* (955 spp), *Rhynchospora* (361 spp.), *Fimbristylis* (321 spp.), *Eleocharis* (295 spp.), *Scleria* (258 spp.) and *Bulbostylis* (220 spp.) comprising together ca. 80% of the total number of species in the family (Escudero & Hipp, 2013). The major radiation of these genera occurred when the climate was becoming drier (Escudero & Hipp, 2013) which may be associated with the acquisition of some morphological traits that in the right ecological or genetic condition, may have improved their reproductive success (Ronse de Craene *et*

al., 2003; Endress, 2010a, 2011; Escudero & Hipp, 2013). Curiously, for these seven genera, except for *Carex* and *Cyperus*, a noticeable reproductive feature is shared: a thickening at the base of style right on top of the ovary apex, known as "stylopodium". Such structure occurs in non-phylogenetic related genera (Muasya *et al.*, 2009; Hinchliff & Roalson, 2013; Semmouri *et al.*, 2019). It becomes distinct in the mature flower and may be persistent (as a beak) in the mature nutlet (Cyperaceae fruit) or detach from the nutlet with the rest of the style (Vrijdaghs *et al.*, 2004, 2009; Gonzalez & López, 2010; Reutemann *et al.*, 2012; Monteiro *et al.*, 2017). The origin of the stylopodium is unclear and its function has been a matter of debate, mainly associated with a possible role in facilitating water entrance for seed germination, but never tested before. Such questions led to the second chapter of this thesis:

2. Development and function of the stylopodium in Cyperoideae (Cyperaceae): a potential adaptative innovation – This chapter aimed to analyze the stylopodium of five Cyperoideae species to assess the ontogenetic origin, identify the mechanism of detachment and any structure related to water supply. Moreover, we investigate the physiological influence of the stylopodium in seed germination. We intend to submit this study to *International Journal of Plant Sciences*.

SPICOID AND FLORAL VARIATION IN MAPANIOIDEAE

Mapanioideae exhibit a considerably lower number of genera and species compared to Cyperoideae (Goetghebeur, 1998; Govaerts *et al.*, 2018) and the latter comprises the vast majority of studies in terms of development of flower, inflorescence and phylogenetic relationship (i.e. Vrijdaghs *et al.*, 2004, 2005a, 2005b, 2007, 2009, 2010, 2011; Muasya *et al.*, 2009; Reutemann *et al.*, 2012; Hinchliff & Roalson, 2013; Monteiro *et al.*, 2017; Semmouri *et al.*, 2019). Therefore, many gaps remain towards the knowledge of Mapanioideae considering the nature and morphological variation of the

7

spicoids. Some aspects in this matter are remarkable such as: 1) It is unclear if a pattern of development is shared among Mapaniodeae spicoids. 2) Little is known about the morphological variation of the spicoids among genera and their significance in an evolutionary perspective.

Development patterns on Mapanioideae spicoids

Considering the patterns on floral development, in Cyperoideae despite the wide diversity in floral morphology, the floral development was reported as following a general scirpoid ontogenetic pattern, in which the first organs to appear are the stamen primordia, followed by gynoecium primordium and later on, if present, perianth parts (Vrijdaghs *et al.*, 2009). For Mapanioideae, developmental studies mainly focus on the debated nature of the spicoids as flowers or inflorescences (Richards *et al.*, 2006; Prychid & Bruhl, 2013; Monteiro *et al.*, 2016), lacking a comparative approach among the development processes in such species. Taking that in account, it led to the third chapter of the thesis:

3. Spicoid ontogeny in *Diplasia* (Cyperaceae): an approach on the developmental processes operating in Mapanioideae spicoids – This chapter has a complete ontogenetic analysis of the spicoid of the monotypic genus *Diplasia* and compare these data with other developmental studies for Mapanioideae (Richards *et al.*, 2006; Prychid & Bruhl, 2013; Monteiro *et al.*, 2016) in order to elucidate the variability of developmental features and their influence in the final morphology of Mapanioideae spicoids. Furthermore, we discuss evidence that may help to interpret *Diplasia* spicoid as a flower or inflorescence. This is study was developed in The Royal Botanic Gardens, Kew (Doutorado sanduíche – PDSE/ CAPES) and is under review in *Plant Systematics and Evolution*.

Morphological variation on Mapanioideae spicoids: an evolutionary perspective

General Introduction

It is notable the importance of understanding the morphological features of spicoids for each Mapanioideae genera, for which most of the information are restrict to taxonomic descriptions (i.e. Holttum, 1948; Kern, 1974; Eiten, 1976; Bruhl, 1995; Simpson, 1992; Goetghebeur, 1998; Alves *et al.*, 2001). The lack of an evolutionary perspective towards variation in the spicoid morphology also reflects in the high level of uncertainty in the phylogeny of Mapanioideae (Simpson *et al.*, 2007; Muasya *et al.*, 2009; Hinchliff & Roalson, 2013; Spanlik *et al.*, 2016; Semmouri *et al.*, 2019). The value of reproductive traits to establish relationships led to the fourth chapter of this thesis:

4. Spicoid morphology of Mapanioideae (Cyperaceae): an evolutionary perspective

– In this chapter we analyzed 49 species from the 11 genera of Mapanioideae, corresponding to ca. 27% of the total number of species from the subfamily. We focused on stable and variable features of the spicoids and mapped such features in the molecular phylogeny (Semmouri *et al.*, 2019), through a character reconstruction. Thus, we could infer about the ancestral spicoid morphology and their significance in an evolutionary perspective. This is study was developed at The Royal Botanic Gardens, Kew (Doutorado sanduíche – PDSE/ CAPES) and we intend to submit it to *American Journal of Botany*.

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Chapter 1

Chapter 1

The effect of spatial constraints on changes in floral development of

Cyperoideae spikelets (Cyperaceae)

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ABSTRACT

Spikelets in Cyperoideae vary mainly in the glume phyllotaxis and floral sexuality. Based in these features we investigated the floral development and anatomy of three cyperoid species to understand the possible influence of spatial constraints on floral development. The species showed different flower primordium shapes suggesting a relation with the glume subtending the flower. In *Pleurostachys sparsiflora*, the spirodistichously disposed glumes subtend triangular-shaped flower primordia, flattened adaxially and laterally, and rounded abaxially. Similar flower primordium shape is observed in *Cyperus eragrostis*. However, the latter species exhibit female flowers reduced to a single pistil, what could be related with particular features as distichous glumes and epicaulescent growth, possibly restricting organs emergence abaxially and laterally. Restriction on available space in the spherical flower primordium of *Carex brasiliensis* may explain the fusion of stamens sharing a single filament. Changes in the gynoecium morphology appears to be mainly influenced by mechanical constraints of surrounding organs on the abaxial side, such as for Cyperus and Pleurostachys. Thus, we suggest a relation between glume phyllotaxis, flower primordium geometry and available space for organs emergence, which together with genetic and biochemical interactions, influence the variation in number and position of organs in each floral whorl.

ADDITIONAL KEYWORDS: Cyperaceae – Cyperoideae – flower primordium – mechanical constraint – spikelet – sedges.

INTRODUCTION

The Cyperaceae is a large, widespread family, with ca. 5500 species in 87 genera (Govaerts *et al.*, 2018) distributed in two well established subfamilies Mapanioideae and Cyperoideae, which differ in the structure of their reproductive parts (Eiten, 1976; Bruhl, 1995; Goetghebeur, 1998; Simpson *et al.*, 2007; Muasya *et al.*, 2009, Hinchliff & Roalson, 2013; Semmouri *et al.*, 2019). While Cyperoideae exhibits flowers with a typical Bauplan of two trimerous perianth whorls, one whorl of stamens and a trimerous gynoecium (Vrijdaghs *et al.*, 2009), Mapanioideae exhibits a reproductive unit named spicoid that has a debatable nature of flower or inflorescence (Simpson, 1992; Richards, Bruhl & Wilson, 2006; Prychid & Bruhl, 2013; Monteiro, Scatena & Oriani, 2016).

Cyperaceae inflorescences are described as compound panicles of spikelets, the functional units of the whole inflorescence (Snell, 1936; Holttum, 1948; Eiten, 1976; Dahlgren, Clifford & Yeo, 1985; Vegetti, 2003; Vrijdaghs *et al.*, 2009). The spikelet in Cyperoideae is reported to be an open spike, with an acropetal maturation, composed of an axis (rachilla) and several bracts (glumes) that may or may not subtend a flower (Eiten, 1976; Bruhl, 1995; Goetghebeur, 1998; Vegetti, 2003). The variation among spikelets is mainly related to the arrangement of the glumes along the axis (phyllotaxis), their fertility and floral sexuality (Dahlgren *et* al., 1985).

Considering the sexuality of the flowers, their distribution along the spikelet is quite variable not only at species level but often in the same individual (Vrijdaghs *et al.*, 2009, 2010, 2011; Reutemann, Vegetti & Pozner, 2015; Lucero, Vegetti & Reinheimer, 2014). Such variation was related to the pollination mode in Cyperoideae (Friedman & Barrett, 2008, 2009; Wragg & Johnson, 2011). However, little is known about the processes occurring during the floral development, especially at the beginning of organ appearance, that may affect such floral features.

Regarding the factors acting within developing flowers, the spatial constraint applied by external organs reflect the development and shape of organs primordia, as well as their position and further changes in the beginning of development (Ronse De Craene, 2018). Also, such spatial constraint has influence later in development, affecting the shape and size of the inner floral organs, and consequently the final floral morphology (Ronse De Craene, 2018). For many Angiosperms this increasing constraint is applied by the perianth parts or subtending bracts, influencing the flower primordium shape in their axils and this is potentially reflected in the morphological variation of mature flowers (Ronse De Craene, 2016, 2018). In Cyperaceae, the perianth is usually reduced or absent (Vrijdaghs et al., 2009) and the flowers are enveloped by well-developed and condensed glumes, which are distributed mainly in two different phyllotaxis patterns along the spikelet axis: spirally or distichously (Vrijdaghs et al., 2009, 2010, 2011). It is known that the glume phyllotaxis not only affects the morphology of the spikelet itself, but also seems to influence the flowers as well (Vrijdaghs & Smets, 2017). Features such as distichous glumes with their bases fused to the rachilla and epicaulescent growth, a process where the margins of the glume of a flower expand and is positioned between the opposite flower and glume, playing a role not only in the spikelet morphology but also in the flower development (Vrijdaghs et al., 2011; Vrijdaghs & Smets, 2017).

The morphological variation in cyperoid flowers affects all whorls. The perianth exhibits a wide range of modifications from foliar to reduced bristle-like structures (Vrijdaghs *et al.*, 2009, 2010). The androecium usually consists of two latero-adaxial and one abaxial stamen (Vrijdaghs *et al.*, 2009, 2010) and the gynoecium varies in shape from trimerous triangular to dimerous dorsiventrally flattened or laterally flattened pistils (Vrijdaghs *et al.*, 2009, 2010; Reynders *et al.*, 2012).

The development of flowers of Cyperoideae was reported as following a scirpoid ontogenetic pattern, in which development starts with the appearance of stamen primordia, followed by gynoecium primordium and later on, if present, perianth parts (Vrijdaghs *et al.*, 2009). Nevertheless, it is expected that changes in the beginning of development, mainly related to available space on the flower primordium, may influence the position and time of floral organs origin, stablishing variations in developmental patterns at certain times during evolution.

Considering that changes in the beginning of development influence directly the final floral morphology, we have analyzed the floral development and anatomy of species that exhibit the major features that vary among Cyperoideae spikelets: phyllotaxis and floral sexuality. Therefore, we selected one species with spikelets composed of spirodistichously arranged bisexual flowers (*Pleurostachys sparsiflora* Kunth), one species with mostly unisexual, distichously arranged flowers (*Cyperus eragrostis* Lam.) and one species with spirally arranged unisexual flowers disposed in distinct inflorescences (*Carex brasiliensis* A.St.-Hil.), to answer the question: What is the influence of spatial constraints on the further development of flower primordia in Cyperoideae? It is expected that elucidating the relation of spatial constraints and available space at the beginning of the floral development will contribute to a better understanding of the morphological factors that appears to be related with changes in each floral whorl.

MATERIAL AND METHODS

MORPHOLOGICAL SAMPLING - Spikelets of *Pleurostachys sparsiflora* were collected in the Reserva Florestal Adolpho Ducke (Manaus, Amazonas, Brazil), in the humid understorey of the Amazon rainforest. Spikelets of *Cyperus eragrostis* were collected in a secondary forest at the University of São Paulo campus (São Paulo, SP, Brazil). The spikelets in *Cyperus* can be grouped in fascicles, which are of three types: prophyllar, serial and mixed (Guarise & Vegetti, 2008) and for this work, we used spikelets from prophyllar fascicles (spikelets in axis that exhibit a prophyll in its base). Spikelets of *Carex brasiliensis* were collected in the Altitude Fields (with grassland vegetation) in the Parque Nacional do Itatiaia (Itatiaia, Rio de Janeiro, Brazil). For each species, we sampled at least five individuals, from at least two populations. Vouchers were deposited in the herbarium SPF (University of São Paulo – Brazil) (*Pleurostachys sparsiflora –* MM/AG/JE 248; *Carex brasiliensis –* MM/LL 257; *Cyperus eragrostis –*MM 259).

LIGHT AND SCANNING ELECTRON MICROSCOPY - Inflorescences and flowers at different developmental stages were fixed in FAA (formaldehyde, glacial acetic acid, 50% ethanol, 1:1:18 v/v) (Johansen, 1940) and stored in 70% ethanol for morphological and anatomical studies. Inflorescences with mature flowers were dissected under a Leica EZ4 stereomiscroscope (Leica Microsystems, Wetzlar, Germany) and photographs were taken using a Leica DFC 320 camera device (Leica Microsystems, Wetzlar, Germany) coupled to stereomicroscope Leica MZ8 (Leica Microsystems, Wetzlar, Germany), using Scan System Images (IM50).

Fixed spikelets at different developmental stages were dissected under a stereomicroscope Zeiss Stemi SV6 (Zeiss, Oberkochen, Germany). The material was dehydrated through an ethanol-acetone series, critical-point dried using CO₂ in a k850 KPD critical-point drier (Quorum Technologies, Kent, UK), coated with platinum using an Emitech k575x Sputter Coater (Quorum Technologies, Kent, UK) and examined with a LEO Supra 55VP scanning electron microscope (Zeiss, Oberkochen, Germany). In addition, transverse anatomical sections of the flowers were made, the samples were subjected to a *tert*-butyl alcohol dehydration series and embedded in Paraplast (Leica

18

Microsystems Inc., Heidelberg, Germany). The embedded material was sectioned at 5-7 µm on a rotary microtome (Leica Microsystems, Wetzlar, Germany), stained with astra blue and safranin (Gerlach, 1984) and mounted on slides with Permount. Light microscope micrographs were taken using a Leica DFC 320 camera (Leica Microsystems, Wetzlar, Germany) coupled to a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany), using Scan System Images (IM50).

The diagrams illustrating the spikelet architecture and floral vasculature of each species were made using the program Corel Drawn Graphics Suite X7.

RESULTS

Pleurostachys sparsiflora - Lateral spikelets in this species are composed of an indeterminate rachilla in the axil of a subtending glume, where the most proximal phyllome of the axis is a sterile prophyll followed by five to nine sterile glumes and the further distal glumes with flowers arising in their axils (Fig. 1A, B). The flowers are bisexual and possess a perianth consisting of two whorls of three bristles each, one whorl of three stamens and a dimerous dorsiventrally flattened pistil (Fig 1C).



Figure 1. Structure of spikelets and cross-section of flowers of cyperoid species. A-C *Pleurostachys sparsiflora*. A, Spikelet with glumes showing the presence of a subtending bract (sb) and a prophyll (pr). B, Axial scheme of a spikelet showing the subtending bract (sb) and prophyll (pr). C, Bisexual flower with outer (arrow) and inner (triangle) perianth parts, the abaxial outer perianth part (asterisk) placed closed to the abaxial inner one, three stamens and a gynoecium. D-G *Cyperus eragrostis*. D, Spikelet with glumes showing the presence of a prophyll (pr), the arrow points to a single bisexual flower among the female flowers. E, Axial scheme of a spikelet showing the subtending bract (sb) and the prophyll (pr). F, Female flower; note the region of fusion of glume wings with the rachilla (asterisks). G, Female flower subtended by a fertile glume (fg). fg, fertile glume; ov, ovary; pr, prophyll, s, stamen; sb, subtending bract. Scale bars: 1mm (A, D); 100μm (C, F, G).

The glumes emerge in a spirodistichous sequence on the rachilla (Fig. 2A). The flower primordium is flattened at adaxial and lateral sides and rounded at abaxial side, acquiring a triangular shape (Fig. 2A – coloured) in the axil of the glume that subtends it

(Fig. 2A - numbered). Each glume partially envelops the subsequent glume (Fig. 2A). Two lateral-adaxial perianth primordia appear followed by the third one in abaxial position on the early developing flower that keeps the triangular shape (Fig. 2B – arrows). The abaxial one remains undeveloped during the appearance of subsequent organs (Fig. 2C, D, F – arrow). The outer perianth is followed by the emergence of a larger adaxial inner perianth primordium (Fig. 2B – triangle), followed by two less developed abaxial ones (Fig. 2C-E – triangle). Two adaxial stamen primordia appear slightly before the abaxial one (Fig. 2B-D). With the inner perianth whorl and stamen primordia appearing, both whorls become apparently compressed into a single whorl (Fig. 2E, F). The development of the inner perianth whorl is delayed compared to the stamens (Fig. 2E, F), and the perianth parts are visible as small floral appendages. The abaxial stamen also has a delayed development as compared to the abaxial stamens (Fig. 2H). The abaxial outer perianth part does not develop (Fig. 2F – arrow) or is strongly delayed and visible as a small appendage at maturity (Fig. 2I – asterisk).

After the initiation of perianth and stamens, the floral apex differentiates into an elliptical ovary wall primordium surrounding a central depression from which a central ovule develops, and two stigmas appear as two lateral projections (Fig. 2F, G). The gynoecium assumes a dorsiventrally flattened shape during development. Later, the perianth parts develop into papillose bristles (Fig. 2H, I).



Figure 2. Scanning electron micrographs of the bisexual flower of *Pleurostachys sparsiflora* at different developmental stages. A, Spikelet axis with spirodistichous glumes, each glume (numbered) and subtending a floral primordium (fp - coloured). B, Initiation of the outer perianth parts (arrow), the adaxial inner perianth parts (triangle) and the adaxial stamen primordia. C-D, Initiation of the abaxial inner perianth parts (triangle) and simultaneous inception of the abaxial stamen primordium. Note that the abaxial outer perianth part is delayed relative to the adaxial ones. E, Stamen primordia surrounding a central raised area. F-G, Development of the ovary wall primordium surrounding the central ovule. H, Mature flower without glume showing stamens with basifixed anthers and papillose structures on the perianth parts. I, Mature flower showing the outer (arrow) and inner (triangle) perianth parts and a weakly developed abaxial outer perianth part (asterisk). c, carpel lobe; fp, flower primordium; s, stamen primordium. Scale bars: 20µm (A); 10µm (B-H); 100 µm (I).

Cyperus eragrostis - The spikelets of *Cyperus eragrostis* are composed of a hyaline prophyll, smaller and thinner than the distal fertile glumes (Fig. 1D, E). Most of the

flowers are female, composed of one pistil (Fig. 1F, G); bisexual flowers, composed of one stamen and one pistil, occur randomly in the spikelet (Fig. 1D - arrow).

The glumes are distributed in a distichous arrangement on the rachilla (Fig. 3A). The flower primordium arises in the axil of a glume in which the wings expand at the base and not only envelop but also fuse to the rachilla (Figs 1F – asterisk; 3A, B – arrow). In the bisexual flower, a lateral stamen develops concomitantly with the gynoecium (Fig. 3C). During development, the glumes elongate and partially envelop the alternate new formed glume (Fig. 3D – arrow). In the female flower, the floral development starts with the appearance of an elliptical flower primordium, flattened adaxially and rounded laterally and abaxially (Fig. 3D) that differentiates into a circular ovary wall primordium surrounding a central depression from which a central ovule develops (Fig. 3E). On this ring the stigmas appear as three projections, the abaxial one seems to be a little less developed than the latero-adaxial ones (Fig. 3E, F - triangle). The stigmas are lifted upwards by the growth of the ovary wall, progressively enclosing the central ovule (Fig. 3F). Papillose protuberances appear along the developed stigmas (Fig. 3G).



Figure 3. Scanning electron micrographs of the female flower of *Cyperus eragrostis* at different developmental stages. A, Spikelet axis with distichously arranged glumes (numbered) enveloping the flower primordia (coloured) and axis (asterisk), with expanded margins enveloping each other and positioned between lower flower primordium and its glume (arrow). B, Lateral view of the spikelet, showing the base of the glume fused to the rachilla and expanding towards the lower flower primordium (arrow). C, Bisexual flower showing a single lateral stamen and the trimerous pistil. D, Flower primordium (fp) subtended by a fertile glume (fg) showing the expanded margins of the opposite new glume formed in contact with the laterals of the flower primordium (arrow). E, Carpel lobes developing in the ovary wall surrounding the central ovule, note the glume (fg) subtending it. F, Development of the ovary wall and stigmas. Note that the abaxial stigma is less developed than the adaxial ones (triangle). G, Mature flower without glume showing papillose structures on the stigmas. c, carpel lobe; fg, fertile glume; fp, flower primordium; s, stamen. Scale bars: 10µm (A, C-F); 20 µm (B); 100µm (G).

Carex brasiliensis - Two to three female inflorescences surround a terminal male spikelet (Fig. 4A). The male flower is subtended by a glume (Fig. 4B) while the female flower is surrounded by a glume and a modified prophyll (Fig. 4C – dotted circle) and the whole structure represents a spike of spikelets (Fig. 4C). In both cases, the glumes are arranged spirally (Fig. 4D, E).



Figure 4. Diagram and cross section of the spikelet and mature flowers of *Carex brasiliensis*. A, Male spikelet in the center (ms) surrounded by female inflorescences (fi). B, Axial scheme of a male spikelet (ms) showing the fertile glumes subtending the flowers (fg). C, Axial scheme of a female inflorescences (fi) showing the individual spikelets (dotted circle) with their prophylls (pr) enveloping each flower and the subtending bracts (sb). D, Male spikelet with flowers spirally arranged. E, Female inflorescence with spikelets spirally arranged. fg, floral glume; fi, female inflorescence; ms, male spikelet; pr, prophyll; sb, subtending bract. Scale bars: 1mm (A); 200 μ m (D, E).

In the male flowers, the number of stamens varies between four (Fig. 5A), five (Fig. 5B) and six stamens (Fig. 5C), which may be free (Fig. 5A) or fused by their
filaments (Fig. 5B, C - arrow). Independently of the number of stamens, the initial divergence of the vascular traces starts with a vascular complex in the receptacle (Fig. 5D – circle). At a successive level, from the vascular complex, one vascular trace diverges to the glume (Fig. 5D- blue arrow) and two vascular traces to the lateral stamens (Fig. 5D- black arrows). In flowers with the lower number of stamens (four) (Fig. 5A), the anatomy shows the presence of four vascular traces in the receptacle, two lateral diverging to distinct filaments and remaining central abaxial and adaxial vascular traces (Fig. 5E), that later splits in another two distinct filaments, each with one vascular bundle (Fig. 5F) that will supply the anther (Fig. 5G). In flowers with a higher number of stamens (i.e., five stamens), the divergence of the vascular traces follows the same initial pattern, diverging first to the lateral stamens, leaving a central vascular system with one abaxial and two adaxial vascular traces (Fig. 5H). At a higher level four filaments are observed, the adaxial one with two vascular bundles (Fig. 5H, I - triangle). This filament with two vascular traces divides in separate stamens at the level of the anthers (Fig. 5J – triangle, K).



Figure 5. Morphology and cross section of mature male flowers of *Carex brasiliensis*. A, Mature male flowers with four stamens. B-C, Mature male flowers with five (B) and six (C) stamens; note the fused filaments (arrow). D, Cross section of a mature male flower showing that the initial divergence of the vascular traces starts with a vascular complex in the receptacle (circle). At a more developed level, one vascular trace diverges from the vascular complex to the glume (blue arrow) and two vascular traces to the lateral stamens (black arrows). E-G, Male flower with four stamens showing the floral receptacle splitting first to the lateral stamens, leaving a center with an abaxial and adaxial vascular trace (E), which will split in four filaments, each with a single vascular bundle (F) that will supply each anther (G). H-K, Cross section of a mature male flower with five stamens showing the lateral stamens, leaving a center with an abaxial and two adaxial vascular traces (triangle) (H), which will split in four filaments, one of them with two vascular bundles (triangle) (I); filaments remain attached until the level of the anthers (J), before completely separating in five stamens (K). Scale bars: 1mm (A-C); 200µm (D, I-K); 100µm (E-H).

The male spikelet exhibits a rachilla with flowers developing in the axil of glumes, which exhibit a narrowing apex (Fig. 6A, B - arrows). The flower primordium is spherical, rounded abaxially and adaxially, from which two lateral primordia appear first (Fig. 6B), followed by an abaxial and an adaxial primordium (Fig. 6C). All observed male flowers start the development with four stamen primordia (Fig. 6B, C) that will develop and may (or not) split at the level of the anthers resulting in flowers with four or more stamens.

On the female spikelets, the spherical spikelet primordium is rounded abaxially and adaxially, arising in the axil of a glume (Fig. 6D - arrow). A prophyll primordium appears as a broken ring expanding around the spherical flower primordium (Fig. 6E – coloured yellow). The two margins of the prophyll become connected on the abaxial side (Fig. 6F) and expand as a tube around the developing flower (Fig. 6G-J). The floral apex differentiates into an annular ovary wall primordium and the stigmas appear as two lateroadaxial and one abaxial projections (Fig. 6F). These projections surround a central ovule that develops before being completely enclosed by the ovary wall (Fig. 6F, G). The prophyll develops as a tubular structure, enclosing the entire flower, except for the tips of stigmas (Fig. 6H). When removing the prophyll, it is possible to observe a reduced rachilla that never exceeds the prophyll height (Fig. 6I). Later in development, the style and stigmas emerge beyond the tubular prophyll (Fig. 6J).



Figure 6. Scanning electron micrographs of the male flower and female spikelet of *Carex brasiliensis* at different developmental stages. A-C, Male Flower. A, Spikelet axis with glumes subtending the stamens, not the narrow tip of the glumes (arrow). B, Initiation of two staminal primordia from the flower primordium subtended by a glume (arrow). C, Flower primordium with four staminal primordia. D-J, Female spikelet. D, Spikelet primordium in the axil of a subtending bract (arrow). E, Initiation of the prophyll or utricle (yellow) as a ring around the flower primordium, all subtended by a bract (arrow). F, Gynoecium developing as the ovary wall surrounding the central ovule; the prophyll is highlighted in yellow and the subtending glume is indicated by an arrow. G-H, Growth of the carpel and the prophyll around the flower (yellow). I, Prophyll removed showing an undeveloped rachilla and the ovary wall. J, Mature female spikelet

with enclosing prophyll (yellow) and the subtended bract removed (arrow). c, carpel; ov, ovary wall; s, stamen primordium; sm, spikelet meristem; st, stigma; ra, undeveloped rachilla. Scale bars: 1mm (A); $20\mu m$ (B-I); $200\mu m$ (J).

DISCUSSION

Several factors throughout the development modulate the final morphology of the flowers, as for instance mechanical constraints applied by surrounding organs (Ronse De Craene, 2018). It is noteworthy the relation between how these constraints are distributed and the geometry of the flower primordium. Although the shape of flower primordium is affected by gene interactions regulating the identity of floral organs, mechanical constraints of surrounding organs (i.e. bracts) are also fundamental in influencing at different sites and affecting its geometry (Chandler, 2014, Ronse De Craene, 2018). We observed different shapes of flower primordium in the studied species and will here discuss their relation to the glumes subtending the flowers and how they seem to influence the floral development.

Shape of the flower primordium

In *Pleurostachys sparsiflora*, the glumes are spirodistichously arranged, each subtending a flower primordium which is flattened in the laterals and adaxially, and rounded abaxially, acquiring a triangular shape. In *Cyperus eragrostis* the flower primordium is similar to *Pleurostachys sparsiflora*, flattened adaxially and rounded abaxially, however the former is also rounded laterally, acquiring an elliptical shape developing in the axil of a distichous glume. In *Carex brasiliensis*, both male and female flowers exhibit spherical flower primordia that are rounded abaxially and adaxially arising in the axil of spirally arranged glumes. The round shape is more distinct in the female spikelets which is encircled by a tubular prophyll. The changes in the shape of the flower primordium is possibly one of several criteria affecting the available space for

organs to emerge and therefore, playing a fundamental role in guiding the development in each floral whorl (Claßen-Bockhoff & Meyer, 2016; Ronse De Craene, 2018).

Perianth

The perianth is known as the whorl with the greatest range of changes in Cyperoideae flowers (Vrijdaghs *et al.*, 2009, 2010). In the studied species, *Pleurostachys sparsiflora* is the only species with perianth, exhibiting two whorls of three perianth parts each. Also, it is the species with the triangular flower primordium that is expanded laterally and adaxially, which means more available space for organs emergence. The triangular shape of such primordium allows a greater available area at adaxial and lateral sides, when compared to the abaxial area and this is reflected in the unidirectional initiation of floral organs starting from the adaxial side to the abaxial side. This restriction in space on the abaxial site is possibly a matter of spatial constraint applied by the glume in direct contact with this site. This leads, for instance, in the outer abaxial perianth part being weakly developed and displaced, which appears to have five perianth parts as reported in taxonomic descriptions (Thomas & Alves, 2008; Alves & Thomas, 2015), instead of six as observed here.

The sequence of development of the perianth in the species starts with the perianth parts appearance before all stamens are formed, as was also observed for *Oreobolus* (Mora-Osejo, 1967). Although the short plastochrons between the appearance of floral parts, which arise nearly simultaneously, may hamper the interpretation of the ontogenetic sequence, it is possible to observe an ontogenetic sequence in the perianth of *Pleurostachys sparsiflora* different from the typical pattern, where the formation of perianth parts starts after stamens are formed (Vrijdaghs *et al.*, 2009, 2010).

The perianth in Cyperoideae exhibits a wide range of modifications, not only in development (Vrijdaghs *et al.*, 2009, 2010), but the presence/absence of perianth in

cyperoid flowers is quite labile (Appendix 1) and its absence seems to be consistent for tribes as Cypereae (Vrijdaghs et al., 2005b, 2009, 2011; Bauters et al., 2014; Reutemann et al., 2014), Cariceae (Smith, 1966; Smith & Faulkner, 1976; Vrijdaghs et al., 2009, 2010; Gehrke et al., 2012) and Abildgaardieae (Reutemann et al., 2015). Despite this variability, it is notable that species which exhibit perianth whorls also exhibit a primordium similar to *Pleurostachys sparsiflora*, that is flattened adaxially and expanded in the laterals with less space abaxially, acquiring a triangular shape subtended by rimlike glumes, usually spirally arranged (Appendix 1), as observed for species in tribe Dulicheae, Scirpeae (Vrijdaghs et al., 2005a), Schoeneae (Vrijdaghs et al., 2007), Rhynchosporeae (Lucero et al., 2014; Monteiro, Scatena & Oriani, 2017), Fuireneae (Vrijdaghs et al., 2004) and Eleocharideae (Vrijdaghs et al., 2009; San Martin, 2014). This could be linked to the availability of space for those organs to develop. In tribe Rhynchosporeae, where *Pleurostachys sparsiflora* belongs, the flower primordium with a triangular shape is a consistent feature among genera and species, as well as the presence of perianth, trimerous androecium and dimerous dorsiventrally flattened pistil (Appendix 1) (Lucero et al., 2014; Monteiro et al., 2017).

It is notable in the studied species, comparing the flower primordium of *Pleurostachys sparsiflora* with *Cyperus eragrostis*, that both exhibit flower primordia that are flattened adaxially and rounded abaxially. However, when comparing the final morphology, flowers in the former species exhibit the trimerous tetracyclic Bauplan, while female flowers in the latter species exhibit a single pistil. Among the genetic and morphological factors acting in such flowers, the divergences in the phyllotaxis and morphology of the glumes subtending their primordia is remarkable and may be affecting the available space for organs to emerge.

Chapter 1 – Floral variation in Cyperoideae

In the female flowers of *Cyperus eragrostis* the flower primordium shape may be affected by a process called epicaulescent growth which occurs due the fusion of the base of the new formed glume to the apex of the rachilla. This rachilla grows and the new formed glume is lifted. Moreover, the margins of this glume (attached to the rachilla) expand and partially envelop the lower flower primordium. Thus, the expanded margins of the new formed glume are positioned between the lower flower primordium and its glume (Vrijdaghs *et al.*, 2009, 2010, 2011; Vrijdaghs & Smets, 2017) (Fig. 3D – arrow). Therefore, for the flower primordium in *Cyperus eragrostis*, spatial constraints from the surrounding bract influence the abaxial side and also the lateral sides (due to the expanded margins of the opposite glume), which may be one of the reasons restricting the available space for organs to be formed. This is different from *Pleurostachys sparsiflora* in which the glumes are arranged spirodistichously and their margins do not expand or fuse to the rachilla, thus constraint on the flower primordium is possibly greater in the median plane in direct contact with the glume and lower in the lateral plane, with flowers showing reductions tendencies restrict to the abaxial side.

Such relation between glume phyllotaxy and shape of the flower primordium is also observed in other Cyperoideae. Spikelet with distichous phyllotaxis, where glumes are fused to the rachilla at the base is a common feature found in some genera of Cypereae clade as *Cyperus*, *Kyllinga* and *Pycreus* (Appendix 1) (Vrijdaghs *et al.*, 2009, 2010, 2011; Muasya, Musili & Vrijdaghs, 2012). In these genera, at the beginning of floral development, the flower primordium is in contact with the surrounding glumes in the same pattern as *Cyperus eragrostis*, not only abaxially but also in the laterals. Curiously, such species exhibit flowers reduced in number of organs, mainly found at tribe Cypereae, such as *Cyperus luzulae*, *Ascolepis brasiliensis*, *Lipocarpha rehmanii* and species of *Pycreus* (Vrijdaghs *et al.*, 2011; Reutemann *et al.*, 2014) (Appendix 1). Although *Ascolepis* and *Lipocarpha* do not have spikelets with distichously arranged glumes, in *Ascolepis brasiliensis* the glume completely envelop the flower primordium abaxially and adaxially, encircling it (Reutemann *et al.*, 2014). In *Lipocarpha* the spikelets are single-flowered and the flower primordium is not only subtended by a glume, but also by a prophyll and reductions are reported in the number of floral organs (Vrijdaghs *et al.*, 2010; Bauters *et al.*, 2014).

The presence of a glume and a prophyll surrounding the primordium was also observed here for female spikelets of *Carex brasiliensis*. Since the beginning of development, a rounded spikelet primordium arises, from which a modified-tubular prophyll appears first. The prophyll appears to constraints uniformly in all sites on the flower primordium, as a ring, maintaining a rounded shape abaxially and adaxially, which means less available space for organs to be formed. The constraint could explain the reduction on the number of floral organs (perianth and androecium), exhibiting flowers composed of a single pistil. The rounded shape primordium seems to be a common feature in species exhibiting glume and prophyll surrounding it, such as in *Carex brasiliensis*, *Ascolepis brasiliensis* and some species of *Lipocarpha* (Appendix 1) (Vrijdaghs *et al.*, 2010; Bauters *et al.*, 2014; Reutemann *et al.*, 2014).

For the male flowers of *Carex brasiliensis*, the flower primordium varies from other Cariceae species (Appendix 1), and the flowers are composed of only stamens, varying from four to six stamens per flower. Among several factors, this variation in number of stamens may be linked to the absence of a perianth, which is known to influence and regulate the formation, position and number of organs in inner whorls (Endress, 1989; Ronse De Craene, 2008, 2016, 2018).

It is notable that when describing *Carex brasiliensis* female flowers the term "spikelet primordium" is used. This is because in the species the tubular prophyll envelop

a female flower and an undeveloped rachilla (Fig. 6I), therefore each prophyll with a female flower and an undeveloped rachilla is considered a spikelet, composed of a single flower, as described by Dahlgren et al. (1985). Such characterize each flower as a single spikelet and the whole inflorescence as a spike of spikelets (Snell, 1936; Kukkonen, 1984; Reznicek, 1990; Molina, Carmen & Llamas, 2012).

Androecium

Similar to the perianth, the number of stamens is one of the most common features varying in Cyperoideae flowers, which can commonly range from zero to four stamens per flower (Vrijdaghs *et al.*, 2009, 2010, 2011). In the studied species only *Pleurostachys* maintain the trimerous condition on the androecium, with reduction tendencies in the abaxial side (abaxial stamen less developed), while in *Cyperus eragrostis* flowers are mostly female. However, bisexual flowers may occur in the spikelet of *Cyperus eragrostis* composed of one stamen and one pistil, a pattern previously reported for the species, with no correlation between sex and position of flowers in the spikelet (Barnard, 1957). Such differences in the number of stamens may be related, among other factors, with the differences in the available space for such organs to be formed, as above discussed for both species. Flowers with reduction and even absence of stamens, as in *Cyperus eragrostis*, is found mainly at tribe Cypereae, observed in species with distichous arranged glumes such as *Cyperus luzulae*, *Ascolepis brasiliensis*, *Lipocarpha rehmanii* and species of *Pycreus* (Vrijdaghs *et al.*, 2011; Reutemann *et al.*, 2014) (Appendix 1).

However, it is noteworthy the fact that in male flowers of *Carex brasiliensis* the number of stamens varies from four to six whilst in *Pleurostachys sparsiflora* the number of stamens is restricted to three. This statement contrast with the fact that *Pleurostachys sparsiflora* with an expanded floral primordium shows a lower number of stamens than the rounded shape flower primordium in male flowers of *Carex brasiliensis*. This raise

question towards other morphological factors that may be acting in such variation besides available space, as the influence of other floral whorls.

In *Pleurostachys sparsiflora*, the flowers exhibit all floral whorls and the trimerous condition of the androecium may be influenced by the space restrictions of perianth parts developing at the edges of the floral primordium, regulating the position and number of stamens to be formed. A relation in presence of perianth parts and stability in stamens number is reported for angiosperms, showing that the first organs formed at the edges of the flower primordium are fundamental in regulating the inner floral organs number and position (Endress, 1989; Ronse De Craene, 2008, 2016, 2018). Although genetic factors play an important role in affecting the space of organs formation in the flower primordium, mechanical constraints are linked with changes in the number and position of such organs (Chandler, 2014). This is exactly what is observed here, while the trimerous condition in the perianth of *Pleurostachys sparsiflora* regulate the trimerous condition of the androecium in such flowers, in male flower of *Carex brasiliensis*, where perianth parts are absent, there is a chaotic and lability in the number of stamens to be formed, possibly due to the fact that stamens are the only floral organs in such flowers.

In tribe Cariceae, male flowers in genera such as *Carex* and *Uncinia* usually exhibit three stamens (Smith, 1966; Smith & Faulkner, 1976; Vrijdaghs *et al.*, 2010; Gehrke *et al.*, 2012) (Appendix 1). However, this number is quite variable in male flowers of *Carex brasiliensis* which may exhibit four to six stamens. During development of male flowers of *Carex brasiliensis*, although the variation on stamens number, the presence of two lateral stamens primordia emerging slightly before two median stamens is consistent among the flowers. No report of equal division of one primordium was here observed, even in flowers exhibiting more than four stamens in maturity. Such features raise question towards considering the increase on number of stamens being a result of

dédoublement, since this process is described when a single primordium is divided into two equal daughter primordium (Ronse De Craene & Smets, 1993a), what was not observed for male flowers of *Carex brasiliensis*.

The anatomy of these male flowers showed that the vascular traces diverge from the central vascular system of the receptacle first to the glume, followed by the two lateral stamens, the same sequence in which these organs emerge. From that level, it is possible to observe the vascular traces of the abaxial and adaxial stamens, again the same sequence of stamens emergence during development. This sequence of vascular divergence and primordium emergence is the same for all flowers, independent of the number of stamens. The difference relies that in the flowers with more than four stamens, the abaxial or adaxial stamens receive more than one vascular trace. The presence of one filament with two or three vascular bundles is an evidence that more than one stamen is fused, since stamens are known to be vascularized by a single bundle (Puri, 1951). Furthermore, it is possible to observe in the mature flowers, that the variation in the number of stamens is observed in the level of the anthers, since the fused stamens shares the same filament.

Therefore, based on our anatomical data and compared to what we observed in the development of these flowers, we believe that: 1) The increase of the number of stamens is due to an unequal division of stamen primordia, resulting in some stamens sharing the same filament; 2) Such instability on stamens number may be related to the absence of other floral whorls regulating its number and position, such as perianth parts; 3) the fusion of stamens is possibly triggered by space restrictions in the flower primordium thus, some stamens keep fused until the level of the anthers; 4) no case of *dédoublement* is reported, since no equal division of a single primordium was observed.

Smith (1966) reported that male flowers of some *Carex* species start their development growing apically first and then dividing in two unequal primordia, one

developing in one stamen and the other develops later, showing in the vasculature two stamens united by their filaments. Smith & Faulkner (1976) showed the same pattern of male flowers in *Carex flacca, Carex nigra* and *Carex panicea* with filaments of two stamens united and interpreted this as a delay in the second division of the primordium, as here observed.

Gynoecium

The influence of available space and spatial constraints do not only operate on the outer whorls, but also affect the center of the flower and consequently the shape of the gynoecium ((Ronse De Craene, Linder & Smets, 2002; Ronse De Craene, 2016; 2018).

The gynoecium in Cyperoideae flowers develops from a ring primordium surrounding a central meristematic zone (corresponding to the floral apex) which can be considered in the course of evolution as resulting from a congenital fusion of carpels (Reynders *et al.*, 2012). The annular ovary wall primordium was interpreted by these authors as the factor allowing shifts in the number and position of stigmas and as responsible for the variation in pistil types in Cyperoideae (trimerous, dorsiventrally or laterally flattened dimerous). Although shifts in pistil morphology often occurs in Cyperoideae (Reynders *et al.*, 2012), it is notable that most species exhibit trimerous pistils (Appendix 1) and since the position of styles and stigmas is strongly fixed and predictable in the floral meristem (Ronse De Craene *et al.*, 2002; Ronse De Craene, 2010), we believe that new gynoecium morphs are more likely to be a matter of mechanical constraint of the surrounding organs and available space for development.

Although most of Cypereae species exhibit a trimerous pistil, as in *Cyperus eragrostis*, it is notable that the abaxial stigma is slightly less developed than the adaxial ones. Furthermore, for the tribe, genus as *Pycreus* is the only one exhibiting dimerous laterally flattened pistils (Vrijdaghs *et al.*, 2011). In Cypereae, a combination of

distichously arranged glumes, fusion of the margins with the rachilla and metatopic displacements create very particular spikelets (Vrijdaghs *et al.*, 2011) and perhaps with some of the factors modulating the possible reductions at the gynoecium.

The presence of a subtending glume and a tubular prophyll observed in female spikelets of *Carex brasiliensis* are consistent features among female inflorescences in tribe Cariceae, found in genera as *Carex* and *Uncinia* where most of the female flowers have a trimerous pistil (Appendix 1) (Smith, 1966; Smith & Faulkner, 1976; Vrijdaghs *et al.*, 2009, 2010; Gehrke *et al.*, 2012). It is noteworthy that the trimerous gynoecium in *Carex brasiliensis*, different from *Cyperus eragrostis*, exhibits no clear evidence of mechanical constraints in the stigma development, possibly due to the fact that the modified tubular prophyll surrounds the flower primordium as a ring, exercising a uniform constraint at all sites. This suggest that the way constraints from surrounding organs is distributed in the primordium may influence different changes in the floral organs.

This is also observed in the variation of the pistil morphology in Cariceae species such as *Carex elata, Carex pendula* and species of *Schoenoxiphum* that have dimerous dorsiventrally flattened pistils (Vrijdaghs *et al.*, 2010; Gehrke *et al.*, 2012), the latter genus having bisexual flowers, an unusual feature among Cariceae (Gehrke *et al.*, 2012). For those species the flower primordium is not surrounded by a ring prophyll, as in *Carex brasiliensis*. In *Carex pendula*, the prophyll lacks the tubular morphology and forms a glume-like structure (Vrijdaghs *et al.*, 2010), in *Carex elata* (Smith & Faulkner, 1976) and *Schoenoxiphum* species (Gehrke *et al.*, 2012), the prophyll is develops late or is absent and the flower primordium is wrapped by a rim-like subtending glume. Such variation may change the sites of the flower primordium affected by spatial constraint of the glume-like prophyll, which is in contact with the median plane of the primordium and

possibly being one of the factor influencing the changes in abaxial side of the gynoecium of the species mentioned above.

In Pleurostachys sparsiflora, only two lateral carpel lobes are formed. Therefore, we believe that one of the possible causes for the dimerous dorsiventrally flattened pistil is the potential loss of a third carpel in the *Pleurostachys sparsiflora* gynoecium, as also reported for Rhynchospora species (Monteiro et al., 2017). This is possibly influenced by the spatial constraints of surrounding organs, causing the eventual sterilization of the abaxial carpel and its loss. This spatial constraint was suggested as one of the possible causes for the formation of a dimerous dorsiventrally flattened pistil in Cyperoideae. However, according to Reynders et al. (2012) a loss of carpels cannot be used as an explanation for the laterally flattened pistil and the shift in stigmas position is due to the annular ovary wall primordium that allows changes in number and position of organs. Evidence for a progressive reduction of the abaxial carpel exists in a number of Restionaceae (Linder, 1992; Ronse De Craene et al., 2002) and the transition from trimerous to dimerous ovaries rarely follows a stepwise pattern of sterilization preceding loss. Instead, it often jumps straight to the completely loss of a carpel, as reported for other Restionaceae species (Linder, 1992; Ronse De Craene et al., 2002; Fomichev et al., 2019).

Species of tribe Rhynchosporeae (Lucero *et al.*, 2014; Monteiro *et al.*, 2017), Dulichieae (Vrijdaghs *et al.*, 2005a) and some species of Eleocharideae (Vrijdaghs *et al.*, 2009; San Martin, 2014) with dimerous dorsiventrally flattened pistils, represent trimerous tetracyclic flowers, with all floral whorls present (Appendix 1). This may suggest that, together with genetic and biochemical factors, a higher number of floral organs and whorls possibly increases the spatial constraint towards the center of the flower, changing the morphology of the gynoecium since it is usually the last whorl to develop.

Changes in the geometry of flower primordium, external and internal constraints and timing of organs initiation are factors operating changes during development of flowers (Ronse De Craene, 2018) and the expansion of the flower primordium as a trigger to the appearance of new organs was previously reported in angiosperms (Claßen-Bockhoff & Meyer, 2016). Therefore, our data suggest that there is a relation between the arrangement of the glumes as a fundamental constraint operating in the flower primordium geometry. Reutemman *et al.* (2015) reported for Abildgaardieae species a change in the shape of the shoot apical meristem according to changes in leaf initiation arrangement showing a connecting between phyllotaxis and shape of the meristem. Moreover, our results suggest that changes in the flower primordium geometry seems to affect the available space for organs emergence, and together with genetic and biochemical interactions, influencing the variation on the number and position of floral organs to be formed in each floral whorl.

CONCLUSION

The results show distinct shapes of the flower primordium in each species and suggest that mechanical constraints from the glumes possibly influence such changes and seem to affect the development in each floral whorl. A flower primordia with a triangular shape, expanded adaxially and laterally was associated with more available space to organ emergence, leading to flowers with all floral whorls as *Pleurostachys*. Restrictions at lateral sides and spherical flower primordia were related to reduction in number of floral organs. Considering the number of stamens, the absence of other floral whorls as perianth appears to be one of the factors regulating the trimerous condition of the androecium in

Pleurostachys sparsiflora. The unequal division of the stamen primordium and available space may be one of the factors triggering the variation on the number of stamens in *Carex brasiliensis*. Considering the predictable position of stigmas in the meristem, we believe changes in the gynoecium appears to be mainly influenced by mechanical constraints of surrounding organs, especially in the abaxial side. Therefore, the present study suggest that there is a relation between the arrangement of the glumes as a fundamental constraint operating in the flower primordium geometry, which seems to affect the available space for organs emergence, and together with genetic and biochemical interactions, influencing the variation towards the number and position of organs in each floral whorl.

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AUTHOR CONTRIBUTIONS

MMM, AO and DD designed the research, MMM carried out the ontogenetic and anatomical lab work. MMM, AO, LRDC and DD integrated and interpreted the results on development and anatomy. All authors contributed with further discussion, suggestions and writing of the manuscript.

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Chapter 1 – Floral variation in Cyperoideae

Appendix 1. Survey of ontogenetic features in the main clades of Cyperoideae (Cyperaceae) base on ontogenetic research papers. (*) The shape of the flower primordium was interpreted based on the images in the literature consulted, since the description of the flower primordium shape is missing in some of these studies. (?) Missing description and images. Pres.= presence; Abs.= absence.

		Reference	Phyllotaxy (glumes)	Glume morphology	Change of flower	Perianth		— Androssium	
Tribe	Species				primordium*	Pres./ Abs.	Number	(number)	Gynoecium
	Ascolepis			Europhania dha	*C				D
	brasiliensis (Kunth) Donth	Doutomonn		Enveloping the	*Spherical (rounded				Dimerous
Cynerese	(Kullul) Dellul. ex C B Clarke	at al 2014	Spiral	faces of primordium	adaxially and abayially)	Absent	0	1	flattened
Cyperede	ex C.D. Clarke	<i>ei ui.</i> , 2014	Spirai	races of prinordium	abaxiany)	nosent	0	1	Hattened
									Trimerous
	Lipocarpha				*Spherical (rounded				(abaxial
~	humboldtiana	Reutemann			adaxially and		0	3 (2 laterals	stigma less
Cypereae	Nees	<i>et al.</i> , 2014	Spiral	Glume + prophyll	abaxially)	Absent	0	and I abaxial)	developed)
									Trimerous
									(eventually
	Lipocarpha				*Spherical (rounded				dimerous
~	rehmannii (Ridl.)	Bauters et	~	~	adaxially and				dorsiventrally
Cypereae	Goetgh.	al., 2014	Spiral	Glume + prophyll	abaxially)	Absent	0	0-1	flattened)
	Linocarnha nana				*Spherical (rounded				
	(A Rich)	Vriidaghs <i>et</i>			adaxially and				
Cypereae	Cherm.	<i>al.</i> , 2010	Spiral	Glume + prophyll	abaxially)	Absent	0	2	Trimerous
<i>.</i> 1		*	×					3 (2 laterals	
	Kyllinga			Expanded wings				and 1 abaxial	Dimerous
~	mbitheana	Muasya <i>et</i>		fused to the rachilla				less	laterally
Cypereae	Muasva	al., 2012	Distichous	by the base	?	Absent	0	developed)	flattened

Cypereae	Isolepis setacea (L.) R. Br.	Vrijdaghs <i>et</i> al., 2005b	Spirotristich ous	Rim-like	*Elliptical (Flattened abaxially and rounded adaxially)	Absent	0	3 (2 laterals and 1 abaxial)	Trimerous (presence of gynophore)
Cypereae	<i>Isolepis</i> antarctica (L.) Roem. & Schult.	Vrijdaghs <i>et</i> al., 2005b	Spiral	Rim-like	*Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	3 (2 laterals and 1 abaxial)	Trimerous (presence of gynophore)
Cypereae	<i>Ficinia</i> minutiflora C.B. Clarke	Vrijdaghs <i>et</i> al., 2005b	Spiral	Rim-like	*Spherical (rounded adaxially and abaxially)	Absent	0	3 (2 laterals and 1 abaxial)	Trimerous (presence of gynophore)
Cypereae	<i>Ficinia brevifolia</i> Nees ex Kunth	Vrijdaghs <i>et</i> al., 2005b	Spirotristich ous	Rim-like	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	3 (2 laterals and 1 abaxial)	Trimerous (presence of gynophore)
Cypereae	<i>Ficinia zeyheri</i> Boeckeler	Vrijdaghs <i>et</i> <i>al.</i> , 2005b	Spiral	Rim-like	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	3 (2 laterals and 1 abaxial)	Trimerous (presence of gynophore)
Cypereae	Ficinia gracilis Schrad.	Vrijdaghs <i>et</i> <i>al.</i> , 2005b	Spiral	Rim-like	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	3 (2 laterals and 1 abaxial)	Trimerous (presence of gynophore)
Cypereae	Cyperus luzulae (L.) Rottb. ex Retz.	Vrijdaghs et al., 2011	Distichous	Rim-like, expanded wings fused to the rachilla by the base	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	0-1	Trimerous
Cypereae	Cyperus capitatus Vand.	Vrijdaghs <i>et</i> al., 2011	Distichous	Rim-like, expanded wings fused to the rachilla by the base	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	3 (2 laterals and 1 abaxial)	Trimerous

Cypereae	Cyperus laevigatus L.	Vrijdaghs <i>et</i> <i>al.</i> , 2009, 2011	Distichous	Rim-like, expanded wings fused to the rachilla by the base	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	3 (2 laterals and 1 abaxial)	Dimerous dorsiventrally flattened
Cypereae	Pycreus pumilus (L.) Nees	Vrijdaghs <i>et</i> <i>al.</i> , 2011	Distichous	Rim-like, expanded wings fused to the rachilla by the base	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	0-2 (number varies within same spikelet)	Dimerous laterally flattened
Cypereae	<i>Pycreus</i> <i>pelophilus</i> (Ridl.) C.B. Clarke	Vrijdaghs <i>et</i> <i>al.</i> , 2011	Distichous	Rim-like, expanded wings fused to the rachilla by the base	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	2 (lateral)	Dimerous laterally flattened
Cypereae	Pycreus polystachyos (Rottb.) P. Beauv.	Vrijdaghs <i>et</i> <i>al.</i> , 2011	Distichous	Rim-like, expanded wings fused to the rachilla by the base	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	1-2 (lateral)	Dimerous laterally flattened
Cypereae	Pycreus bipartitus (Torr.) C.B. Clarke	Vrijdaghs <i>et</i> <i>al.</i> , 2011	Distichous	Rim-like, expanded wings fused to the rachilla by the base	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	2 (lateral)	Dimerous laterally flattened
Cypereae	Pycreus flavescens (L.) P. Beauv. ex Rchb.	Vrijdaghs <i>et</i> al., 2011	Distichous	Rim-like, expanded wings fused to the rachilla by the base	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	3 (2 laterals and 1 abaxial)	Dimerous laterally flattened
Cypereae	Pycreus sanguinolentus (Miq.) H.B. Naithani & S. Biswas	Vrijdaghs <i>et</i> <i>al.</i> , 2011	Distichous	Rim-like, expanded wings fused to the rachilla by the base	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	3 (2 laterals and 1 abaxial)	Dimerous laterally flattened

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Chapter 1 – Floral variation in Cyperoideae

		Smith.							
	Carex pendula	1966/			* Elliptical (flattened				
	Huds.(male	Vrijdaghs <i>et</i>			adaxially and rounded				
Cariceae	flower)	al., 2010	Spiral	Glume-like	abaxially/laterally)	Absent	0	3	Absent
		Smith							
	Carex pendula	1966/		Not completely					Dimerous
	Huds (female	Vriidaghs <i>et</i>		tubular glume-like					dorsiventrally
Cariceae	flower)	<i>al.</i> , 2010	Spiral	adaxially	Conical	Absent	0	Absent	flattened
)	Smith,	~ F	j			Ū		
	Carex flacca	1966/Smith							
	Schreb. (male	& Faulkner,							
Cariceae	flower)	1976	Spiral	Glume-like	Conical	Absent	0	3	Absent
		Smith,							
	Carex flacca	1966/Smith							
	Schreb. (female	& Faulkner,		Modified tubular					
Cariceae	flower)	1976	Spiral	prophyll	Conical	Absent	0	Absent	Trimerous
		Smith,							
	Carex nigra (L.)	1966/Smith							
A .	Reichard (male	& Faulkner,	a · 1	C1 1'1		A.1 (0	2	A.1 /
Cariceae	flower)	1976 Smith	Spiral	Glume-like	Conical	Absent	0	3	Absent
	Canon miana (I.)	Smith,							
	Carex nigra (L.) Reichard (female	8 Faulkner		Modified tubular					
Cariceae	flower)	1976	Spiral	nronhvll	Conical	Absent	0	Absent	Trimerous
Carleeae	nower)	Smith	Spirar	propityii	Conteat	nosem	0	rosent	millious
		1966/Smith							
	Carex panicea L.	. & Faulkner.							
Cariceae	(male flower)	1976	Spiral	Glume-like	Conical	Absent	0	3	Absent
		Smith,	I	-					
		1966/Smith							
	Carex panicea L.	. & Faulkner,		Modified tubular					
Cariceae	(female flower)	1976	Spiral	prophyll	Conical	Absent	0	Absent	Trimerous

Cariceae	<i>Carex elata</i> All.(female flower)	Gehrke <i>et</i> <i>al.</i> , 2012	Spiral	Modified tubular prophyll (developing later than the flower primordium)	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	Absent	Dimerous dorsiventrally flattened
Cariceae	<i>Uncinia rubra</i> Colenso ex Boott (male flower)	Vrijdaghs <i>et</i> <i>al.</i> , 2009, 2010	Spiral	Glume-like	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	3	Absent
Cariceae	<i>Uncinia rubra</i> Colenso ex Boott (female flower)	Vrijdaghs <i>et</i> <i>al.</i> , 2009, 2010	Spiral	Modified tubular prophyll	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	Absent	Trimerous
Cariceae	<i>Schoenoxiphium lehmannii</i> (Nees) Kunth ex Steud.	Gehrke <i>et</i> <i>al.</i> , 2012	Spiral	Glume-like	?	Absent	0	Absent	Dimerous doriventrally flattened
Cariceae	<i>Schoenoxiphium burkei</i> C.B. Clarke	Gehrke <i>et</i> <i>al.</i> , 2012	Spiral	Glume-like	?	Absent	0	Absent	Dimerous doriventrally flattened
Abildgaardieae	<i>Fimbristylis</i> <i>autumnalis</i> (L.) Roem. & Schult.	Reutemann et al., 2015	Spiral	Rim-like	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	2 (lateral)	Trimerous
Abildgaardieae	<i>Fimbristylis</i> squarrosa Vahl	Reutemann et al., 2015	Spiral	Rim-like	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	1 (lateral)	Dimerous dorsiventrally flattened

Abildgaardieae	Bulbostylis capillaris (L.) Kunth ex C.B. Clarke	Reutemann et al., 2015	Spiral	Rim-like	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	2 (lateral)	Trimerous
Abildgaardieae	Bulbostylis conifera (Kunth) C.B. Clarke	Reutemann et al., 2015	Spiral	Rim-like	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	3 (2 laterals and 1 abaxial)	Trimerous
Abildgaardieae	Bulbostylis juncoides (Vahl) Kük. ex Osten	Reutemann et al., 2015	Spiral	Rim-like	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	3 (2 laterals and 1 abaxial)	Trimerous
Abildgaardieae	<i>Abildgaardia ovata</i> (Burm. f.) Kral	Reutemann et al., 2015	Spiral	Rim-like	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	3 (2 laterals and 1 abaxial)	Trimerous
Fuireneae	Fuirena pubescens (Poir.) Kunth	Vrijdaghs <i>et</i> <i>al.</i> , 2004	Spiral	Rim-like, midrib protude	*Spherical (rounded abaxially and adaxially)	Present	6(3 outer and 3 inner parts)	3 (2 laterals and 1 abaxial)	Trimerous
Fuireneae	Fuirena abnormalis C.B. Clarke	Vrijdaghs <i>et</i> al., 2004	Spiral	Rim-like, midrib protude	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Absent	0	2-3	Trimerous
Fuireneae	Fuirena ciliaris (L.) Roxb.	Vrijdaghs <i>et</i> <i>al.</i> , 2004	Spiral	Rim-like, midrib protude	* Triangular(flattened adaxially/laterally and rounded abaxially)	Present	6(3 outer and 3 inner parts)	3 (2 laterals and 1 abaxial)	Trimerous
Fuireneae	Fuirena leptostachya Oliv.	Vrijdaghs <i>et</i> <i>al.</i> , 2004	Spiral	Rim-like, midrib protude	* Triangular(flattened adaxially/laterally and rounded abaxially)	Present	6(3 outer and 3 inner parts)	2 (lateral)	Trimerous

Eleocharideae	Eleocharis montana (Kunth) Roem. & Schult.	San Martin, 2014	Spiral	Rim-like	* Triangular(flattened adaxially/laterally and rounded abaxially)	Present	5 (2 outer and 3 inner parts)	1-2	Dimerous dorsiventrally flattened or trimerous
Eleocharideae	Eleocharis filiculmis Kunth	San Martin, 2014	Spiral	Rim-like	* Triangular(flattened adaxially/laterally and rounded abaxially)	Present	5 (2 outer and 3 inner parts)	3 (2 laterals and 1 abaxial)	Trimerous
Eleocharideae	<i>Eleocharis</i> <i>geniculata</i> (L.) Roem. & Schult.	San Martin, 2014	Spiral	Rim-like	* Triangular(flattened adaxially/laterally and rounded abaxially)	Present	6(3 outer and 3 inner parts)	3 (2 laterals and 1 abaxial)	Trimerous
Eleocharideae	Eleocharis acutangula (Roxb.) Schult.	San Martin, 2014	Spiral	Rim-like	* Triangular(flattened adaxially/laterally and rounded abaxially)	Present	6(3 outer and 3 inner parts)	3 (2 laterals and 1 abaxial)	Trimerous
Eleocharideae	<i>Eleocharis</i> palustris (L.) Roem. & Schult.	Vrijdaghs <i>et</i> al., 2009	Spiral	Rim-like	* Triangular(flattened adaxially/laterally and rounded abaxially)	Present	4 (3 outer and 1 inner parts)	3 (2 laterals and 1 abaxial)	Dimerous dorsiventrally flattened
Scirpeae	Scirpus sylvaticus L.	Vrijdaghs <i>et</i> al., 2005a	Spiral	Rim-like	* Triangular(flattened adaxially/laterally and rounded abaxially)	Present	6(3 outer and 3 inner parts)	3 (2 laterals and 1 abaxial)	Trimerous
Scirpeae	Scirpoides holoschoenus (L.) Soják	Vrijdaghs <i>et</i> <i>al.</i> , 2005a	Spiral	Rim-like	* Triangular(flattened adaxially/laterally and rounded abaxially)	Absent	0	3 (2 laterals and 1 abaxial)	Trimerous

Scirpeae	Eriophorum latifolium Hoppe	Vrijdaghs <i>et</i> <i>al.</i> , 2005a	Spiral	Rim-like	* Triangular(flattened adaxially/laterally and rounded abaxially)	Present	Perigonial zone with many hairs	3 (2 laterals and 1 abaxial)	Trimerous
Dulicheae	Dulichium arundinaceum (L.) Britton	Vrijdaghs <i>et</i> <i>al.</i> , 2005a	Distichous	Rim-like with lateral wings	* Elliptical (flattened adaxially and rounded abaxially/laterally)	Present	Perigonial zone (2 lateral, 3 adaxial and 3 abaxial parts)	3 (2 laterals and 1 abaxial)	Dimerous dorsiventrally flattened
Rhynchosporeae	Rhynchospora corymbosa (L.) Britton	Lucero <i>et</i> <i>al.</i> , 2014	Spirodistich ous	Rim-like	* Triangular(flattened adaxially/laterally and rounded abaxially)	Present	6(3 outer and 3 inner parts)	3 (2 laterals and 1 abaxial)	Dimerous dorsiventrally flattened
Rhynchosporeae	Rhynchospora riedeliana C.B. Clarke	Lucero <i>et</i> <i>al.</i> , 2014	Spirodistich ous	Rim-like	* Triangular(flattened adaxially/laterally and rounded abaxially)	Absent	0	2 (lateral)	Dimerous dorsiventrally flattened
Rhynchosporeae	Rhynchospora brittonii Gale	Lucero <i>et</i> <i>al.</i> , 2014 Lucero <i>et</i>	Spirodistich ous	Rim-like	* Triangular(flattened adaxially/laterally and rounded abaxially)	Present	6(3 outer and 3 inner parts)	3 (2 laterals and 1 abaxial)	Dimerous dorsiventrally flattened
Rhynchosporeae	Rhynchospora consanguinea (Kunth) Boeck	<i>al.</i> , 2014; Monteiro <i>et</i> <i>al.</i> , 2017	Spirodistich ous	Rim-like	* Triangular(flattened adaxially/laterally and rounded abaxially)	Present	5 (2 outer and 3 inner parts)	3 (2 laterals and 1 abaxial)	Dimerous dorsiventrally flattened
Schoeneae	Schoenus nigricans L.	Vrijdaghs <i>et</i> al., 2007	Spirodistich ous	Rim-like with lateral wings	* Triangular(flattened adaxially/laterally and rounded abaxially)	Present	6(3 outer and 3 inner parts)	3 (2 laterals and 1 abaxial)	Trimerous
Schoeneae	Lepidosperma tetraquetum Nees	Vrijdaghs <i>et</i> <i>al.</i> , 2009	Distichous	Rim-like, distant from flower primordium	?	Present	4	4	Tetramerous

Chapter 2

Chapter 2

Development and function of the stylopodium in Cyperoideae (Cyperaceae): a potential adaptive innovation

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ABSTRACT

Premise of the study The stylopodium, a thickening at the base of the style, right on top of the ovary apex, is found in some Cyperoideae genera. This structure may be deciduous or persistent in the nutlet as a beak; however, little is known regarding its origin and function, being related to water uptake for seed germination but never tested before. Thus, we analyzed the stylopodium of five Cyperoideae species to assess the ontogenetic origin, identify any abscission layer related to its detachment from the nutlet and any structure related to the water supply. Moreover, we investigate the physiological influence of the stylopodium in seed germination.

Methodology Fixed material of five species, from floral bud to nutlet, were analyzed through scanning electron and light microscopy. Furthermore, germination tests in different water availability conditions were carried out.

Pivotal results The results showed that the stylopodium in all species originate from the enlargement of the style base. The detachment from the nutlet is mostly associated with the extension of the contact between stylopodium and nutlet apex since no abscission layer was found. The presence of tracheoids spread in the stylopodium parenchyma was observed in all species. The germination tests revealed higher germination rates at the lower osmotic potential for species with stylopodium.

Conclusions The stylopodium is homologous among the species and the extension of connection between it and nutlet apex regulates its detachment. The presence of tracheoids is related in facilitating water entrance and germination tests suggest a possible influence of the stylopodium in promoting seed germination. The presence of stylopodium in the most diverse genera of Cyperaceae suggests that its acquisition, together with further morphological and genetic features, possibly influence some aspects of their reproductive success to changing environmental conditions, raising questions whether it could be considered a possible key innovation.

Keywords: Cyperaceae; Cyperoideae; gynoecium development; style base; ontogeny; sedges.

INTRODUCTION

Cyperaceae exhibit a cosmopolitan distribution and the highest diversity on the Neotropics (Govaerts et al. 2018). Such wide distribution reflects their importance as economical and ethnobotanical source, with several genera being vital for regional and local incomes (Simpson and Inglis 2001; Simpson et al. 2011). Furthermore, Cyperaceae genera are commonly the dominant components of some vegetations, playing a role in conservation and environmental studies (Simpson et al. 2011). The family is recovered in two subfamilies (Cyperoideae and Mapanioideae) (Simpson et al. 2007; Muasya et al. 2009; Hinchliff and Roalson, 2013; Spanlik et al. 2016; Semmouri et al. 2019), from which 40% of the genera are well represented in Brazilian vegetation, most of them belonging to Cyperoideae (Alves et al. 2009). Cyperoideae is the most diverse in terms of species richness and morphological modifications, especially regarding size, number and shape of perianth parts, stamens and carpels, which has led to a highly variable floral morphology (i.e., Vrijdaghs et al. 2009, 2011; Reutemann et al. 2012, 2015; Lucero et al. 2014; Monteiro et al. 2017, submitted). Alternatively, early diverging Mapanioideae exhibit most of the genera with restrict distribution (Govaerts et al. 2018).

Some of the most species-rich genera of Cyperoideae in Brazil occurs mainly in the Cerrado vegetation (Kauffman et al., 1994; Borba-Roschel et al., 2005; Alves *et al.*, 2009), as *Rhynchospora* Vahl., *Bulbostylis* Kunth, *Fimbristylis* Vahl and *Eleocharis* R.Br. (Araújo *et al.*, 2003; Alves *et al.*, 2009) which are considered some of the most diverse genera of Cyperaceae (Escudero and Hipp, 2013). Curiously, although the previous genera do not belong to the same clade (Simpson et al. 2007; Muasya et al. 2009; Hinchliff and Roalson, 2013; Spanlik et al. 2016; Semmouri et al. 2019), they share a noticeable reproductive feature: a thickening at the base of style right on top of the ovary
apex (Vrijdaghs et al. 2004, 2009; Gonzalez and López, 2010; Reutemann *et al.*, 2012; Monteiro et al. 2017).

The thickening at the style base, generically known as "stylopodium" (Kukkonen, 1984; Gonzalez and López, 2010; Monteiro *et al.*, 2017) is mainly distinct in the mature flower, and remains in the mature nutlet as a beak, after the rest of the style detaches in *Rhynchospora, Eleocharis* and *Bulbostylis*. However, for genera such as *Fimbristylis* and *Abildgaardia* Vahl, the stylopodium is observed until the initiation of nutlet maturation and style detach entirely from the mature nutlet (Gonzalez and López, 2010; Reutemann *et al.*, 2012; Monteiro et al. 2017). The term "nutlet" describes the Cyperaceae fruits, referring to a "small nut" with a single seed and developed from a superior ovary, as a more accurate term to describe than "achene" (Vrijdaghs 2006). Although the family has an achene-type fruit classified due to the indehiscent characteristic (Goetghebeur 1998), achenes originate from inferior ovaries (Font Quer 1953; Lawrence 2000) which is not observed in Cyperaceae (Bruhl 1995; Goetghebeur 1998; Vrijdaghs 2009, 2011).

The origin of the stylopodium has been a matter of debate, interpreted either as the result of the enlargement of the base of the style (Gonzalez and López, 2010; Reutemann *et al.*, 2012; Monteiro et al. 2017) or as a constriction of the apical portion of the ovary (Vrijdaghs *et al.*, 2004). Despite the uncertainty around its origin, it has been used for systematics implications, for instance, as a synapomorphy of Abildgaardieae (Reutemann *et al.*, 2012) and as a diagnostic feature for subsections of *Rhynchospora* (Strong 2006) and *Pleurostachys* (Alves and Thomas, 2015). Furthermore, anatomical studies reported the presence of cells with helical, reticulated and scalariform wall thickenings (tracheoids) in the stylopodium of *Bulbostylis, Abildgaardia, Fimbristylis* and *Rhynchospora* species (Gonzalez and López, 2010; Reutemann *et al.*, 2012; Monteiro *et al.*, 2017), which was interpreted as an evidence that the stylopodium may function in

facilitating the water entrance for seed germination, since the nutlets in Cyperaceae retains the seed, functioning as a single unit (Gonzalez and López, 2010). However, the influence of this structure in seed germination has never been physiologically tested before.

Regarding the lack of understanding on the ontogenetic origin of the stylopodium among the genera of Cyperaceae, combined to its possible role in the germination fitness, we aim to analyze the stylopodium in species of *Bulbostylis*, *Eleocharis*, *Fimbristylis*, *Pleurostachys* and *Rhynchospora* to answer the questions: a) Is the stylopodium ontogenetically homologous among these genera? b)What cause the detachment of the entire stylopodium from the mature nutlet in some species?; c) Does the presence of stylopodium influence seed germination? This is addressed by an integrative approach of ontogeny, anatomy and physiology to 1) analyze the development of the stylopodium to establish whether it is ontogenetically homologous in all species; 2) study the anatomy of this structure to identify any abscission layer related to its detachment and any type of tissue or cell that potentially indicate a strategy to water supply; 3) investigate the influence of the stylopodium in the rate and speed of germination through germination tests.

MATERIAL AND METHODS

Material

Samples of *Bulbostylis hirtella* (Schrad. ex Schult.) Nees ex Urb., *Eleocharis filiculmis* Kunth, *Fimbristylis autumnalis* (L.) Roem. & Schult. and *Rhynchospora albiceps* Kunth were collected in an area of Cerrado (savanna) vegetation in Itirapina (São Paulo, SP-Brazil). Samples of *Pleurostachys sparsiflora* Kunth were collected in the Reserva Florestal Adolpho Ducke (Manaus, Amazonas, Brazil), in the moist understory

of the Amazon rainforest. Vouchers were deposited in the spirit collection of the herbarium SPF (University of São Paulo). All the studied species exhibit stylopodium.

Structural analyses

Inflorescences with flowers in different developmental stages and fruits were fixed in FAA (formaldehyde, glacial acetic acid, 50% ethanol, 1:1:18 v/v) (Johansen 1940) and stored in ethanol 70% for morphological and anatomical studies.

For the anatomical study, flowers at different developmental stages and nutlets were subjected to a *tert*-butyl alcohol dehydration series and embedded in Paraplast (Leica Microsystems Inc., Heidelberg, Germany). The embedded materials were sectioned at 13–15 µm on a rotary microtome (Leica Microsystems, Wetzlar, Germany), stained with astra blue and safranin and mounted on slides with Permount. Photomicrographs were obtained using an image-capturing device coupled to a microscope Leica DMLB (Leica Microsystems, Wetzlar, Germany), using the software Scan System Images (IM50).

To assess the ontogenetic homologies among the species, the development of the stylopodium was carried out, from floral buds to mature nutlets, under the scanning electron microscope (SEM). Samples were dissected in 70% alcohol under a stereomicroscope. The materials were dehydrated through an ethanol series, critical-point dried, coated with gold and examined in a 1.5 Supra 55VP SEM.

Germination tests

The germination test was carried out with species that grow in similar environmental conditions and have the two different states: with and without stylopodium in the mature nutlet. Nutlets of *Eleocharis filiculmis* (persistent stylopodium in the nutlet) and *Fimbristylis autumnalis* (deciduous stylopodium in the nutlet) were collected from the natural environment and kept on paper bags at room temperature. Previous tests were carried out to compare the germination rates of both species with seven and fifteen days after collection, and the latter was chosen for showing better response.

The nutlets of both species were exposed to four water stress conditions for 10 days, induced by different osmotic potential levels: 0 (control-distilled water) and -0.2; -0.4; -0.8 MPa of polyethylene glycol 6000 (PEG 6000). For each condition, five replications were made, each with 20 seeds. The nutlets were placed in dishes plates, wrapped in plastic to keep them moist and taken to a growth chamber at approximately 250 μ mol m⁻² s⁻¹, 12 h photoperiod, air temperature of 27°C day / 22°C night and air humidity of 60% day / 80% night, and monitored daily. The criterion for germination was the emergence of the radicle. The number of germinated seeds were calculated for each species, as well as the germination speed index (GSI), calculated by the formula GSI= Σ (NGS/DAS) (NGS = noncumulative number of germinated seeds/ DAS= number of days after the test started) (Maguire, 1962). The variances homoscedasticity was homogenous, and the data were analyzed by a variance analysis (ANOVA) and Tukey test. Differences were considered as statistically significant within each species when p<0,05. All statistical analyses were performed using PAST software version 2,17c.

RESULTS

Development of the stylopodium

The base of the style in the floral bud does not present any thickening in the species (figs. 1*A*; 2*A*, *B*; 3*A*; 4*A*, *B* - arrow), except for *Fimbristylis autumnalis* (fig. 5*A*). For this species a thickening in the base of the style is observed at early stages (fig. 5*A*). The width of the stylopodium increases from floral bud to nutlet maturation in all species (Table 1). Furthermore, the extension of the connection between stylopodium and ovary/nutlet apex increases in all species, except for *Bulbostylis hirtella* and *Fimbristylis*

autumnalis. For *Bulbostylis hirtella* a decrease in the extension of such connection occurs during anthesis and in the nutlet, for *Fimbristylis autumnalis* the connection of stylopodium with ovary apex decreases significantly from floral bud to the early stages of nutlet development (table 1).



Fig. 1 Scanning electron micrographs of the flowers and nutlet of *Eleocharis filiculmis* at different developmental stages. *A*, Floral bud with glumes removed to show the gynoecium with flat style, without thickening (arrow). *B*, Pre-anthetic flower in the beginning of style base expansion (yellow). *C*, Thickening style base (yellow) in the mature flower. *D*, Stylopodium in the nutlet showing a distal constriction at the apex from which the rest of the style will detach (arrowhead). *E*, Stylopodium (yellow) in the apex of the nutlet. *F*, Detail of the stylopodium in the apex of the nutlet, note the connection of stylopodium with nutlet surface. Scale bars = $200 \mu m (A, E)$, $100 \mu m (B, C, D)$, $20 \mu m (F)$.



Fig. 2 Scanning electron micrographs of the flowers and nutlet of *Bulbostylis hirtella* at different developmental stages. *A*, Floral bud and *B*, pre-anthetic flower showing a flat style, without thickening (arrow). *C*, Beginning of style base expansion (yellow). *D*, Mature flower with a constriction distally in the stylopodium from which the rest of the style will detach (arrowhead). *E*, Mature flower at the later stage of development and *F*, nutlet showing a distal (white arrowhead) and proximal (black arrowhead) constriction in the stylopodium. *G*, Stylopodium (yellow) in the apex of the nutlet. (F) Detail of the stylopodium (yellow) in the apex of the nutlet. Scale bars = $50\mu m (A, C, D)$, $100\mu m (B, E-H)$.



Fig. 3 Scanning electron micrographs of the flowers and nutlet of *Rhynchospora albiceps* at different developmental stages. *A*, Floral bud with glumes removed to show the gynoecium with flat style, without thickening (arrow). *B*, Pre-anthetic flower in the beginning of style base expansion (yellow). *C*, Thickening style base (yellow) in the mature flower. *D*, Stylopodium in mature flower showing a distal constriction at the apex from which the rest of the style will detach (arrowhead). *E*, Stylopodium (yellow) in the apex of the nutlet. *F*, Detail of the stylopodium in the mature nutlet, note the triangular shape and the connection of stylopodium with nutlet surface. Scale bars = A: $20\mu m$ (*A*), $100\mu m$ (*B*, *D*, *E*, *F*), $20\mu m$ (*C*).



Fig. 4 Scanning electron micrographs of the flowers and nutlet and transverse section of nutlet of *Pleurostachys sparsiflora* at different developmental stages. *A*, Floral bud and *B*, pre-anthetic flower showing a flat style, without thickening (arrow). *C*, Stylopodium in mature flower. *D*, Detail of the stylopodium in the mature flower, note the subtle expansion and the absence of distal or proximal constrictions in the stylopodium. *E*, Transverse section of a nutlet showing the continuity of stylopodium and nutlet surface, limited by two narrow depressions (arrowhead), one in each edge, marking the connection of stylopodium and nutlet surface. sty, stylopodium. Scale bars = 100μ m (*A-D*), 0,5mm (*E*).



Fig. 5 Scanning electron micrographs of the flowers and nutlet of *Fimbristylis autumnalis* at different developmental stages. *A*, Floral bud with glumes removed to show the gynoecium with a thick style (yellow). *B*, Pre-anthetic flower (yellow). *C*, Thickening style base (yellow) in the mature flower. *D*, Stylopodium detaching (yellow) from the nutlet. *E*, Closer detail of the stylopodium detaching from the apex of the nutlet (yellow), note the shorter connection of stylopodium with nutlet surface. *F*, Mature nutlet without stylopodium (arrow). Scale bars = $100 \mu m (A, B, C, F)$, $200 \mu m (D)$; $20 \mu m (E)$.

Table 1 Measurements of the width of the stylopodium and the width of the connection between stylopodium and ovary/nutlet apex in the studied species at different developmental stages: floral bud, pre-anthesis, anthesis and nutlet.

Species	Width of the stylopodium				Width of the connection stylopodium and ovary/nutlet apex			
	Floral bud	Pre- anthesis	Anthesis	Nutlet	Flora bud	l Pre- anthesis	Anthesis	Nutlet
<i>Bulbostylis</i> <i>hirtella</i> (Schrad. ex Schult.) Nees ex Urb.	73,3µm	83,9µm	104,8µm	169,5µm	83µm	1 89,4µm	78,5µm	105,5µm
Eleocharis filiculmis Kunth	81,18µm	132,2µm	221,3µm	461,3µm	90,17µ	m 134,2µm	182,8µm	427,9µm
<i>Fimbristylis autumnalis</i> (L.) Roem. & Schult.	141µm	153,35µm	204,5µm	223µm	110µr	n 115,5µm	138µm	95,8µm
Pleurostachys sparsiflora Kunth	96,4µm	102,5µm	241µm	268µm	104,7µ	m 113µm	266µm	270µm
Rhynchospora albiceps Kunth	81,01µm	172,6µm	308,8µm	401,5µm	131µr	n 164,2µm	254µm	354µm

For Bulbostylis hirtella, Eleocharis filiculmis, Pleurostachys sparsiflora and Rhynchospora albiceps the enlargement of the stylopodium starts at the base of the style, right up the ovary apex, after the elongation of the style and concomitantly with the elongation of the ovary, during pre-anthesis (figs. 1B, C; 2C-E; 3B, C; 4C, D). In Bulbostylis hirtella, a constriction appears first at the distal part of the stylopodium (fig. 2D – arrowhead) and further on it is observed on both distal and proximal parts (fig. 2E, F – arrowhead) whilst in Eleocharis filiculmis and Rhynchospora albiceps it is only observed in the distal part from where the style will detach (figs. 1D; 3D – arrowhead).

For all these previous species, the stylopodium is persistent in the mature nutlet (figs. 1*E*, *F*; 2*G*, *H*; 3*E*, *F*). For *Pleurostachys sparsiflora* the enlargement of the stylopodium is more subtle and less evident when compared to the other species, without any visible constrictions in distal and proximal parts in the flower (fig. 4*C*, *D*). The stylopodium remains in the nutlet and two narrow depressions, one in each edge, mark the connection of stylopodium and nutlet surface (fig. 4*E* - arrowhead).

The stylopodium morphology in the mature nutlet varies among the species. In *Eleocharis filiculmis* it exhibits a triangular shape with angular edges that touch the ovary and nutlet surface as they slightly curve upwards (fig. 1*E*, *F*). In *Bulbostylis hirtella* it has a disc-like shape with rounded edges, and the attachment is restricted to the median region of the stylopodium (fig. 2*G*, *H*). In *Rhynchospora albiceps* the stylopodium is completely attached to the ovary and nutlet surface forming a cap-like structure (fig. 3*E*, *F*) and in *Pleurostachys sparsiflora* appears as a continuous structure to the nutlet surface with a distinct beak, more evident anatomically (fig. 4*E*).

For *Fimbristylis autumnalis*, the base of the style is already expanded at the early stages of development (fig. 5*A*, *B*) and the distinction of a triangular structure becomes more evident in the mature stages (fig. 5*C*). A proximal constriction, between stylopodium and the ovary apex is formed (fig. 5*D*, *E*), from which the entire style (together with stylopodium) falls off the mature nutlet (fig. 5*F* - arrow).

Anatomy of the stylopodium

Anatomically, the stylopodium in all species exhibit a mesophyll with parenchymatous tissue exhibiting numerous idioblasts with phenolic compounds (figs. 6A-D; 7A-F – arrowhead), as well as the presence of cells with reticulate to scalariform wall thickening (tracheoids) scattered throughout the stylopodium (figs. 6A-D; 7A-F – arrow). The species exhibit tracheoids with thin cell walls (figs. 6B; 7B-D), as well as tracheoids with thicker cell walls (figs. 6*D*; 7*E*, *F*), both spread throughout the stylopodium. Although the stylopodium is deciduous in *Fimbristylis autumnalis*, no abscission layer or a line of rupture was observed (fig. 6C – yellow circle).



Fig. 6 Anatomical aspects of flowers and nutlet of *Eleocharis filiculmis* (*A-B*) and *Fimbristylis autumnalis* (*C-D*). *A*, *C*, Longitudinal section (LS) of the stylopodium. *C*, Note the absence of abscission layer in the proximal part of the stylopodium of *Fimbristylis autumnalis* (yellow circle). *B*, *D*, Transverse section (TS) of the stylopodium, showing the tracheoids (arrow) and cells containing phenolic compounds (arrowhead). Scale bars = $50 \,\mu$ m (*A-C*), $100 \,\mu$ m (*D*).



Fig. 7 Anatomical aspects of flowers and nutlet of *Rhynchospora albiceps* (*A*, *B*), *Bulbostylis hirtella* (*C*, *D*) and *Pleurostachys sparsiflora* (*E*, *F*). *A*, *C*, *E*, Longitudinal section (LS) of the stylopodium and *B*, *D*, *F*, Transverse section (TS) of the stylopodium, showing the tracheoids (arrow) and cells containing phenolic compounds (arrowhead). Scale bars = 50 µm (*A*-*D*), 250 µm (*E*), 100 µm (*F*).

Germination tests

Seeds of *Eleocharis filiculmis* (persistent stylopodium in the nutlet) showed no significant difference (p=0.574) in the number of germinated seeds among the water stress conditions during the 10-days experiment (fig. 8*A*). Within each condition by the end of the experiment the average of germinated seeds was: Control= 68%; -0.2Mpa= 72%; -0.4Mpa= 63%; -0.8MPa= 65%. For *Fimbristylis autumnalis* (deciduous

stylopodium in the nutlet) there was a significant decrease in the number of germinated seeds at the -0.4Mpa (p=0.045) and -0.8MPa (p=0.035) conditions, when compared to the control and -0.2Mpa, with a more significant difference between the -0.4Mpa and -0.2Mpa conditions (fig. 8*B*). By the end of the experiment, the average of germinated seeds of *Fimbristylis autumnalis* was: Control= 64%; -0.2Mpa= 74%; -0.4Mpa= 50%; -0.8MPa= 49% of the seed germinated.



Fig. 8 Graphics showing the percentage of seed germination after 10 days in different osmotic potential (0 MPa – control; -0.2 MPa.; -0.4MPa.; -0.8Mpa). (A) *Eleocharis filiculmis.* (B) *Fimbristylis autumnalis.* Germination speed index after 10 days in different osmotic potential (0 MPa – control; -0.2 MPa.; -0.4MPa.; -0.8Mpa). (C) *Eleocharis filiculmis.* (D) *Fimbristylis autumnalis.* Different letters above bars indicate significant differences (p<0.05) among conditions.

Considering the germination speed index, *Eleocharis filiculmis* seeds began to germinate in the second day of experiment and there was no significant difference (p=0.0815) of the germination speed among the conditions (fig. 8*C*). Within each condition by the end of the experiment the average of germination speed index was: Control= 2.5; -0.2Mpa= 3; -0.4Mpa= 2.2; -0.8MPa= 2.4. For *Fimbristylis autumnalis*, the seeds began to germinate in the fifth day of experiment, with a significant decrease in the germination speed at the -0.4Mpa (p=0.012) and -0.8MPa (p=0.013) conditions, when compared to the control and -0.2Mpa, with a more significant difference between the - 0.4Mpa and -0.2Mpa conditions (fig. 8*D*). Within each condition by the end of the experiment the average of germinated seeds was: Control= 1.78; -0.2Mpa= 2.23; -0.4Mpa= 1.34; -0.8MPa= 1.34.

DISCUSSION

Development and morphology of the stylopodium

The stylopodium in all species originated as a result of the expansion of the style base, therefore it is an ontogenetically homologous structure appearing several times during Cyperoideae evolution, as previously reported (Monteiro et al. 2017). Such structure was reported as an important feature on the delimitation of tribe Abildgaardieae (Gordon-Gray 1971; Kern 1974; Reutemann et al. 2012), as well as *Rhynchospora* (Thomas 1984; Strong 2006), particularly concerning two main aspects: the length of the stylopodium compared to the nutlet (Thomas 1984) and the duration of the stylopodium in the nutlet (persistent or deciduous) (Goetghebeur 1998; Kern 1974). Although the stylopodium expansion in *Pleurostachys* is more discrete when comparing to *Rhynchospora*, the presence of such structure in both genera allow to consider the presence of stylopodium as a synapomorphy for Rhynchosporeae clade (Simpson et al.

2007; Muasya et al. 2009; Hinchliff and Roalson, 2013; Semmouri et al. 2019). Furthermore, since the structure is also observed in *Eleocharis* we consider it one of the morphological features that group Eleocharideae and Abildgaardieae clades as sister clades (Simpson et al. 2007; Muasya et al. 2009; Hinchliff and Roalson, 2013; Semmouri et al. 2019). In order to further clarify the phylogenetic significance of the stylopodium, an extensive study, mainly regarding the development of the stylopodium in more species, is needed.

The presence or absence is not the only variation related to the stylopodium in Cyperoideae genera (Vrijdaghs et al. 2004, 2009; Gonzalez and López 2010; Reutemann et al. 2012; Monteiro et al. 2017). The persistence or deciduousness of the stylopodium in the mature nutlet was reported as an important feature to distinguish genera such as Bulbostylis, Fimbristylis and Abildgaardia, where it is shown to be persistent in the nutlet in most of the species of Bulbostylis and deciduous in the latter two genera (Gonzalez and López 2010; Reutemann et al. 2012). Among the studied species, only the stylopodium of Fimbristylis autumnalis detached entirely from the nutlet. The process of detaching has been associated with three main causes: 1) the presence of an abscission layer located in the proximal part of the stylopodium (Gordon-Gray 1971; Bruhl 1995); 2) a result of mechanical shear effects caused by a lower number of cells with thickening walls at the base of the stylopodium, decreasing the stylopodium support (Reutemann et al. 2012); and 3) A lower or greater extension of connection of stylopodium and nutlet apex (Gordon-Gray 1971). Considering the first hypothesis of the abscission layer, our results do not support such interpretation since we did not observe any type of an abscission layer in distal or proximal parts of the stylopodium of Fimbristylis autumnalis. For the second hypothesis, we agree that some tracheoids acquire a thicker cell wall, which is related to the support of the structure as well, however our results show that these tracheoids are spread all over the stylopodium in all species, with no concentration in a certain region and no line of abscission observed, regardless of its persistence or detachment. Such statement hampers the interpretation of duration of stylopodium associated with a lower number of cells with thicker walls at the base of the stylopodium (Reutemann et al. 2012). The third hypothesis of the extension of the connection between stylopodium and nutlet apex as regulating its detachment (Gordon-Gray 1971) is supported here.

The duration of the stylopodium seems to be a matter of the extension of the contact between stylopodium and the nutlet apex. Our results showed that in *Rhynchospora, Pleurostachys* and *Eleocharis* the enlargement of the stylopodium follows the expansion of the ovary and nutlet apex throughout development. For *Bulbostylis*, a slight decrease in such extension is observed only in the stages of the mature flower and nutlet. Instead, in *Fimbristylis* the stylopodium is larger in early stages of development. However, it does not follow the expansion of ovary and nutlet and becomes much thinner, decreasing the contact of the surface of the stylopodium with the nutlet apex, which becomes more fragile and possibly causing its easy detachment from the nutlet.

The influence of the stylopodium in seed germination

Besides the homologous ontogenetic origin of the stylopodium, another feature shared by all species is the presence of tracheoids spread in the stylopodium parenchyma. This structure is often related to facilitating water entrance and storage in seeds of Orchidaceae (Prutsch et al. 2000) and resembles the structure of transfusion tissue in leaves of gymnosperms, as a structure increasing the capacity of water transference in a particular direction (Esau 1975). The presence of such cells in the stylopodium raised questions towards their functional significance (Gonzalez and López 2010; Reutemann et al. 2012; Monteiro et al. 2017), reported as possible evidence of the role of the stylopodium in aiding in the water uptake for seed germination (Gonzalez and López 2010).

For Cyperaceae, the process of seed germination involves numerous factors especially because nutlet and seed behave as a unity, characteristic of fruits of achene type (Goetghebeur 1998). The complexity of such structure shows that Cyperaceae seeds present a wide range of conditions to germinate; however, a few circumstances such as moist environment seems to influence more than other factors (Baskin and Baskin 1978; Larson 1997; Demeda et al. 2018). Thus, a structure aiding in the water uptake could significantly increase the success of germination. Furthermore, species that grow in similar environments usually require similar conditions to germinate (Baskin and Baskin 1978; Larson 1997).

Eleocharis and *Fimbristylis*, as commonly observed for Cyperaceae, are found in wet or seasonally flooded habitats, mostly in the borders of seasonal ponds of grasslands vegetation (Moreira et al. 2011; Barret 2013). *Eleocharis* usually occurs emergent in association with water bodies (Cook et al. 1974; Gil and Bove 2004, 2007; San Martin 2014) whilst *Fimbristylis* is usually distributed at humid soils but not flooded (Chauhan and Johnson 2009). Considering our results on germination rates of both species, *Eleocharis filiculmis* and *Fimbristylis autumnalis* exhibit similar rates in treatments with higher osmotic potential. However, following the decrease of water availability, *Eleocharis filiculmis* with persistent stylopodium in the nutlet, showed no significant changes on the number of germinated seeds in all variations of osmotic potential. Alternatively, *Fimbristylis autumnalis* with deciduous stylopodium, showed a significant decrease in the number of germinated seeds at the lower osmotic potential. Although the tests show a considerable standard deviation that might be reduced by a larger sampling, the results suggest that the stylopodium may be influencing the better germination

response in *Eleocharis filiculmis* by aiding the water entrance when the availability of water decreases.

Furthermore, the stylopodium seems to influence not only the number of germinated seeds but also the speed of germination. Seeds of *Eleocharis filiculmis* started to germinate faster than *Fimbristylis autumnalis*, with no significant decrease under lower water availability. For *Fimbristylis autumnalis*, despite the standard deviation, the statistic tests showed a significant difference in the germination speed index in conditions with lower water availability. Although seed germination involves several aspects such as temperature and oxygen fluctuations, especially in species growing in seasonal wet environments (Bell and Clarke 2004; Leck and Schütz 2005), our results shed light in a notable relationship between the presence of a stylopodium as a structure possibly increasing the germination success of the species.

Insights on the role of the stylopodium and its possible relevance in the germination success of Cyperoideae

Some notable facts may be related to the functional significance of the stylopodium such as 1) species with persistent stylopodium in the nutlet possibly increase the germination rates and speed, as suggested by our results; 2) genera exhibiting a high cover in grassland vegetation exhibit stylopodium (Strong 2006; Alves et al. 2009; Moreira et al. 2011); 3) the stylopodium may be necessary not only for seed germination but also for dispersion, especially in wetland species (floatation) (Vrijdaghs et al. 2009).

Genera such as *Fimbristylis, Rhynchospora* and *Bulbostylis* occurs mainly in grasslands formation characterized by extended annual dry seasons (Strong 2006) however, it is notable that *Fimbristylis* is usually found near water bodies where soils are humid whilst *Rhynchospora* and *Bulbostylis* occurs on a broader range of dry environments, exhibiting a high cover in grasslands (Strong 2006; Barbosa da Silva et al.

2020) and sometimes dominating the stratum (Alves et al. 2009; Moreira et al. 2011). The germination success of the latter two genera may be related, among other factors, with the presence of a stylopodium in the nutlet increasing the capacity of water transference for seed germination in contrasting seasonal regimes. The absence of stylopodium in *Fimbristylis* added to water availability as a crucial factor for germination in Cyperaceae (Baskin and Baskin 1978; Larson 1997; Demeda et al. 2018) suggest that the occurrence of this genus mostly in wet soils may be a strategy to increase the seed germination success, possibly limiting the species distribution to seasonal and drier environments.

It its noteworthy that for *Eleocharis* species, water availability is not a limited condition, reinforcing that the stylopodium role might go beyond facilitating water uptake for germination, as well as functioning possibly as a floating structure for diaspore dispersion together with other floral structures on nutlet such as perianth bristles, which are known to promote dispersion (Robinson 1962; Bruhl 1995; Goetghebeur 1998; Leck and Schütz 2005; Vrijdaghs et al. 2009; Barret et al. 2013). Such structures are vital for species growing emergent in water bodies reducing the chances of seed loss to adverse locations (Goetghebeur 1998; Leck and Schütz 2005). Furthermore, it is remarkable that whilst most of Cyperaceae species exhibit inflorescences with a high number of spikelets and flowers (Bruhl 1995; Goetghebeur 1998; Vrijdaghs et al. 2009, 2010), *Eleocharis* species exhibit a single terminal spikelet (Goetghebeur 1998) decreasing considerably the number of nutlets and seeds per individual and therefore, a structure to increase the success in germination and dispersion of these diaspores appears to be fundamental.

It is well known for angiosperm families that some floral features have a remarkable evolutionary role on adaptive radiation (i.e., Ronse De Craene et al. 2003; Endress 2010a; 2011) and when it comes to the stylopodium, it is notable that it is present in at least four of the seven most species-rich genera of Cyperaceae (*Eleocharis*,

81

Fimbristylis, Rhynchospora, Bulbostylis, Carex, Cyperus and *Scleria*), which together correspond to ca. 80% of Cyperaceae species (Escudero and Hipp 2013). Escudero and Hipp (2013), studying the shifts in diversification rates in Cyperaceae, pointed out these seven genera are part of early-divergent lineages with high species richness, with their major radiation occurring during the transition from the Paleoceno to the Eoceno, when the climate was becoming drier.

The appearance of a new morphological trait may be associated with changes in environment conditions (Escudero and Hipp 2013). When the right ecological and genetic conditions are present, such new morphological traits may be critical to the reproductive success of the species, thus being considered as a key innovation (Givnish 1997; Ronse de Craene et al. 2003; Endress 2010a; 2011; Escudero and Hipp 2013). Considering the presence of stylopodium in the most diverse genera of Cyperaceae, we believe that the acquisition of such structure should be investigated as a possible key innovation, possibly influencing some aspects of the species reproductive success, such as germination fitness, to a variety of environmental conditions and contributing to the wide diversification of these genera.

CONCLUSION

Based on the developmental study of Cyperoideae species, we observed that the stylopodium originated as a result of the enlargement of the style base. It seems a homologous structure appearing several times during Cyperoideae evolution. Our results show that the stylopodium detachment from the mature nutlet is associated with a particularity of the development towards a decrease in the contact of the surface of the stylopodium with the nutlet apex, which becomes more fragile and easily detaching from the nutlet. Considering the role of the stylopodium, the presence of tracheoids spread in

the stylopodium parenchyma was associated with facilitating water entrance for seed germination and our results suggest a possible influence of the stylopodium in improving the rate and speed of seed germination. Untangle the ontogeny and role of stylopodium of Cyperoideae species will have major implication to the understanding of its relevance on the adaptation of Cyperaceae to changing environments. It is notable that the major radiation of some of the most diverse genera of Cyperaceae occurred when the climate was becoming drier and these genera also exhibit a stylopodium, which together with further morphological and genetic features, possibly influence some aspects of their reproductive success to changing environmental conditions. Further analyses comprising more Cyperoideae species should focus on the stylopodium to tackle the question of whether it is a key-innovation that thrive Cyperaceae lineages through dry environments.

Author Contribution Statement

MMM, AO LF and DD designed the research, MMM carried out the ontogenetic and anatomical lab work. MMM and LF carried out the germination tests. All authors integrated and interpreted the results and contributed with further discussion, suggestions and writing of the manuscript.

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Chapter 3

Chapter 3

Spicoid ontogeny in *Diplasia* (Cyperaceae): an approach on the developmental processes operating in Mapanioideae spicoids

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ABSTRACT

Mapanioideae (besides Cyperoideae the other subfamily of Cyperaceae) is divided in two tribes (Hypolytreae and Chrysitricheae) in which the reproductive units (spicoids) are interpreted either as flowers or as inflorescences. Little is known about the ontogenetic factors operating during the spicoid development, how they may affect their final morphology and their stability within and among genera. In this context we studied the development of the monotypic genus Diplasia and compare it with developmental studies in Mapanioideae. Our results show that the main developmental variations are related to the sequence of organ formation, size and shape of the spicoid primordium and the way constraints of the subtending bract is distributed in such primordium. Considering the sequence of organ formation, Chrysitricheae genera follow a basipetal sequence, different from the acropetal sequence in Hypolytreae genera. Furthermore, species with a bigger and adaxially flattened primordium show a higher number of organs in the spicoids, different from the rounded and smaller primordium of *Hypolytrum schraderianum*, which exhibit the most reduced spicoid in number of organs. Moreover, for Chrysitricheae genera, reduction of structures usually occurs adaxially and for Hypolytreae genera, abaxially. Diplasia shares more developmental features with Chrysitricheae, supporting its positioning in the tribe. We favour the interpretation of the spicoid in *Diplasia* as a reduced inflorescence based on the extra-floral nature of the lateral bracts and the appearance of the inner bracts alternating with stamens, which hamper to interpret the bracts as perianth parts, since the perianth in Cyperoideae is exclusively extrastaminal.

KEYWORDS: Mapanioideae; spicoid; mapaniid; *Diplasia karatifolia*; spatial constraints, pseudanthium.

INTRODUCTION

Reproductive features, especially concerning floral traits, are crucial to establish relationships among angiosperm taxa (c.f. Ronse De Craene et al. 2003; Endress 2006; Chartier et al. 2014; Sauquet et al. 2017; Reyes et al. 2018). In Cyperaceae each subfamily shows particular features in its reproductive units: in Cyperoideae the spikelets are formed of true flowers with a typical trimerous tetracyclic bauplan (Bruhl 1995; Goetghebeur 1998; Vrijdaghs et al. 2009), whereas in Mapanioideae the nature of the spicoids has been a matter of debate for a long time and authors have described it either as flower or as inflorescence (see Koyama 1971; Kern 1974; Simpson 1992; Bruhl 1995; Goetghebeur 1998; Prychid and Bruhl 2013; Monteiro et al. 2016).

Mapanioideae comprise 11 genera distributed in two tribes (Hypolytreae and Chrysitricheae) (Simpson et al. 2007; Muasya et al. 2009, Hinchliff and Roalson 2013; Semmouri et al. 2019). The reproductive units of the subfamily (spicoids) are describe as consisting of two large lateral bracts enclosing two lateral stamens and a central gynoecium. Between the lateral stamens and the central gynoecium, a variable number of additional stamens may be found which may be intervened by inner bracts (Simpson 1992; Bruhl 1995; Goetghebeur 1998; Richards et al. 2006; Prychid and Bruhl 2013; Monteiro et al. 2016). This peculiar morphology raised the debate about the euanthial versus pseudanthial hypothesis in this subfamily (Eiten 1976; Koyama 1971; Kern 1974; Dahlgren et al. 1985; Bruhl 1995; Goetghebeur 1998; Richards et al. 2006; Vrijdaghs et al. 2006; Prychid and Bruhl 2013; Monteiro et al. 2016). The most common interpretations of the spicoids in Mapanioideae are: 1) The unit is a flower, where the bracts are considered perianth parts, and the presence of bracts between stamens were interpreted as perianth parts relocated between androecium and gynoecium during development (Kern 1974; Eiten 1976; Goetghebeur 1998); 2) The reproductive unit is a reduced inflorescence formed of two lateral bracts, male flowers

composed of single stamens, each subtended by their own bract (inner bracts) and a terminal female flower composed of a pistil (Bruhl 1995; Simpson 1992; Dahlgren et al. 1985).

The extremely reduced and condensed morphology of the structures in the mature spicoids hampers their interpretation. Therefore, developmental studies have been carried out focusing on the debated nature of the spicoids as flowers or inflorescences (Richards et al. 2006; Prychid and Bruhl 2013; Monteiro et al. 2016). Richards et al. (2006) studied the development of spicoids in the monotypic genus *Exocarya* Benth. (tribe Chrysitricheae) and proposed that the reproductive unit of *Exocarya sclerioides* (F. Muell.) Benth. is rather a spikelet than a flower. The spicoid in this species is subtended by a glume and composed of two lateral bracts adaxially positioned (leaf-like structures), two lateral stamens, two ad- and abaxial inner bracts, the latter with an inner stamen and a dimerous dorsiventrally flattened pistil (Fig. 1a).



Fig. 1. Simplified spicoid diagrams of Mapanioideae species based on previous developmental studies. Tribe Chrysitricheae (**a**) *Exocarya sclerioides* (Richards et al. 2006). (**b**) *Lepironia articulata* (Prychid

and Bruhl 2013). (c) *Mapanya pycnostachya* and (d) *Hypolytrum schraderianum* (Monteiro et al. 2016). ib, inner bract; lb, lateral bract; sb, subtending glume.

Prychid and Bruhl (2013) studied the monotypic genus *Lepironia* Pers. (tribe Chrysitricheae) and showed the reproductive unit of *Lepironia articulata* (Retz.) Domin in the axil of a subtending glume and composed of two lateral bracts (prophyll-like units), two lateral stamens, two whorls of inner bracts (leaf-like structures) alternating with 12-15 inner stamens (five adaxial and a variable number abaxially) (Fig. 1b). Each inner stamen is subtended by an inner bract of the outer whorl, whilst the inner whorl of bracts surrounds a dimerous dorsiventrally flattened pistil (Fig. 1b). The ontogeny and the localization of floral gene proteins in the spicoid structures of this species favored the inflorescence interpretation (Prychid and Bruhl 2013).

Monteiro et al. (2016) studied the development of *Mapania pycnostachya* (Benth.) T. Koyama (Fig. 1c) and *Hypolytrum schraderianum* Nees (Fig. 1d) (tribe Hypolytreae), the most diverse genera of Mapanioideae in number of species. They showed that the species share the most reduced spicoids in number of structures, with *Mapania pycnostachya* composed of two lateral bracts (prophyll-like units), two lateral stamens, two inner bracts (leaf-like structures) between stamens and the dimerous dorsiventrally flattened pistil (Fig. 1c). *Hylpolytrum schraderianum* differs from the previous species only by the absence of inner bracts (Fig. 1d). Based on anatomical and developmental differences of these reproductive units with Cyperoideae flowers, the authors favored the inflorescence interpretation as well (Monteiro et al. 2016). Although the previous studies are consistent towards the inflorescence interpretation, none of them are conclusive in excluding the possibility of a flower interpretation as well, mainly due to the lack of studies regarding other Mapanioideae genera (Richards et al. 2006; Prychid and Bruhl 2013; Monteiro et al. 2016). Furthermore, little is known about the factors operating

during spicoid development in most of the genera and how they may affect the spicoid's final morphology, as well as how consistent they are within and among genera.

From the 11 genera of Mapanioideae, five are monotypic and for some of them information is restricted to the taxonomic descriptions. This is the case of *Diplasia* Rich. (Clarke 1909; Bentham 1877; Simpson 2006), a genus restricted to tropical South America (Simpson 2006). The phylogenetic placement of *Diplasia* has been controversial. It emerged within the tribe Hypolytreae (Muasya et al. 2009), as sister-group to Hypolytreae and Chrysitricheae (Spalink et al. 2016), found it in an unresolved placement (Hinchliff and Roalson 2013) and most recently, within the tribe Chrysitricheae (Semmouri et al. 2019). Studying the origin of the spicoid structures in *Diplasia* may not only contribute to the discussion of the spicoid nature, but also clarify inconsistences in taxonomic descriptions (Bentham 1877; Clarke 1909; Simpson 2006). The complexity in understanding the position and origin of organs in the mature spicoids, added to the inconsistency in the spicoid description in *Diplasia* and the unresolved phylogenetic position of the genus highlights the importance of morphological and developmental studies to provide informative data for comparative analyses and to better understand phylogenetic intergeneric relationships.

Here we aim to present a complete ontogenetic analysis of the spicoid of *Diplasia karatifolia* Rich. and compare these data with other developmental studies for Mapanioideae (Richards et al. 2006; Prychid and Bruhl 2013; Monteiro et al. 2016) in order to answer the following questions: 1) What are the main developmental features that varies among these species? 2) How may such features influence the final spicoid morphology? 3) How consistent and informative are the developmental features among Mapanioideae genera? Based on our results and a comparison with other Mapanioideae genera, we finally discuss evidence that may help to interpret *Diplasia* spicoid as a flower or as inflorescence. Thus, we expect to expand the

understanding of the spicoid morphology of *Diplasia*, as well as to grasp the variability of developmental features and their relation to the morphology of Mapanioideae spicoids.

MATERIAL AND METHODS

Morphological sampling – Spikes of *Diplasia karatifolia* were collected in the Reserva Florestal Adolpho Ducke (Manaus, Amazonas, Brazil), in the moist understory of the Amazon rainforest. Vouchers (AG 1658) were deposited in the herbarium SPF (University of São Paulo – Brazil). Light and scanning electron microscopy – Spikes at different developmental stages were fixed in FAA (formaldehyde, glacial acetic acid, 50% ethanol, 1:1:18 v/v) (Johansen 1940) and stored in 70% ethanol for morphological and anatomical studies.

For scanning electron microscopy, fixed spikes were dissected under a Leica Wild M8 stereomicroscope to expose the spicoids at different developmental stages. The samples were then dehydrated, and critical-point dried using CO₂ in an Autosamdri-815B (Tousimis Research, Rockville, Maryland, USA). Dried material was mounted onto aluminum stubs using nail polish, coated with platinum using a Quorum Q-150-T sputter coater (Quorum Technologies, East Grinstead, UK) and examined with a Hitachi S-4700-II cold field emission scanning electron microscope (Hitachi High Technologies, Tokyo, Japan).

Terminology

Spike: The entire inflorescence that holds the spicoids in the axil of spirally arranged subtending bracts.

Spicoid: The reproductive unit of Mapanioideae.

Subtending bract: a large bract that subtends the entire spicoid (bracts + stamens + gynoecium). *Lateral bracts*: a pair of lateral bracts, subtending a lateral stamen each. Usually the first organ to appear during development, also named prophyll-like units (Prychid and Bruhl 2013).

98
Inner bracts: bracts placed within the spicoid, between the outer and inner whorls of stamens, or between the androecium and gynoecium, also named leaf-like structures (Richards et al. 2006; Prychid and Bruhl 2013).

RESULTS

The *Diplasia* spicoids arise in the axil of a subtending bract and are composed of two lateral bracts subtending two lateral stamens, two inner bracts subtending three stamens each and a central dimerous dorsiventrally flattened pistil. The subtending bracts arise in a spiral phyllotaxis on the indeterminate spike axis (Fig. 2a). Each spicoid primordium is flattened adaxially, expanded in the laterals and rounded abaxially (Fig. 2a - coloured; the subtending bract was removed in Fig. 2b (asterisk) onwards). The development of each spicoid starts with the appearance of two triangular lateral bracts (Fig. 2b – dark green), which enlarge, leaving a central oval apex (Fig. 2c). Subsequently, the apex expands laterally acquiring an elliptical shape and two stamen primordia appear in the lateral position, simultaneously with the appearance of a dome-shaped gynoecium primordium is followed by the formation of two additional inner bracts in adaxial and abaxial positions (Fig. 2e – light green). The former seems to be slightly delayed compared to the abaxial one (Fig. 2e, f – light green).



Fig. 2. Scanning electron micrographs of the spicoid of *Diplasia karatifolia* at successive developmental stages. (a) Spike showing the spicoids (coloured in a gradient sequence from blue (older) to yellow (younger)). (b-c) Formation of the two lateral bracts (dark green); the subtending bract was removed (asterisk). (d) Emergence of two lateral stamens and a central rounded gynoecium primordium. (e) Formation of the two inner bracts (light green). (f) Three inner stamen primordia initiate in abaxial position (arrowheads) concomitantly with the adaxial ones (arrows). g, gynoecium; ls, lateral stamen primordium; sa, spike axis. Bars = $100 \,\mu m$ (a); $50 \,\mu m$ (b-f).

Shortly after the emergence of the inner bracts, three inner stamen primordia arise in abaxial position (Fig. 2f – arrowheads) concomitantly with the three adaxial inner stamen

primordia (Figs. 2f; 3a-c - arrows). The adaxial inner stamen primordia appear slightly delayed (Fig. 3d – arrows) when compared to the abaxial ones (Fig. 3d, e – arrowheads). This slight difference disappears soon and no distinction between the six inner stamens is discernible in later developmental stages (Fig. 3f-h). While the lateral bracts are keeled and eventually fully enclose the lateral stamens (Fig. 3d-g – dark green), the abaxial and adaxial inner bracts are flat, membranaceous, subtending the inner stamens and partially the lateral ones as well (Fig. 3h). Later in development, the spicoid is completely covered by bracts (Fig. 4a – inner bracts marked in light green; the lateral bracts were removed – yellow arrow).

Although the gynoecium primordium arises before the inner bracts (Fig. 2d), its development is delayed until the appearance of all stamens (Fig. 3a-c). Only then an annular ovary wall primordium develops surrounding a central depression, and the two stigmas arise as two lateral projections (Fig. 3d-f). The projections elongate upward (Fig. 3g) and the developing ovary wall progressively enclose the central ovule (Fig. 3h), forming a dimerous dorsiventrally flattened gynoecium. In older spicoids, the lateral stamens are about the same length as the gynoecium, whilst the six inner stamens are shorter (Fig. 4b-d). Each stamen differentiates into a basifixed anther and a filament (Fig. 4c, d). The ovary and style elongate by intercalary growth and papillose protuberances appear along the stigmas (Fig. 4d).



Fig. 3. Scanning electron micrographs of the spicoid of *Diplasia karatifolia* at successive developmental stages. (**a-c**) Ongoing growth of the stamen primordia in adaxial position (arrows), as well as the abaxial ones (arrowheads). (**d-e**) Elongation of the lateral bracts (dark green) that partially enclose the lateral stamens. The gynoecium development continues with the emergence of two lateral stigma primordia. (**f-g**) The lateral bracts (dark green) completely cover the lateral stamens. The young stigmas grow upward. (**h**) The inner bracts (light green) elongate to the sides and envelop the inner stamens and partially the lateral stamens. The ovary starts to close over the central ovule. g, gynoecium; ls, lateral stamen; ov, ovule; ow, ovary wall; st, stigma. Bars = 50 μ m (**a-f; h**); 100 μ m (**g**).



Fig. 4. Scanning electron micrographs of the spicoid of *Diplasia karatifolia* at successive developmental stages. (**a-b**) Lateral views of the spicoid. (**a**) The lateral bracts were removed (yellow arrow) to show the inner bracts (light green) elongated and enclosing the spicoid. (**b**) The lateral (yellow arrows) and inner (yellow arrowheads) bracts were removed to show the distinction in the height of the lateral stamens and

gynoecium, compared to the inner stamens. (c) The mature stamens are differentiated into a basifixed anther and a filament. (d) Mature spicoid with the bracts and three abaxial inner stamens removed to show the dimerous dorsiventrally flattened gynoecium with papillose stigmas. ant, anther; fil, filament; ls, lateral stamen; ov, ovary; st, stigma; sty, style. Bars = $100 \,\mu m \, (a-b)$; 0,5 mm (c-d).

Table 1 shows our results for Diplasia compared to the developmental data obtained for

other species of Mapanioideae.

Tab 1. Developmental and morphological traits of spicoids of *Diplasia karatifolia* and other Mapanioideae species. References: *Lepironia articulata* (Prychid and Bruhl, 2013); *Exocarya scleriodes* (Richards et al., 2006); *Mapania pycnostachya* and *Hypolytrum schraderianum* (Monteiro et al. 2016).

	Species				
Features	Diplasia karatifolia Rich.	Exocarya scleriodes (F. Muell.) Benth	<i>Lepironia</i> <i>articulata</i> (Retz)Domin	Hypolytrum schraderianum Nees	<i>Mapania</i> pycnostachya (Benth.) T.Koyama
Width(µm) of spicoid primordium	128.3	78	221.3	72.6	95.8
Height (µm) of spicoid primordium	98.9	41.1	87.6	36.8	56.1
Sequence of organ appearance	Basipetal	Basipetal	Basipetal	Acropetal	Acropetal
Spicoid primordium shape	Flattened adaxially and rounded abaxially	Flattened adaxially and rounded abaxially	Flattened adaxially and rounded abaxially	Rounded adaxially and abaxially	Flattened adaxially and rounded abaxially
Number of lateral bracts	2	2	2	2	2
Number of lateral stamens	2	2	2	2	2
Number of inner bracts	2	2	20-28	0	2
Number of inner stamens	6	1	12-15	0	0

DISCUSSION

A comparison of our results for *Diplasia* with other developmental studies for Mapanioideae (including species of *Exocarya* (Richards et al. 2006), *Lepironia* (Prychid and Bruhl 2013), *Mapania pycnostachya* and *Hypolytrum schraderianum* (Monteiro et al. 2016)), shows that the main developmental variations among the species are related to the sequence of organ appearance, the size and shape of the spicoid primordium and the way constraints of the subtending bract is distributed in such primordium, which is possibly some of the factors leading to changes in the number of organs to be formed. The distribution of such features among the species will be discussed here.

Variation of developmental features and their influence on the spicoid morphology

Temporal sequence of organ formation

Concerning the sequence of organ appearance, it is important to highlight that in *Diplasia* the spicoids shows centrifugal/basipetal tendencies, because the inner bracts and the six inner stamens in ab- and adaxial positions are formed after the gynoecium and the two lateral stamens. The basipetal sequence was also found in the spicoid development of *Exocarya* (Richards et al. 2006) where the lateral stamens and gynoecium develop before the inner bracts and inner stamens. For *Lepironia articulata*, the lateral and inner stamens emerge simultaneously (Prychid and Bruhl 2013), and a distinction in their timing of appearance is not as clear as in *Diplasia* and *Exocarya*. However, later in development, the lateral stamens show a distinct bigger size than the inner ones (Prychid and Bruhl 2013).

Although for *Lepironia* the stamens and inner bracts emerge early in the spicoid primordium, giving the impression of a centripetal sequence of appearance, a primordial gynoecium becomes apparent before all other organs, remaining undeveloped until all stamens and bracts start to develop (Prychid and Bruhl 2013). Therefore, although the extremely short

timing interval of organs emerging may hamper the clear understanding towards the sequence that they appear, the early development of the gynoecium primordium in *Diplasia*, *Exocarya* and *Lepironia*, suggest that organs are formed in a basipetal sequence (table 1).

Such sequence is quite distinct when comparing the spicoid development of the above cited genera with *Hypolytrum* and *Mapania* spicoids (tribe Hypolytreae). For the latter genera, the spicoid development follows a centripetal/acropetal sequence of organ formation (Monteiro et al. 2016), where the lateral bracts are the first to appear, followed by the stamens, inner bracts and at last, the gynoecium. For such species, no sign of gynoecium primordium is seen before the emergence of stamens and inner bracts (Monteiro et al. 2016). Such sequence was also observed in Cyperoideae flowers (Vrijdaghs et al. 2009, 2010).

Despite the differences, for all species the lateral bracts are the first organs to appear, with the longest timing of emergence between the organs, which was interpreted as an evidence that they are more likely to be foliar structures of an axis than floral parts (Richards et al. 2006; Prychid and Bruhl 2013; Monteiro et al. 2016). This was reinforced by the absence of floral proteins in the lateral bracts of *Lepironia*, which therefore were classified as extra-floral structures (Prychid and Bruhl 2013).

Moreover, we can verify that the inner bracts in *Diplasia karatifolia* are two solid structures enveloping the inner stamens, as stated by Clarke (1909). *Diplasia* spicoids were described either as having two inner bracts (Clarke 1909) or as having six inner bracts that fuse three by three and give only the impression of being two bracts in the mature spicoid (cf. Bentham 1877; Simpson 2006). The hypothesis of such bracts being a result of a fusion of initially free bracts (Bentham 1877; Simpson 2006) was not supported by our results, where no fusion of parts was observed during development.

Spicoid primordium

For Mapanioideae, we observed that the size and shape of the spicoid primordium varies among the genera (Table 1). *Diplasia, Lepironia, Exocarya* and *Mapania pycnostachya* show primordia that are laterally expanded, adaxially flattened and abaxially rounded, which means more available space for organs to initiate (Richards et al. 2006; Prychid and Bruhl 2013, Monteiro et al 2016). The number of organs to be formed is known to be influenced by the existing space on the primordium (Classen-Bockhoff and Meyer 2016). This is notable for the above cited genera, in which the spicoid shows a considerable increase in the number of organs when compared to *Hypolytrum schraderianum* (table 1). Most likely due to the available space in the primordium, the spicoid in *Diplasia* possess eight stamens, and in *Lepironia* (Prychid and Bruhl 2013), spicoids are composed of 12-15 stamens and two whorls of 20-28 inner bracts (table 1). Although *Exocarya* and *Mapania pycnostachya* exhibit a spicoid primordium similar to *Diplasia* and *Lepironia*, the size of their primordium is considerably smaller (table 1), which may reflect in the lower number of organs in *Exocarya* and *Mapania pycnostachya* (table 1).

In *Hypolytrum* the spikes are reduced in size and show strongly condensed subtending bracts (Alves et al 2001; Monteiro et al. 2016). This may increase the pressure on the spicoid primordium, giving it a round shape ab- and adaxially without lateral expansions (table 1), with less available space for organs to form. It is notable that *Hypolytrum schraderianum* and *Exocarya* spicoid primordia exhibit similar sizes (table 1); however, the spicoid in *Exocarya* exhibit a higher number of organs when compared to *Hypolytrum* (Richards et al. 2006; Monteiro et al. 2016). This could possibly be due to the shape of the primordium, which is adaxially flattened in *Exocarya* with more space for organs to appear in the laterals, while in *Hypolytrum* the rounded primordium possibly constricts the available space where organs can be formed, possibly resulting in a reduced number of organs in the spicoid. Thus, it is notable that there is a relation in the number of organs in the spicoids with the size and shape of the

spicoid primordium, suggesting that both features together seems to influence the number of organs to be formed.

Although the shape of flower primordium is affected by gene interactions regulating the identity of floral organs, the spatial constraints applied by surrounding organs such as bracts are also fundamental in influencing the primordium geometry and may lead to new morphs (Chandler 2014; Ronse De Craene 2018). In Cyperaceae, inflorescences are commonly composed of several reproductive units arising in the axil of glume-like structures, which grow to envelop the whole unit (Bruhl 1995; Goetghebeur 1998; Vrijdaghs et al. 2009). The contact of such bracts with the developing primordium may have a relation with its shape and size, as reported for Cyperoideae (Monteiro et al. submitted), possibly leading to spatial restrictions on the formation of floral organs.

In Cyperoideae flowers, spatial restriction on organs formation commonly occurs in abaxial position (i.e. Lucero et al. 2014; Vrijdaghs et al. 2009; Monteiro et al. 2017). Regarding Mapanioideae species, the site of the primordium where reduction or loss of structures occur is quite variable (Fig. 5). In *Exocarya*, the only inner stamen present is the abaxial one (Richards et al. 2006) and for *Lepironia*, a higher number of inner bracts and stamens is reported on the abaxial side, whilst the adaxial side always shows only five stamens each with its own bract (Prychid and Bruhl 2013). The latter authors considered that in the beginning of spicoid development in *Lepironia*, the primordium expands laterally, and a restriction of growth is observed in the adaxial side, leading to the flattened adaxial side. However, in *Hypolytrum scraderianum* and *Mapania pycnostachya*, reduction and loss occur mainly at the abaxial side, in which the organs emerge in a latero-adaxial position and abaxial stamen and inner bract are absent (Monteiro et al. 2016).





Fig. 5. Simplified cladogram of Cyperaceae, adapted from Semmouri et al. (2019), showing the relationships among Mapanioideae genera. The species highlighted in blue correspond to tribe Hypolytreae and the species highlighted in yellow correspond to tribe Chrysitricheae. The arrow downwards represents the presence of spicoids with basipetal organ appearance and the upwards arrow represents the spicoids with acropetal organ appearance. The shape of the spicoid primordium is represented by a circular shape and a form that is flattened adaxially and rounded abaxially, the circle filled with a cross above the forms indicate the position of the axis. The reduction and/or loss of organs in the adaxial side is represented by a circle without the upper half and reductions in the abaxial side, by a circle without the bottom half.

Therefore, it is notable that added to the shape and size of spicoid primordium, the way constraints from surrounding organs is distributed in the primordium may influence different changes in the organs, as for instance when comparing *Exocarya* and *Mapania pycnostachya*. For both species it is remarkable that the only difference between their spicoid morphology is the presence of an abaxial stamen in *Exocarya* (table 1). In both species the primordium is flattened adaxially and rounded abaxially and considering the size of the primordium, *Mapania*

pycnostachya exhibit a larger primordium when compared to *Exocarya*, even though the latter species has an extra stamen. The fact that *Mapania pycnostachya* exhibits a lower number of organs, even though having a bigger primordium, is possibly due among other factors, to the contact of the subtending bract operating on the abaxial side of its primordium. Thus, although genetic factors certainly play a role in fixing the position of organs (Ronse De Craene et al. 2002; Ronse De Craene 2010), the morphological aspects related to the size and shape of the spicoid primordium, added to the influence of the spatial constraint of the bract subtending it, may also be crucial in influencing the spicoid morphological variation in Mapanioideae.

Developmental features and phylogenetic relation in Mapanioideae

Taking together our results for *Diplasia*, the developmental studies of spicoids in other genera (Richards et al. 2006; Prychid and Bruhl 2013; Monteiro et al. 2016), and the phylogenetic relationships among these genera (Semmouri et al. 2019), some features appear to be consistent among genera of the same tribe (Fig. 5).

In Chrysitricheae, *Diplasia*, *Lepironia* and *Exocarya* show spicoids with the same sequence of organ formation, following a basipetal emergence. Furthermore, *Diplasia* and *Lepironia* have a larger spicoid primordium, that can be related to a higher number of organs in such spicoids, compared to other Mapanioideae. Moreover, for *Lepironia* and *Exocarya*, a higher number of stamens is observed on the abaxial side of the primordium, thus reduction or loss of organs seems to occur commonly at the adaxial side. The presence of spicoids with a higher number of stamens and inner bracts is a notable feature in Chrysitricheae genera, with genus such as *Chrysitrix* L. exhibiting spicoids with ca. 150 stamens (Clarke 1909). Considering the developmental similarities of *Diplasia* with *Exocarya* and *Lepironia*, our results support the systematic placement of *Diplasia* close to both genera, in Chrysitricheae (Semmouri et al. 2019).

In Hypolytreae, *Hypolytrum schraderianum* and *Mapania pycnostachya* exhibit spicoids with organs appearing in an acropetal sequence, occupying mainly the latero-adaxial side of the primordium, and reduced or lost organs commonly on the abaxial side. Species exhibiting spicoids with a lower number of stamens and inner bracts is a common feature in Hypolytreae genera, as for instance in *Paramapania* Uittien with spicoids composed of two lateral bracts, ca. three inner bracts and one inner stamen, and a trimerous pistil (Vrijdaghs et al. 2006), as well as in *Principina* Uittien with spicoids composed of two lateral bracts, two-three stamens and a trimerous pistil (Mesterhazy and Browning 2014).

While the genera of each tribe seem to share developmental features, variation is found within the tribes, as for instance *Mapania pycnostachya* that shares developmental features with *Hypolytrum scharderianum* (Monteiro et al. 2016), as well as with *Exocarya* (Richards et al. 2006). Therefore, gaps of unstudied genera are in need to be filled to get a more comprehensive picture.

Interpretations of the nature of the Diplasia spicoid

The spicoid in *Diplasia* can be interpreted as a flower with a tetramerous bauplan (Fig. 6a-b). Alternatively, it can be interpreted as a reduced inflorescence with different architectures (Fig. 6c-d). The development of *Diplasia* spicoids favor the inflorescence interpretation and here we will discuss the pros and cons of both hypothesis (flower and inflorescence).



Fig. 6. The distinct hypothesis of interpretation of *Diplasia karatifolia* spicoid as flower or inflorescence. Floral interpretation (**a**) A tetramerous flower with four perianth parts (two outer parts – dark green and two inner parts – light green), eight stamens (blue) and one dimerous dorsiventrally flattened pistil (red). (**b**) A tetramerous flower enveloped by two halves of a split glume (lateral bracts – grey) and composed of two perianth parts (green), eight stamens (blue) and one dimerous dorsiventrally flattened pistil (red). Inflorescence interpretation (**c**) A reduced inflorescence composed

of a terminal bisexual flower subtended by a split bract (gynoecium and two lateral stamens subtended by the lateral bracts – dark grey) and two male flowers subtended by a bract (each with three stamen subtended by the inner bracts – white) in lateral positions. (d) A reduced inflorescence composed of a central female flower (gynoecium – dark grey), two lateral male flowers subtended by their bracts (lateral stamens and bracts – light grey) and one adaxial and one abaxial male flowers, subtended by their bracts (each with three stamens subtended by the inner bract - white). (e) A general Cyperoideae flower, based on Vrijdaghs et al. (2009), showing the usual morphology with a subtending bract (grey) enveloping a flower with three outer perianth parts (dark green), three inner perianth parts (light green), one whorl of three stamens (blue) and a trimerous gynoecium (red).

Diplasia spicoid as a flower

Regarding the floral interpretation, the spicoid can be interpreted in two different ways: 1) As a flower with four perianth parts (two outer - green and two inner parts – light green), eight stamens and one dimerous dorsiventrally flattened pistil (Fig. 6a). Alternatively, 2) instead of four perianth parts, the flower would be enveloped by two halves of a glume (lateral bracts grey) and possess two perianth parts (inner bracts – light green) (Fig. 6b). Although Cyperaceae usually exhibit trimerous flowers (Fig. 6e) (Goetghebeur 1998), shifts to tetramery occur and this is known as a result of an increase in meristem size (Ronse De Craene 2016; 2018).

In favor of the floral interpretation, features as: the absence of foliar structures (bracts) between the stamens and the gynoecium; and the absence of a vestige of a rachilla suggest that the spicoid in *Diplasia* could be interpreted as a flower. However, a few aspects raise questions towards the interpretation of *Diplasia* spicoid as a flower: the nature of the lateral bracts and the ontogenetic sequence of the inner bracts (interpreted as perianth parts).

Considering the hypothesis number one, the lateral bracts are interpreted as outer perianth parts, however during development such bracts show a relative long difference in timing of appearance when compared to the other organs, suggesting their extra-floral nature. This is reinforced by the absence of floral identity in such organs as reported by Prychid and Bruhl (2013) for *Lepironia*. Thus, it is more likely to interpret them as bracts. Considering the hypothesis number two, the lateral bracts could be interpreted as two halves of a split glume, subtending the tetramerous flower. However, no splitting is observed during development and

the lateral bracts arise as distinct and separate organs. Thus, since in Cyperaceae each single flower is subtended by one glume (Eiten 1976; Bruhl 1995; Goetghebeur 1998), it hampers the interpretation of the whole unit as a flower subtended by two glumes (lateral bracts).

Furthermore, the sequence of development of stamens and inner bracts (interpreted as perianth parts) in *Diplasia* is quite different from perianth development in Cyperoideae flowers. In the *Diplasia* spicoid, the development follows the sequence: first the lateral stamens + gynoecium, followed by inner bracts and at last, inner stamens. Thus, the emergence of the inner bracts alternates with the emergence of lateral and inner stamens. When comparing to flowers in Cyperoideae, the perianth follows two different developmental patterns: either before the appearance of the stamens (Monteiro et al. submitted; Mora-Osejo 1967) or after the appearance of the stamens (Vrijdaghs et al. 2005a, 2006, 2009). However, the perianth in Cyperoideae is always positioned extrastaminally with no report of intrastaminal perianth parts (Mora-Osejo 1967; Vrijdaghs et al. 2005a, 2006, 2009). This hampers the interpretation of the spicoid as a single flower, since inner bracts are positioned between lateral and inner stamens. Therefore, we found no data in *Diplasia* development that support the floral interpretation of the spicoid.

Diplasia spicoid as a reduced inflorescence

The spicoid could be interpreted in two different possible inflorescence architectures: 1) An inflorescence composed of a terminal bisexual flower (gynoecium and two lateral stamens), subtended by two halves of a bract (lateral bracts) and two male flowers (each with three stamen) in median positions, each subtended by one bract (inner bracts) (Fig. 6c). 2) An inflorescence composed of a central female flower (gynoecium), two lateral male flowers (composed of one lateral stamen), each subtended by a bract (lateral bracts) and two male flowers in median position (each with three stamens), each subtended by a bract (inner bracts) (Fig. 6d). The features in favor of interpreting the spicoid as a reduced inflorescence are the difference in timing of the appearance of the lateral bracts compared to the remaining organs, reinforce their extra-floral nature, possibly as bracts of the reduced inflorescence. This is also true for the inner bracts, due to the temporal sequence of bract formation, alternating with the formed stamens. This favors their interpretation as foliar structures of a reduced inflorescence.

Although the development of *Diplasia* spicoid favors the inflorescence interpretation, it raise a question towards the architecture of such reduced inflorescence: are the lateral stamens and gynoecium part of a terminal bisexual flower or should they be considered each an unisexual flower (two male and one central female)?

Considering the sequence of organs appearance, the simultaneous emergence of the lateral stamens and the gynoecium primordium before the remaining organs allows to interpret them as a bisexual flower in the axil of a splitting prophyll (Holttum 1948), favoring the first hypothesis of reduced inflorescence. The problem with this hypothesis is again the presence of a split bract, when no splitting is observed during development. Thus, the bisexual terminal flower would be subtended by two bracts, unlike what is commonly found in Cyperaceae (Bruhl 1995; Goetghebeur 1998). Thus, the second hypothesis is more likely, interpreting the spicoid as a reduced inflorescence composed of a central female flower, two lateral male flowers, each subtended by a bract and two male flowers in median position (each with three stamens), each subtended by a bract (Fig. 6d).

Spicoid interpretation in other genera

Concerning other Mapanioideae genera, the interpretation of spicoids as inflorescence is favored in spicoids with a high number of stamens, as in *Diplasia, Lepironia, Scirpodendron, Capitularina* J. Kern and *Chrysitrix*, the latter exhibiting ca. 150 stamens per spicoid, each stamen subtended by its own bract (Clarke 1909). This differs from the typical Cyperoideae morphology where flowers commonly have zero to four stamens (Vrijdaghs et al. 2009, 2010, 2011). Even for genera with reduced spicoids, which resemble a single flower with two stamens and a gynoecium, as in *Hypolytrum* and *Mapania*, features as stamens developing at different levels, phyllotaxy and organization of vascular tissues resembling an inflorescence axis favor the inflorescence interpretation of such units, which possibly have undergone reduction and loss of structures during evolution (Monteiro et al. 2016).

Differences in the developmental sequence of bracts in *Exocarya* and perianth parts in Cyperoideae flowers favored the interpretation of *Exocarya* spicoids as reduced inflorescences (Richards et al. 2006). Based on the same developmental differences, the absence of floral protein in the lateral bracts and the loss of such protein in the mature inner bracts of *Lepironia*, Prychid and Bruhl (2013) favored the inflorescence interpretation as well.

CONCLUSION

Our results on *Diplasia* spicoids, compared to studies on other Mapanioideae (*Lepironia, Exocarya, Hypolytrum* and *Mapania*), show that the main developmental variations among the species are related to the sequence of organ appearance and the size and shape of the spicoid primordium, which in time may be influencing the spicoid's morphological variation in Mapanioideae.

Diplasia, Lepironia and Exocarya exhibit similarities concerning the temporal sequence of organ formation in a basipetal sequence, as well as adaxially flattened and abaxially rounded spicoid primordia, possibly leading to spicoids with higher number of bracts and stamens. For *Hypolytrum scraderianum* and *Mapania pycnostachya* the organs start to form from lateral to the center in an acropetal sequence and *Hypolytrum scraderianum* exhibit the smaller primordium, with a rounded shape ab- and adaxially, possibly restricting space for organs to be formed. The spatial constraint of the subtending bract seems to influence not only the geometry of the spicoid primordium, but also changes in reduction sites. The developmental features of the spicoids appear to be consistent among genera of the same tribe and support the phylogenetic placement of *Diplasia* in the tribe Chrysitricheae, as it shares more developmental patterns with *Lepironia* and *Exocarya* than with Hypolytreae species. On-going research will bring more details on the morphology of spicoids among Mapanioideae in an evolutionary perspective.

Concerning the nature of the *Diplasia* spicoids as flower or inflorescence, we argue in favour of a reduced inflorescence due to the sequence of bracts emerging alternate with the stamens, distinct from solitary flowers in Cyperoideae that exhibit exclusively extrastaminal perianth parts. Furthermore, the presence of two lateral bracts arising as separate structures, with no splitting during development, hampers their interpretation as two halves of a single glume, as in Cyperoideae flower. At last, for Mapanioideae spicoids, the number of stamens and developmental sequence of organs is quite different from flowers in Cyperoideae, hampering their interpretation as flowers.

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COMPLIANCE WITH ETHICAL STANDARDS

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Chapter 4

Chapter 4

Spicoid morphology of Mapanioideae (Cyperaceae): an evolutionary

perspective

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ABSTRACT

Premise of the study: Mapanioideae (Cyperaceae) exhibit reproductive units named spicoids, which has been interpreted by some studies as flower and sometimes as inflorescence. Little is known concerning the morphological variation of such spicoids in an evolutionary perspective. Thus, combining morphological and evolutionary analysis, we aimed to understand the variation of spicoids morphological features and their evolutionary significance in the phylogenetic relationships within Mapanioideae.

Methods: Spicoids of 49 species of Mapanioideae were analyzed. The spicoids were partitioned in eight characters, from which five were shown variable, potentially informative and selected for downstream comparative analyses.

Key results: The results show that the spicoid morphology exhibit some stable features among the species. Nevertheless, the presence of keels in the lateral bracts, the number of lateral stamens, inner bracts, inner stamens and stigmas shown to be variable among genera, but consistent within genera. The possible ancestral spicoid morphology was recovered resembling the current morphology of *Hypolytrum* spicoids and base on the most recurrent changes for each character, the acquisition of structures seems as a recurrent process among genera.

Conclusions: We demonstrated that the variable morphological features of the spicoids are informative at generic level. Furthermore, the ancestral character reconstruction allowed us to infer that evolutionary changes in the subfamily were mostly driven by the acquisition of structures, contradicting previous reductional hypothesis. The notable inconclusive relations in the phylogeny of Mapanioideae highlight the need of further studies combining molecular and morphological traits to elucidate the phylogenetic relationships within Mapanioideae.

KEYWORDS: ancestral reconstruction, mapaniid, Hypolytreae, Chrysitricheae, spicoid.

INTRODUCTION

Inflorescences in Cyperaceae has been always a matter of controversy and misinterpretation especially due to the variety and complexity of reduced structures that can be found (Bruhl, 1995; Goetghebeur, 1998; Vrijdaghs et al., 2009). Such statement applies in different context for the two well established subfamilies of Cyperaceae: Cyperoideae and Mapanioideae (Simpson et al., 2007; Muasya et al., 2009; Hinchliff and Roalson, 2013; Spanlik et al., 2016; Semmouri et al., 2019).

Cyperoideae hold the vast majority of genera of Cyperaceae (ca. 76 genera) and have lateral axes of the compound panicles terminate in spikelets, the smallest unit in the whole inflorescence. The spikelet is composed of an axis holding several bracts that may or may not subtend a flower (Eiten, 1976; Bruhl, 1995; Goetghebeur, 1998; Vegetti, 2003). This subfamily has been intensely investigated in terms of development of flower, inflorescence and phylogenetic relationship (i.e. Vegetti, 2003; Vrijdaghs et al., 2004, 2005a, 2005b, 2009, 2010, 2011; Muasya et al., 2009; Hinchliff and Roalson, 2013; Reutemann et al., 2014, 2015; Lucero et al., 2014; Monteiro et al., 2017; Semmouri et al., 2019). On the other hand, Mapanioideae is less diverse (11 genera) (Govaerts et al., 2018) and many gaps are still to be filled especially concerning the understanding of their particular reproductive units named spicoid (Simpson, 1992).

There is a lack of consensus regarding the nature of the spicoid that was simultaneously interpreted as a single flower (Kern, 1974; Eiten, 1976; Goetghebeur, 1998) as well as a reduced inflorescence according to mature morphology (Bruhl, 1995; Simpson, 1992; Dahlgren et al., 1985). More recently, ontogenetic studies have favored the interpretation of the spicoid as reduced inflorescences (Richards et al., 2006; Prychid and Bruhl, 2013; Monteiro et al., 2016, submitted).

The spicoid is the smallest unit of Mapanioideae inflorescences, but it is presumably not homologous to the Cyperoideae spikelet. In a comparative perspective, the spikelet (Cyperoideae) has an axis holding many flowers (Fig. 1A) while in Mapanioideae this axis belongs to a spike holding many spicoids (Fig. 1B) which in turn hold many flowers (Fig. 1C). The spicoid is described as having a general pattern of two lateral bracts involving two lateral stamens and a central positioned gynoecium (Fig. 1C). Further, the spicoid holds a variable number of stamens and inner bracts that may be present outside the androecium and/or between androecium and gynoecium (Fig. 1C) (Koyama, 1971; Kern, 1974; Simpson, 1992; Bruhl, 1995; Goetghebeur, 1998; Richards et al., 2006; Prychid and Bruhl, 2013; Monteiro et al., 2016). Although the debate between flower versus inflorescence hypothesis are of great importance to the subfamily, a considerable gap remains in the understanding of the morphological features of these spicoids for each Mapanioideae genera, most of them with information restrict to taxonomic and systematic purposes (i.e. Holttum, 1948; Kern, 1974; Eiten, 1976; Bruhl, 1995; Simpson, 1992; Goetghebeur, 1998; Alves et al., 2001).



Figure 1. Simplified schemes of Cyperoideae and Mapanioideae reproductive units. (A) Schematic draw of a general pattern of spikelet of Cyperoideae, based on Bruhl (1995),

Goetghebeur (1998), Vrijdaghs et al. (2009, 2010). (B) Schematic draw of a general pattern of a spike of Mapanioideae. (C) Schematic draw and diagram of a general pattern of a spicoid of Mapanioideae. B-C were based on Simpson (1992), Bruhl, (1995), Goetghebeur (1998), Richards et al. (2006), Prychid & Bruhl (2013), Monteiro et al. (2016). bf, bisexual flower; ib, inner bract; is, inner stamen; g, glume; lb, lateral bract; ls, lateral stamen; sb, subtending bract; spb, spike bract; spc, spicoid.

Mapanioideae is divided in two monophyletic tribes (Hypolytreae and Chrysitricheae) (Simpson et al., 2007; Muasya et al., 2009; Hincliff and Roalson 2013; Semmouri et al., 2019). The most species-rich genera of Mapanioideae, *Mapania* Aubl. (100 species) and *Hypolytrum* Pers. (61 species) are the ones with the greater number of available information (i.e. Simpson, 1992, 1996, 2006; Alves et al., 2001, 2002; Coan et al., 2008, 2010; Monteiro et al., 2016). Further genera are restricted to few species such as *Paramapania* Uittien (seven), *Chorizandra* R.Br. (six), *Chrysitrix* L. (four) and *Scirpodendron* Zipp. Ex Kurz (two) (Govaerts et al., 2018). A remarkable characteristic of Mapanioideae is the significant amount of monotypic genera, five from the 11 genera belonging to the subfamily is composed of one species each, such as *Capitularina* J.Kern, *Lepironia* Pers., *Exocarya* Benth., *Diplasia* Rich. and *Principina* Uittien (Govaerts et al., 2018). The variation in their spicoid morphology also reflects in their high level of uncertainty in the phylogeny (Simpson et al., 2003, 2007; Muasya et al., 2009; Hinchliff and Roalson, 2013; Spanlik et al., 2016; Semmouri et al., 2019).

Considering the value of reproductive traits to establish relationships among angiosperms (i.e. Ronse De Craene et al., 2018; Endress, 2006; Chartier et al., 2014; Sauquet et al., 2017; Reyes et al., 2018), it is remarkable the importance of studies towards the morphology of the spicoids in Mapanioideae genera, focusing on stable and variable features among them and mapping such features in the molecular phylogeny (Semmouri et al., 2019) in order to infer about the ancestral spicoid morphology and their significance in an evolutionary perspective, an indubitable crucial step in the understanding of such particular and little-known subfamily. Thus, we aim through a combined morphological and evolutionary perspective to answer the questions: 1) What are the most stable and most variable morphological traits in the spicoids of Mapanioideae? 2) How informative the spicoid morphology is in terms of evolutionary relationships in Mapanioideae? 3) What are the ancestral traits related to the spicoid? Based on a comprehensive survey of spicoid morphology we expect to expand the understanding of such units and their evolutionary significance in the phylogenetic relationships within Mapanioideae.

MATERIAL AND METHODS

Morphological sampling – The spicoid morphology was assessed from herbarium collection available at K (The Royal Botanic Gardens, Kew), as well as material conserved in 70% ethanol (Appendix 1). A total of 49 species were analyzed, from the 11 genera of Mapanioideae, corresponding to ca. 27% of the total number of species from the subfamily, including the terminals in the phylogeny (Appendix 1). For each species, up to three different individuals were analyzed when available. For the terminals in the phylogeny which material was unavailable, the morphological features were consulted from the literature. These are *Scirpodendron bogneri* (Hooper and Simpson, 1997) and *Mapania meditensis* (Simpson, 1992). The dried spikes were rehydrated in boiling water for 10 minutes, dissected under a stereomicroscope Leica Wild M8 and photographed using a Leica M 165 FC digital compound with a camera device DFC 450 C. The diagrams illustrating the spicoids architecture were made using the software Corel Drawn Graphics Suite X7.

Character reconstruction – Ancestral character reconstruction was performed using the phylogeny from Semmouri et al. (2019). Morphological studies in Cyperaceae commonly use typological classification of the inflorescence in order to classify groups in a particular

architecture (i.e. Eiten, 1976; Dahlgren et al., 1985). However, we believe that considering a typological classification for the spicoid may hamper an evolutionary perspective of morphological variation, since typologies are a combination of several features that can be shared by different types. Therefore, instead to be evaluated as a single unit, the spicoid was tackled in a reductive coding through an interactive process of decomposition. Thus, the spicoid was preliminary partitioned in eight characters (presence/absence of subtending bract; number of lateral bracts; presence/absence of keel in the lateral bracts; number of lateral stamens, number of inner bracts, number of inner stamens; position of pistil; number of stigmas). From these, five were shown variable, potentially informative and selected for downstream comparative analyses. The variable features are: 1) The presence of keels in the lateral bracts (a: present, b: absent); 2) Number of lateral stamens (a: 0, b: 2); 3) Number of inner bracts (a: 0, b: 1-6, c: 7-12, d: 13-19, e: >19); 4) Number of inner stamens (a: 0, b: 1-6, c: 7-14, d: >14); 5) Number of stigmas (a: 2, b: 3). The coding arrangements were performed to optimize the character reconstruction analyses. The parameter "type=discrete" was used since the morphological features were coded as discrete. Bayesian stochastic character (BSC) mapping (Huelsenbeck et al., 2018) was performed using the 'make.simmap' function available in phytools v0.5–64 (Revell, 2012). The analyses were implemented in R (R Core Team, 2018) using the model that best fit to the data ("equal rates" or "all rates different") to recover the evolutionary history of the morphological features under investigation and recover ancestral character state probabilities of the nodes. Outgroup, terminals with more than one accession per taxon and polytomies were removed from the analysis using the function 'drop.tip' from ape to prevent bias in character reconstruction. Stochastic character mapping was performed using 1000 simulations. BSC provide transition rates which were used to build a transition matrix among features as input to the heatmaps depicted using pheatmap v1.0.12.

RESULTS

Morphology of the spicoids in Mapanioideae

For all the analyzed species, the spicoids exhibit a subtending bract which envelops the whole unit, two lateral bracts and a central positioned pistil (Figs 2A-E; 3A-F). A comparative table with morphological features of the spicoids of each analyzed species was performed (Appendix 2). For most of the genera, except for *Chrysitrix* (Fig. 3F), *Chorizandra cymbaria* and some *Mapania* species (Appendix 2), a distinct keel is observed along the midribs of the two lateral bracts. The lateral bracts subtend two lateral stamens in most of the species, except in *Chorizandra cymbaria*, *Capitularina involucrata* (Fig. 3C) and some individuals of *Mapania pycnostachya* (Appendix 2), the latter may exhibit just one lateral stamen (Fig. 2A). The number of inner bracts and inner stamens varies from zero (i.e. most of species of *Hypolytrum*) to > 100 (*Chrysitrix capensis* - Fig. 3C) (Appendix 2). From the 49 species analyzed, 31 exhibit a dimerous dorsiventrally flattened pistil while the remaining 18 species show a trimerous pistil each (Appendix 2).





Figure 2. Mature morphology and schematic draws of spicoids of Mapanioideae, tribe Hypolytreae, based on the phylogeny recovered by Semmouri et al. (2019). (A) *Mapania*. (B) *Hypolytrum*. (C) *Principina*. (D) *Paramapania*. (E) *Scirpodendron*. Arrows indicate an inner bract. Dotted lines indicate that the structure may be present or not. The circle with a cross above the spicoid diagram indicates the position of the axis. lb, lateral bract; ls, lateral stamen; sb, subtending bract. Scale bars: (A) *M. bancana*, 250µm; *M. holttumi*, 1mm; *M. pycnostachya*, 750µm. (B) *H. pulchrum*, 500µm; *H. sylvaticum*, 250µm. (C) *P. grandis*, 500µm. (D) *P. parvibractea*, 500µm. (E) *S. ghaeri*, 750µm.





Figure 3. Mature morphology and schematic draws of spicoids of Mapanioideae, tribe Chrysitricheae, based on the phylogeny recovered by Semmouri et al. (2019). (A) *Diplasia*. (B) *Exocarya*. (C) *Capitularina*. (D) *Lepironia*. (E) *Chorizandra*. (F) *Chrysitrix*. Arrows indicate an inner bract. Dotted lines indicate that the structure may be present or not. The circle with a cross above the spicoid diagram indicates the position of the axis. lb, lateral bract; ls, lateral stamen; p, pistil; sb, subtending bract. Scale bars: (A) D. karatifolia, 500µm. (B) E. sclerioides, 250µm. (C) C. involucrata, 1mm. (D) L. articulata, 750µm. (E) C. enodis, 1mm. (F) C. capensis, 1mm.

Tribe Hypolytreae

Mapania – The spicoids in the genus generally exhibit two lateral bracts usually keeled subtending one lateral stamen each (Fig. 2A). Some individuals of *M. pycnostachya* exhibit a single lateral stamen (Fig. 2A). The adaxial and abaxial inner bracts are sterile in *M. coriandrum*, *M. imeriensis*, *M. macrophylla*, *M. pycnocephala*, *M. pycnostachya* (Appendix 2). For the remaining analyzed species, the adaxial inner bract is sterile and the abaxial one subtends an inner abaxial stamen (Fig. 2A). Furthermore, for *M. holttumi* (Fig. 2A), *M. tenuiscapa*, *M. sylvatica*, *M. sumatrana*, *M. palustris*, *M. meditensis*, *M. macrocephala*, *M. longiflora* and *M. graminea* besides the above-mentioned features, the central pistil is surrounded laterally by two inner bracts (Appendix 2). The morphology of the pistil varies from dimerous dorsiventrally flattened to trimerous in the genus (Appendix 2).

Hypolytrum – The genus general spicoid morphology consists of two keeled lateral bracts, which may be fused abaxially and/or adaxially in species such as *H. sylvaticum* (Fig. 2B). Each lateral bract subtends a lateral stamen (Fig. 2B). For *H. pulchrum* (Fig. 2B), *H. supervacuum* and *H. rigens*, an abaxial inner bract subtending an inner stamen is observed. The genus showed exclusively dimerous dorsiventrally flattened pistil (Appendix 2).

Principina – The spicoid of *Principina grandis* consists of two lateral keeled bracts, which may be fused abaxially, subtending one lateral stamen each (Fig. 2C). An abaxial
inner bract is observed subtending an inner stamen and the pistil is trimerous (Fig. 2C) (Appendix 2).

Paramapania – The spicoid of Paramapania species consists of two lateral keeled bracts subtending two lateral stamens, an abaxial inner bract subtending an inner stamen (Fig. 2D). The species exhibit three inner sterile bracts surrounding the pistil (Fig. 2D) which is trimerous (Appendix 2).

Scirpodendron – Spicoids of *Scirpodendron ghaeri* are composed of two lateral keeled bracts subtending each a single lateral stamen and seven to nine inner stamens, spirally arranged, each subtended by its own inner bract (Fig. 2E). Bracts were not observed surrounding the trimerous pistil (Appendix 2).

Tribe Chrysitricheae

Diplasia – *Diplasia karatifolia* exhibits a spicoid composed of two lateral keeled bracts subtending one lateral stamen each and two inner bracts each subtending three inner stamens (Fig. 3A). The pistil is dimerous dorsiventrally flattened (Appendix 2).

Exocarya – The spicoid of *Exocarya sclerioides* exhibits two lateral keeled bracts subtending a single lateral stamen each (Fig. 3B). Two inner bracts are observed, an adaxial sterile one and an abaxial one. The latter subtends an inner stamen (Fig. 3B). The species exhibit a dimerous dorsiventrally flattened pistil (Appendix 2).

Capitularina – The spicoids in *Capitularina involucrata* are composed of two lateral keeled bracts which are sterile (Fig. 3C). Seven to thirteen inner bracts are observed from which the proximal four are sterile (Fig. 3C). The following three inner envelop three inner stamens each, those in turn are subtended by a single smaller bract each (Fig. 3C). The pistil is dimerous dorsiventrally flattened (Appendix 2).

Lepironia – *Lepironia articulata* spicoids have two lateral keeled bracts each subtending a lateral stamen and 23 inner bracts from which ca. 14 subtend a single stamen each. Nine

inner bracts are sterile and positioned between stamens and the dimerous pistil (Fig. 3D). The number of inner bracts and stamens is greater at the abaxial side while the adaxial side exhibit ca. five inner stamens subtended by their own bract (Fig. 3D). The species exhibits a dimerous dorsiventrally flattened pistil (Appendix 2).

Chorizandra – *Chorizandra* species have spicoids exhibiting two keeled lateral bracts each subtending a lateral stamen (Fig. 3E) except for *Chorizandra cymbaria* in which the lateral bracts are sterile and not keeled (Appendix 2). Nine to more than 20 inner bracts are observed. From those, seven to more than 20 subtend each a single stamen. The remaining inner bracts (two to seven) surround the trimerous pistil (Appendix 2).

Chrysitrix – The spicoid in *Chrysitrix* consists mainly of two lateral bracts with no keels which are sterile followed by several inner bracts (50 to more than 100 inner bracts), the two most proximal ones are sterile and the remaining subtend a single inner stamen (Fig. 3F). About three sterile inner bracts are positioned between stamens and the trimerous pistil (Fig. 3F) (Appendix 2).

Ancestral character reconstruction of spicoid

The ancestral state of the spicoid Mapanioideae was reconstructed as composed of two keeled lateral bracts each subtending a lateral stamen and a central dimerous pistil (Fig. 4A).





Figure 4. Character reconstruction of the spicoid morphology of Mapanioideae. (A) Schematic draw and diagram of the possible ancestral spicoid of Mapanioideae recovered as having a subtending bract, two lateral bracts, two lateral stamens and a central dimerous pistil. The circle with a cross above the spicoid diagram indicates the position of the axis. (B) Character reconstruction of the presence of keels in the lateral bracts in the spicoids of Mapanioideae. The character state "presence" is indicated in blue and "absence" is indicated in red. Pies provide posterior probabilities. Arrow indicates the clade containing *Mapania cuspidata* + *Mapania meditensis* and *Scirpodendron*. Arrowhead indicates the clade containing *Mapania macrophylla* and *Mapania pycnocephala*. (C) Heatmap of transition rates among presence and absence of keels in the lateral bracts, read must follow left to right. The most recurrent changes are indicated by warm colors.

Presence of keels in the lateral bracts – The most recent common ancestral (MRCA) of Mapanioideae was reconstructed as having keels in the lateral bracts (Fig. 4B). The absence of keels in the lateral bracts arose at least twice in Chrysitricheae (*Chorizandra cymbaria, Chrysitrix capensis* and *Chrysitrix dodii*) and once in Hypolytreae (*Mapania macrophylla* and *Mapania pycnocephala*) (Fig. 4B). The reconstruction recovered approximately 4.85 changes on average with the most recurrent changes occurring from presence to absence of keels in the lateral bracts (3.20) (Fig. 4C).

Number of lateral stamens – The MCRA of Mapanioideae was reconstructed as expressing two lateral stamens as well as Hypolytreae (Fig.5A). Lateral stamens were lost at least three times in Chrysitricheae (*Capitularina involucrata, Chorizandra cymbaria, Chrysitrix capensis* and *Chrysitrix dodii*) (Fig.5A). The reconstruction recovered ca. 4.32 changes on average from which most of changes (2.71) occurred toward decreasing the number of lateral stamens (Fig.5B).

Number of inner bracts – The MCRA of Mapanioideae was reconstructed as lacking any inner bracts (zero) (Fig. 5C). The character is highly variable in both tribes specially in tribe Chrysitricheae (Fig. 5C) that was recovered ca. 16.23 changes between stages on average. The most recurrent changes occurred toward increasing the number of bracts, while loss of bracts was seldom (Fig. 5D).



Figure 5. Character reconstruction of the spicoid morphology of Mapanioideae. (A) Character reconstruction of number of lateral stamens in the spicoids of Mapanioideae. The character state "zero" is indicated in blue and "two" is indicated in red. Pies provide posterior probabilities. Arrow indicates the clade containing *Mapania cuspidata* + *Mapania meditensis* and *Scirpodendron*. Arrowhead indicates the clade containing *Mapania macrophylla* and *Mapania pycnocephala*. (B) Heatmap of transition rates among zero and two lateral stamens, read must follow left to right. The most recurrent changes are indicated by warm colors. (C) Character reconstruction of number of inner bracts in the spicoids of Mapanioideae. The character state "zero" is indicated in blue, "one to six" in red, "seven to twelve" in yellow, "thirteen to nineteen" in green and "more than nineteen" in purple. Pies provide posterior probabilities. Arrowhead indicates the clade containing *Mapania meditensis* and *Scirpodendron*. Arrowhead indicates the clade in blue, "one to six" in red, "seven to twelve" in yellow, "thirteen to nineteen" in green and "more than nineteen" in purple. Pies provide posterior probabilities. Arrow indicates the clade containing *Mapania cuspidata* + *Mapania meditensis* and *Scirpodendron*. Arrowhead indicates the clade containing *Mapania macrophylla* and *Mapania pycnocephala*. (D) Heatmap of transition rates among different intervals of the number of inner bracts, read must follow left to right. The most recurrent changes are indicated by warm colors.

Number of inner stamens – The MCRA of Mapanioideae was reconstructed as lacking inner stamens (zero) with the widest range of variation occurring in tribe Chrysitricheae (Fig. 6A). The character was recovered ca. 10.24 changes on average with the most recurrent changes occurring toward increasing the number of stamens. Reversions toward loss of inner stamens were not reported (Fig. 6B).

Number of stigmas – The MCRA of Mapanioideae was reconstructed as having a dimerous pistil while a trimerous pistil evolved at least five times in Mapanioideae (*Chorizandra, Chrysitrix, Mapania cuspidata + Mapania meditensis, Scirpodendron bogneri + Scirpodendron ghaeri* and *Paramapania parvibractea*) (Fig. 6C). The character was recovered ca. 5.45 changes on average and the most recurrent changes occurred from dimerous to trimerous pistil (3.79) (Fig. 6D).



Figure 6. Character reconstruction of the spicoid morphology of Mapanioideae. (A) Character reconstruction of number of inner stamens in the spicoids of Mapanioideae. The character state "zero" is indicated in blue, "one to six" is indicated in red, "seven to fourteen" in yellow and "more than fourteen" in green. Pies provide posterior probabilities. Arrow indicates the clade containing *Mapania cuspidata* + *Mapania meditensis* and *Scirpodendron*. Arrowhead indicates the clade containing *Mapania macrophylla* and *Mapania pycnocephala*. (B) Heatmap of transition rates among different intervals of the number of inner stamens, read must follow left to right. The most recurrent changes are indicated by warm colors. (C) Character reconstruction of number of stigmas in the spicoids of Mapanioideae. The character state "dimerous (two stigmas)" is indicated in blue and "trimerous (three stigmas)" is indicated in red. Pies provide posterior probabilities. Arrow indicates the clade containing *Mapania macrophylla* and *Mapania stigmas* and *Scirpodendron*. Arrowhead indicates the clade containing *Mapania macrophylla* and *Mapania by character* reconstruction of number of stigmas in the spicoids of Mapanioideae. The character state "dimerous (two stigmas)" is indicated in blue and "trimerous (three stigmas)" is indicated in red. Pies provide posterior probabilities. Arrow indicates the clade containing *Mapania macrophylla* and *Mapania pycnocephala*. (D) Heatmap of transition rates among dimerous and trimerous pistil, read must follow left to right. The most recurrent changes are indicated by warm colors.

DISCUSSION

Based on a survey of the spicoid morphology throughout Mapanioideae genera, the results show that the spicoid exhibit some stable features among the species such as a subtending bract, two lateral bracts and a central pistil. Nevertheless, the presence of keels in the lateral bracts, the number of lateral stamens, inner bracts, inner stamens and stigmas shown to be quite variable.

Concerning the presence of keels, it is a usual feature observed in Cyperaceae and reported especially in the prophylls which are normally reported as being two-keeled (see Snell, 1936; Blaser, 1944; Bruhl, 1995). In Mapanioideae only the lateral bracts may present a distinctive keel which is absent in the inner bracts (Simpson, 1992; Bruhl, 1995), as here observed. The variation in the number of stamens and inner bracts is here reported for both tribes in Mapanioideae and appear to have evolved independently in different genera, considered homoplastic features and not informative to characterize the tribes.

The morphological variation in the pistil is related to the number of stigmas branches and although it varies considerably among genera, it is quite stable among species of the same genera, except for *Mapania* as previously reported by Simpson (1992) and for *Chorizandra*. Although quite variable, the morphological characters of the spicoids shown to be informative at generic level and here we will discuss such variable features in an evolutionary perspective.

Spicoid morphology and evolutionary relationships in Mapanioideae

Mapanioideae is divided in two monophyletic tribes according to molecular data: Hypolytreae including *Hypolytrum*, *Mapania*, *Paramapania*, *Principina*, *Scirpodendron* and Chrysitricheae nesting *Capitularina*, *Chorizandra*, *Chrysitrix*, *Diplasia*, *Exocarya* and *Lepironia* (Simpson et al., 2007; Muasya et al., 2009; Hincliff and Roalson, 2013; Semmouri et al., 2019). Particularly, within Hypolytreae a general pattern of reduced

number of structures in the spicoids is recurrent in most genera. This pattern results in a spicoid arrangement that resembles a single flower (Holttum, 1948; Kern, 1974; Goetghebeur, 1998; Simpson, 1992). On the other hand, spicoids of Chrysitricheae tend to exhibit a higher number of structures with a wide range of variation (Holttum, 1948; Kern, 1974; Goetghebeur, 1998).

Considering the relations within Hypolytreae, the phylogeny recovered Mapania as not monophyletic since Scirpodendron is embedded in the genus. As result, Mapania was segregated in a clade containing species of Mapania + Scirpodendron (Figs 4-6 arrow) and other clade containing only species of Mapania (Figs 4-6 - arrowhead) (Simpson et al., 2007; Muasya et al., 2009; Hincliff and Roalson, 2013; Semmouri et al., 2019). This segregation is seemly supported by the morphology of the spicoids since spicoids in Mapania cuspidata and Mapania meditensis share more features with Scirpodendron (keeled lateral bracts, two lateral stamens and a trimerous pistil) than with Mapania macrophylla and Mapania pycnocephala (two lateral stamens and two inner bracts). According to our data, the latter two species seem to share more similarities with Hypolytrum spicoids, i.e. two lateral stamens, absence of inner stamens, and a dimerous dorsiventrally flattened pistil. Previously, morphological similarities among species of Hypolytrum and Mapania raised questions about the need of reinvestigate the boundaries of both genera (Smith et al., 2009). Despite the similarities between the spicoids in such genera, further morphological features (i.e. pollen and embryo type) and molecular reconstruction showed that combining both in a large group seems unlikely (Simpson et al., 2007; Muasya et al., 2009; Hincliff and Roalson, 2013; Semmouri et al., 2019).

Among Chrysitricheae genera, *Chrysitrix* exhibits the most peculiar spicoid morphology. The spicoid of the genus was described by Eiten (1976) as arising from a continuation of the axis of the culm, exhibiting bracts without keels and described as

highly unusual (Goetghebeur, 1998). These "unusual morphology" was showed here to be mostly related with the expression of a wide range of variation in the morphological features of the spicoids when compared to the rest of Mapanioideae. The absence of keels in the lateral bracts, together with the absence of lateral stamens and a notable increase in the number of inner bracts and inner stamens (ca. 100 each) are among the features regulating the morphology of the *Chrysitrix* spicoids. It is remarkable that *Chrysitrix* spicoids are quite variable when compared with other genera but within genus, the spicoids features of *Chrysitrix* are stable, supporting their phylogenetic relationship (Semmouri et al., 2019). Such statement is true for most of the Mapanioideae genera except for *Mapania* and *Chorizandra* that show spicoids with some variation within genus. This morphological variation in *Mapania* is expected, considering that the genus exhibit the highest number of species among Mapanioideae, with a wide distribution, thus morphological changes may have become stablished at certain times during evolution in response to both genetic mechanisms and the interaction with environment (Prusinkiewicz et al., 2007; Prenner et al., 2009).

However, a notable morphological variation also occurs between the studied species of *Chorizandra* which exhibit a restrict number of species (six) as well as a restrict distribution (Govaerts et al., 2018). The genus is a sister group of *Chrysitrix* (Simpson et al., 2007; Muasya et al., 2009; Hincliff and Roalson, 2013; Semmouri et al., 2019) and it is notable that *Chorizandra cymbaria* shares more similarities in the spicoid morphology with *Chrysitrix* species (absence of keeled lateral bracts, absence of lateral stamens and a trimerous pistil) than with other analyzed *Chrorizandra*, with which the only feature they shared was the presence of a trimerous pistil (Appendix 2). The considerable variation in the spicoid morphology among *Chorizandra* species, added to the similarities with spicoids of *Chrysitrix*, raise question whether they should be combined in a single

genus. Based on the reconstruction of the phylogeny using pollen and molecular data, *Chorizandra* and *Chrysitrix* species were previously grouped together (Simpson et al., 2003), although the authors reported that distinct morphological features of the inflorescences hamper a generic relationship between such species (Simpson et al., 2003). We believe that further investigation is needed to clarify the relations between such unknown and restricted distributed genera.

Circumscription of tribes and the included genera was always a matter of consensus in previous studies (Simpson et al., 2003, 2007; Muasya et al., 2009; Hincliff and Roalson, 2013; Spalink et al., 2016; Semmouri et al., 2019) except for Diplasia. The genus was placed within tribe Hypolytreae (Simpson et al. 2003, 2007; Muasya et al., 2009), as a separate group from Hypolytreae and Chrysitricheae (Spalink et al., 2016) and recently it was nested within Chrysitricheae (Semmouri et al., 2019). Concerning the spicoid morphology, it is noteworthy that Diplasia shares many similarities with *Exocarya* such as two lateral bracts, two lateral stamens, two inner stamen and a dimerous pistil. Such similarities are also observed in developmental features of the spicoid (Monteiro et al. submitted). Despite the similarities between Diplasia and Exocarya spicoids, both are considerably distinct from the remaining Chrysitricheae genera, mainly in the lower number of inner bracts and inner stamens, sharing more similarities with Hypolytreae genera. Both genera were previously placed within Hypolytreae (Simpson, 1992; Goetghebeur, 1998) based on morphological features such as poorly differentiated embryos but pollen data has placed both genera in distinct tribes (Simpson et al., 2003) and molecular data have placed Diplasia and Exocarya within Chrysitricheae (Semmouri et al., 2019). Our results based on the morphology of spicoids suggest the placement of Diplasia and Exocarya as sister groups but question their placement within Chrysitricheae.

Evolution of spicoid morphology in Mapanioideae

Based on the ancestral state reconstructions, we can hypothesize that the ancestral morphology of the spicoid is composed of two lateral keeled bracts, two lateral stamens and a dimerous pistil. This architecture resembles what is currently found in *Hypolytrum*. Smith et al. (2009) suggested the close morphological affinity of pollen and infructescence of *Hypolytrum* and *Mapania* with the fossil record of an extinct sedge (*Volkeria messelensis*). Furthermore, from the five characters reconstructed, the most recurrent changes occurred towards an increase in the number of parts (number of inner bracts, number of inner stamens, and number of stigmas). Thus, we believe that changes in the spicoid morphology during evolution occurred both by reduction and acquisition of parts. However, the acquisition seems to be more recurrent and the main factor leading such changes. This interpretation contradicts previous hypothesis which comparing the morphology of the Mapanioideae spicoids, interpreted that changes in the spicoid morphology of the spicoid spicole, interpreted that changes in the spicoid and the main factor leading such changes. This interpretation contradicts previous hypothesis which comparing the morphology of the Mapanioideae spicoids, interpreted that changes in the spicoid morphology occurred mainly due to reduction of organs such as stamens and bracts in an ancestral possibly resembling *Scirpodendron* (Holltum, 1948; Kern, 1974; Dahlgren et al., 1985).

Although we interpreted the evolution of spicoid morphology driven mostly by an increase in number of parts, no evidences allow us to stablish a connection between changes in Mapanioideae spicoids and trimerous flowers in Cyperoideae. However, it is noteworthy that Mapanioideae spicoids exhibit similarities with flowers of Trilepideae, the first branching lineage and sister of the rest of Cyperoideae (Muasya et al., 2009; Hincliff and Roalson, 2013; Semmouri et al., 2019). Blaser (1944) reported similarities of male flower of *Trilepis lhotzkiana* Nees with Mapanioideae spicoids, the former exhibiting a two-keeled bract, an abaxial scale and two lateral smaller scales, the latter three scales subtending one stamen each, raising questions towards the nature of flowers

in Trilepideae. Our study is limited to highlight such morphological similarities and further combined molecular and morphological studies are needed in order to clarify the phylogenetic relation between Trilepideae and Mapanioideae.

CONCLUSION

Considering the morphological features of the spicoids in Mapanioideae, we observed that they are more informative at generic level, since some genera with more than one species share a stable spicoid architecture such as *Chrysitrix*. *Hypolytrum*, *Paramapania* and *Scirpodendron*. Features such as number of inner bracts and inner stamens were the ones with the highest variation within both tribes, mainly in Chrysitricheae. The same was observed for the absence of keeled bracts, lateral stamens and the presence of a trimerous pistil, interpreted in our analysis as homoplastic features. Similarities in the spicoid morphology of different genera were observed, such as in *Diplasia* and *Exocarya*, for species of *Chrysitrix* and *Chorizandra*, as well as for some *Mapania* and *Scirpodendron* species. Also, similarity of Mapanioideae spicoids with Trilepideae flowers, the first branching lineage of Cyperoideae, were observed reinforcing the need of reinvestigate their phylogenetic boundaries combining molecular and morphological data.

Moreover, the ancestral character reconstruction of the spicoid morphology allowed us to infer that the possible morphology of the spicoid in the ancestral of Mapanioideae resembles the morphology of *Hypolytrum* spicoids and base on the most recurrent changes for each character, we infer that evolutionary changes in the subfamily were mostly driven by the acquisition of structures. The notable inconclusive relations in the phylogeny of Mapanioideae, added to a considerable number of monotypic genera of restricted distribution may hamper the access to material and acquisition of molecular data, highlighting the need of expand and combining molecular and morphological studies concerning Mapanioideae reproductive units in order to push forward the knowledge towards such a rich and exceptional subfamily.

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AUTHOR CONTRIBUTIONS

MMM, DD, GP and CP designed the research, MMM performed the research and generated the dataset. MMM, DD, GP, IL and CP integrated and interpreted the results. All authors contributed with further discussion, suggestions and writing of the manuscript.

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DATA ACCESSIBILITY STATEMENT

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Blaser, 1944 - DOI: 10.2307/2437667

Bruhl, 1995 - DOI:10.1071/SB9950125

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Appendix 1. Analyzed species in the current study and respective information of herbarium, collector, number, bar code and collection area. Blank spaces indicate missing data.

Tribe	Species	Herbarium	Collector Number Bar		Barcode	Collection area
Chrysitricheae	Capitularina involucrata (Valck.Sur.) J.Kern	k	E.J.H.Corner	49		Pacific - British Solomon Islands
Chrysitricheae	Capitularina involucrata (Valck.Sur.) J.Kern	k	Sands	6509		New Guinea - Irian Jaya - Indonesia
Chrysitricheae	Chorizandra cymbaria R.Br.	k	S.T.Blake	20998		Australia-Queensland
Chrysitricheae	Chorizandra cymbaria R.Br.	k	K.L.Wilson	9855		Australia-Sydney
Chrysitricheae	Chorizandra enodis Nees	k	M.Visoiu	544427		Australia-Tasmania
Chrysitricheae	Chorizandra enodis Nees	k	P.J.Ainsley; D.J.Duval; G.Verena	200782		Australia - South Australia
Chrysitricheae	Chorizandra sphaerocephala R.Br.	k	J.H.Camfield	22491		Australia-Sydney
Chrysitricheae	Chrysitrix capensis L.	k	W.J.Burchell	600		South Africa - Cape Province
Chrysitricheae	Chrysitrix capensis L.	k	C.B. Clarke	4424		South Africa - Cape Province
Chrysitricheae	Chrysitrix dodii C.B.Clarke	k	Wooley	3549	K000363322	South Africa - Cape Province
Chrysitricheae	Diplasia karatifolia Rich.	k	Basset Maguire; D.B. Fanshawe	22850		Tropical South America- Guyana - Essequibo river
Chrysitricheae	Diplasia karatifolia Rich.	k	H.S.Irwin; G.T.Prance; T.R.Soderstrom; N.Holmgreen	55551	K000062366	Tropical South America- Suriname
Chrysitricheae	Exocarya sclerioides (F.Muell.) Benth.	k	L.A.S.Johnson; E.F.Constable	42307		Australia-New South Wales
Chrysitricheae	Exocarya sclerioides (F.Muell.) Benth.	k	J.R.Croft	68670		New Guinea - Papua
Chrysitricheae	Lepironia articulata (Retz.) Domin	k	T.L.Yao	53090		Malay peninsula - Malaysia
Chrysitricheae	Lepironia articulata (Retz.) Domin	k	C.J.Prychid	13		Australia
Hypolytreae	Hypolytrum amplum Poepp. & Kunth	k	G.S.Jenman	4115		Tropical South America-Guyana
Hypolytreae	Hypolytrum amplum Poepp. & Kunth	k	P.J.Edwards	2912		Tropical South America- Guyana - Corentyne district
Hypolytreae	<i>Hypolytrum compactum</i> Nees & Meyen ex Kunth	k	L.J.Brass	28125		New Guinea - Papua - Rambuso
Hypolytreae	<i>Hypolytrum compactum</i> Nees & Meyen ex Kunth	k	J.M.Simaga	9619		New Guinea - Papua - Milne Bay province

Hypolytreae	Hypolytrum heteromorphum Nelmes	k	H.C.Dawkins	598		British East Africa - Uganda
Hypolytreae	Hypolytrum heteromorphum Nelmes	k	K.A.Lye	22117		British East Africa - Uganda
Hypolytreae	Hypolytrum longifolium subsp. longifolium (Rich.) Nees	k	Jenman	2486		Tropical South America- British Guyana
Hypolytreae	Hypolytrum longifolium subsp. longifolium (Rich.) Nees	k	A.C.Smith	2656		Tropical South America- British Guyana
Hypolytreae	Hypolytrum longifolium subsp. nicaraguense (Liebm.) T.Koyama	k	W.A.Schipp	110		Central America - Honduras
Hypolytreae	Hypolytrum longifolium subsp. rubescens (Huber ex C.B.Clarke) T.Koyama	k	G.T.Prance; C.C.Berg; F.A.Bisby; W.C.Steward; O.P.Monteiro; J.F.Ramos	17798	K001182386	Tropical South America- Brazil-AM
Hypolytreae	<i>Hypolytrum</i> <i>longifolium</i> subsp. <i>sylvaticum</i> (Poepp. ex Kunth) T.Koyama	k	B.V.Rabelo; S.Mori; A.Farias; H.Belo; J.Mitchell; M.R.dos Santos; J.A.Cardoso	2405	K001182406	Tropical South America- Brazil - Amapá
Hypolytreae	<i>Hypolytrum</i> <i>longifolium</i> subsp. <i>sylvaticum</i> (Poepp. ex Kunth) T.Koyama	k	B.V.Rabelo; S.Mori; A.Farias; H.Belo; J.Mitchell; M.R.dos Santos; J.A.Cardoso	2405	K001182407	Tropical South America- Brazil - Amapá
Hypolytreae	Hypolytrum mauritianum Nees ex Kunth	k	L.Gautier	6072		Mascarene islands - Madagascar
Hypolytreae	Hypolytrum nemorum (Vahl) Spreng.	k	P.Goetghebeur; W.Vyverman	6101		New Guinea-Papua - Central Province Varirata
Hypolytreae	Hypolytrum nemorum (Vahl) Spreng.	k	R.Pullen	7134		New Guinea-Papua
Hypolytreae	Hypolytrum pulchrum (Rudge) H.Pfeiff.	k	M.J.Jansen-Jacobs; E.M.Nic Lughadha; B.J.H.ter Welle; D.Gopaul	1427		Tropical South America- Guyana - Essequibo river
Hypolytreae	Hypolytrum pulchrum (Rudge) H.Pfeiff.	k	S.Mori; R.Cardoso	17272	K001182359	Tropical South America- Brazil - Amapá
Hypolytreae	Hypolytrum rigens Nees	k	W.R.Anderson	7152	K001182439	Tropical South America- Brazil - Goiás
Hypolytreae	Hypolytrum schraderianum Nees	k	R.M.Harley	20193	K000189327	Tropical South America- Brazil - Bahia
Hypolytreae	Hypolytrum schraderianum Nees	k	G.T.Prance; D.F.Coelho;O.P.Monteiro	14972	K001182417	Tropical South America- Brazil - AM

Hypolytreae	Hypolytrum sphaerostachyum Boeckeler	k	K.M.Redden	5169		Tropical South America- Guyana - Essequibo river
Hypolytreae	Hypolytrum supervacuum C.B.Clarke	k	R.C.Forzza; M.Magenta; J.V.Coffani-Nunes; C.Kameyama	1599	K001182400	Tropical South America- Brazil - Goiás
Hypolytreae	Hypolytrum testui Cherm.	k	A.Peter	22789		East Tropical Africa - Targaryika
Hypolytreae	Mapania bancana (Miq.) Ridl.	k	W.Griffith	6273		Malay peninsula
Hypolytreae	Mapania bancana (Miq.) Ridl.	k	John Carrick	1497		Malay peninsula - Singapore
Hypolytreae	Mapania bancana (Miq.) Ridl.	k	Md. Nurr	34096		Malay peninsula - Singapore
Hypolytreae	Mapania coriandrum Nelmes	k	L.Aké Assi	7164		West Tropical Africa - Cote d'ivoire - Adiopodoumé
Hypolytreae	Mapania coriandrum Nelmes	k	R.Schnell	5082		West Tropical Africa - Guinee
Hypolytreae	Mapania cuspidata (Miq.) Uittien	k	Ridley	s.n.		Malay peninsula
Hypolytreae	Mapania cuspidata (Miq.) Uittien	k	Kiah	31732		Malay peninsula - Malaysia
Hypolytreae	Mapania graminea Uittien	k	Dewol Sundaling	99466		Malay islands -Tongod Kinabatangan
Hypolytreae	Mapania graminea Uittien	k	Axel D. Poulsen	41		Malay islands - Province Temburong, Brunei
Hypolytreae	Mapania holttumii J.Kern	k	Ridley	16192		Malay peninsula - Malaysia
Hypolytreae	Mapania imeriensis (Gross) T.Koyama	k	B.A.Whitton	72		Tropical South America - Guyana - Potaro
Hypolytreae	Mapania imeriensis (Gross) T.Koyama	k	B.A.Whitton	329		Tropical South America - Guyana - Potaro
Hypolytreae	Mapania insignis Sandwith	k	A.G.H.Daniëls; F.P.Jonker	956		Tropical South America - Surinam
Hypolytreae	Mapania longiflora C.B.Clarke	k	W.Meijer	22604	K000496506	Malay islands - Sandakan/Malaysia
Hypolytreae	Mapania longiflora C.B.Clarke	k	P.C.Boyce	723	K000496505	Malay islands
Hypolytreae	Mapania lorea Uittien	k	Ridley	12315		Malay islands - Malacca/Malaysia
Hypolytreae	Mapania macrocephala (Gaudich.) K.Schum.	k	R.J. Johns	10899		New Guinea - Milne Bay Province

Hypolytreae	Mapania macrocephala (Gaudich.) K.Schum.	k	F.B. Essig	55071	New Guinea - Morobe
Hypolytreae	Mapania macrophylla (Boeckeler) H.Pfeiff.	k	Stockdale	8806	Tropical South America - Guyana - Potaro
Hypolytreae	Mapania macrophylla (Boeckeler) H.Pfeiff.	k	FDBG	2734	Tropical South America - Guyana - Moraballi Ck.
Hypolytreae	<i>Mapania palustris</i> (Hassk. ex Steud.) Fern Vill.	k	Ridley	11003	Malay peninsula - Malaysia
Hypolytreae	<i>Mapania palustris</i> (Hassk. ex Steud.) Fern Vill.	k	W.Griffith	s.n.	Malay peninsula
Hypolytreae	Mapania pycnocephala subsp. fluviatilis (Sandwith) T.Koyama	k	B.A.Whitton	15	Tropical South America - Guyana - Potaro
Hypolytreae	Mapania pycnocephala subsp. fluviatilis (Sandwith) T.Koyama	k	Basset Maguire; D.B. Fanshawe	32093	Tropical South America - Guyana -Mazaruni
Hypolytreae	Mapania pycnostachya (Benth.) T.Koyama	k	G.T.Prance; L.F.Coelho; J.F.Ramos	14744 K001183840	Tropical South America - Brazil - Reserva Ducke/Manaus
Hypolytreae	Mapania sumatrana subsp. pandanophylla (F.Muell.) D.A.Simpson	k	Ridley	1027	Malay peninsula - Merlimau (Malacca - Malaysia)
Hypolytreae	Mapania sumatrana subsp. pandanophylla (F.Muell.) D.A.Simpson	k	Lake & Keball	4093	Malay peninsula - Kwala Kahang (Johor-Malaysia)
Hypolytreae	Mapania surinamensis Uittien	k	A.G.H.Daniëls; F.P.Jonker	956	Tropical South America - Surinam
Hypolytreae	Mapania sylvatica Aubl.	k	FDBG	5513	Tropical South America - Guyana - Bartica/Potaro
Hypolytreae	Mapania tenuiscapa C.B.Clarke	k	Ridley	1571	Malay peninsula
Hypolytreae	Mapania tenuiscapa C.B.Clarke	k	W.Griffith	37- H1261	Malay peninsula - Malaysia
Hypolytreae	Mapania tenuiscapa C.B.Clarke	k	L.H.Fitt	22	Malay peninsula - Malaysia
Hypolytreae	Mapania tepuiana (Steyerm.) T.Koyama	k	B.A.Whitton	72	Tropical South America - Guyana - Potaro

Hypolytreae	Mapania tepuiana (Steyerm.) T.Koyama	k	B.A.Whitton	329	Tropical South America -
Hypolytreae	Mapania theobromina D.A.Simpson	k	D.Sasaki	1787	Tropical South America - Brazil - MS
Hypolytreae	Mapania theobromina D.A.Simpson	k	R. Liesner	16390	Tropical South America - Venezuela - Rio Negro
Hypolytreae	Mapania theobromina D.A.Simpson	k	G.Davidse; J.S.Miller	26944	Tropical South America - Venezuela - Rio Negro
Hypolytreae	<i>Paramapania parvibractea</i> (C.B.Clarke) Uittien	k	M.J.S.Sands	3841	Malay islands - Malaysia
Hypolytreae	<i>Paramapania parvibractea</i> (C.B.Clarke) Uittien	k	H.J.Lam	830	New Guinea - Papua
Hypolytreae	<i>Paramapania parvibractea</i> (C.B.Clarke) Uittien	k	L.J.Brass	27935	New Guinea - Papua
Hypolytreae	Paramapania radians (C.B.Clarke) Uittien	k	B.E.Smythies; G.H.S.Wood; P.S.Ashton	17066	Malay islands - Temburong/Borneo
Hypolytreae	Paramapania radians (C.B.Clarke) Uittien	k	W.Meijer	27745	Malay islands - Sandakan/Borneo
Hypolytreae	Paramapania simplex (Ridl.) Uittien	k	A.M.Polak	724	New Guinea - Irian Jaya - Indonesia
Hypolytreae	Principina grandis Uittien	MAGAB	A. Mesterházy, Süveges, E. Akouangou	64	Woleu-Ntem - Gabon, Mbé Akelayong, Inselberg Milobo
Hypolytreae	Scirpodendron ghaeri (Gaertn.) Merr.	Κ	Kostermans; Anta	822	Malay islands - Bangka
Hypolytreae	Scirpodendron ghaeri (Gaertn.) Merr.	K	P.Buwalda	5861	Malay islands - Kiandarat

-		Lateral	bracts	No	No	No	Pistil
Tribe	Species	Number	Keel	lateral stamens	inner bracts	inner stamens	morphology
Chrysitricheae	Capitularina involucrata (Valck.Sur.) J.Kern	2	Present	2	7-13	9-10	Dimerous dorsiventrally flattened
Chrysitricheae	Chorizandra cymbaria R.Br.	2	Absent	0	9	7	Trimerous
Chrysitricheae	Chorizandra enodis Nees	2	Present	2	13-19	7-10	Trimerous
Chrysitricheae	Chorizandra sphaerocephala R.Br.	2	Present	2	>20	>20	Trimerous
Chrysitricheae	Chrysitrix capensis L.	2	Absent	0	>100	>100	Trimerous
Chrysitricheae	Chrysitrix dodii C.B.Clarke	2	Absent	0	50-80	50-80	Trimerous
Chrysitricheae	Diplasia karatifolia Rich.	2	Present	2	2	6	Dimerous dorsiventrally flattened
Chrysitricheae	<i>Exocarya sclerioides</i> (F.Muell.) Benth.	2	Present	2	2	1	Dimerous dorsiventrally flattened
Chrysitricheae	<i>Lepironia articulata</i> (Retz.) Domin	2	Present	2	20-23	10-14	Dimerous dorsiventrally flattened
Hypolytreae	<i>Hypolytrum amplum</i> Poepp. & Kunth	2	Present	2	0	0	Dimerous dorsiventrally flattened
Hypolytreae	<i>Hypolytrum compactum</i> Nees & Meyen ex Kunth	2	Present	2	0	0	Dimerous dorsiventrally flattened
Hypolytreae	Hypolytrum heteromorphum Nelmes	2	Present	2	0	0	Dimerous dorsiventrally flattened
Hypolytreae	Hypolytrum longifolium subsp. longifolium (Rich.) Nees	2	Present	2	0	0	Dimerous dorsiventrally flattened
Hypolytreae	Hypolytrum longifolium subsp nicaraguense (Liebm.) T.Koyama	2	Present	2	0	0	Dimerous dorsiventrally flattened

Appendix 2. Morphological traits of spicoids of Mapanioideae. Asterisk (*) indicates data from literature: Simpson et al. (1992) (*Mapania meditensis*) and Hooper & Simpson 1997 (*Scirpodendron bogneri*).

Hypolytreae	<i>Hypolytrum longifolium</i> subsp. <i>rubescens</i> (Huber ex C.B.Clarke) T.Koyama	2	Present	2	0	0	Dimerous dorsiventrally flattened
Hypolytreae	Hypolytrum longifolium subsp. sylvaticum (Poepp. ex Kunth) T.Koyama	2	Present	2	0	0	Dimerous dorsiventrally flattened
Hypolytreae	<i>Hypolytrum mauritianum</i> Nees ex Kunth	2	Present	2	0	0	Dimerous dorsiventrally flattened
Hypolytreae	Hypolytrum nemorum (Vahl) Spreng.	2	Present	2	0	0	Dimerous dorsiventrally flattened
Hypolytreae	<i>Hypolytrum pulchrum</i> (Rudge) H.Pfeiff.	2	Present	2	1	1	Dimerous dorsiventrally flattened
Hypolytreae	Hypolytrum rigens Nees	2	Present	2	1	1	Dimerous dorsiventrally flattened
Hypolytreae	Hypolytrum schraderianum Nees	2	Present	2	0	0	Dimerous dorsiventrally flattened
Hypolytreae	Hypolytrum sphaerostachyum Boeckeler	2	Present	2	0	0	Dimerous dorsiventrally flattened
Hypolytreae	Hypolytrum supervacuum C.B.Clarke	2	Present	2	1	1	Dimerous dorsiventrally flattened
Hypolytreae	Hypolytrum testui Cherm.	2	Present	2	0	0	Dimerous dorsiventrally flattened
Hypolytreae	Mapania bancana (Miq.) Ridl.	2	Present	2	2	1	Trimerous
Hypolytreae	Mapania coriandrum Nelmes	2	Present	2	2	0	Dimerous dorsiventrally flattened
Hypolytreae	<i>Mapania cuspidata</i> (Miq.) Uittien	2	Present	2	2	1	Trimerous
Hypolytreae	Mapania graminea Uittien	2	Present	2	4	1	Trimerous
Hypolytreae	Mapania holttumii J.Kern	2	Present	2	4	1	Trimerous

Hypolytreae	<i>Mapania imeriensis</i> (Gross) T.Koyama	2	Present	2	2	0	Dimerous dorsiventrally flattened
Hypolytreae	Mapania insignis Sandwith	2	Absent	2	2	1	Dimerous dorsiventrally flattened
Hypolytreae	Mapania longiflora C.B.Clarke	2	Present	2	4	1	Trimerous
Hypolytreae	Mapania lorea Uittien	2	Present	2	2	1	Trimerous
Hypolytreae	<i>Mapania macrocephala</i> (Gaudich.) K.Schum.	2	Present	2	4	1	Trimerous
Hypolytreae	<i>Mapania macrophylla</i> (Boeckeler) H.Pfeiff.	2	Absent	2	2	0	Dimerous dorsiventrally flattened
Hypolytreae	<i>Mapania palustris</i> (Hassk. ex Steud.) FernVill.	2	Present	2	4	1	Trimerous
Hypolytreae	<i>Mapania meditensis</i> D.A.Simpson*	2	Present	2	4	1	Trimerous
Hypolytreae	<i>Mapania pycnocephala</i> subsp. <i>fluviatilis</i> (Sandwith) T.Koyama	2	Absent	2	2	0	Dimerous dorsiventrally flattened
Hypolytreae	<i>Mapania pycnostachya</i> (Benth.) T.Koyama	2	Absent	1-2	2	0	Dimerous dorsiventrally flattened
Hypolytreae	<i>Mapania sumatrana</i> subsp <i>pandanophylla</i> (F.Muell.) D.A.Simpson	2	Present	2	3	1	Trimerous
Hypolytreae	Mapania surinamensis Uittien	2	Present	2	2	1	Dimerous dorsiventrally flattened
Hypolytreae	Mapania sylvatica Aubl.	2	Present	2	4	1	Trimerous
Hypolytreae	Mapania tenuiscapa C.B.Clarke	2	Present	2	4	1	Trimerous
Hypolytreae	<i>Mapania tepuiana</i> (Steyerm.) T.Koyama	2	Absent	2	2	1	Dimerous dorsiventrally flattened
Hypolytreae	<i>Mapania theobromina</i> D.A.Simpson	2	Present	2	2	1	Dimerous dorsiventrally flattened

Hypolytreae	Paramapania parvibractea (C.B.Clarke) Uittien	2	Present	2	4	1	Trimerous
Hypolytreae	Paramapania radians (C.B.Clarke) Uittien	2	Present	2	4	0	Trimerous
Hypolytreae	Paramapania simplex (Ridl.) Uittien	2	Present	2	4	0	Trimerous
Hypolytreae	Principina grandis Uittien	2	Present	2	1	1	Trimerous
Hypolytreae	<i>Scirpodendron ghaeri</i> (Gaertn.) Merr.	2	Present	2	7-9	7-9	Trimerous
Hypolytreae	Scirpodendron bogneri S.S. Hooper*	2	Present	2	7-9	7-9	Trimerous

General Conclusions

GENERAL CONCLUSIONS

The results obtained in this thesis brings a new perspective towards the understanding of floral traits in Cyperaceae, as well as their evolutionary significance. Furthermore, it raised new questions to impulse the knowledge of such fascinating family. Changes at the beginning of floral development showed to be fundamental in regulating variations in each floral whorl and this remarkable matter was reinforced by our results where we showed a relation between the arrangement of the glumes as a fundamental constraint operating in the flower primordium geometry of Cyperoideae species. This seems to affect the available space for organs emergence, and together with genetic and biochemical interactions, influencing the variation towards the number and position of organs in each floral whorl.

Moreover, the influence of floral traits in the reproductive success and diversity of Cyperoideae genera was approached concerning the stylopodium, which was recovered as a homologous structure, evolving several times during Cyperoideae evolution. The results shed light in a notable relationship between the presence of a stylopodium as a structure possibly increasing the germination fitness of the species. The presence of stylopodium in some of the most diverse genera of Cyperaceae raise questions whether the acquisition of such structure should be investigated as a possible key innovation, possibly promoting the germination success of such species to a variety of environmental conditions and contributing to their wide diversification.

Developmental studies showed to be fundamental to understanding the processes regulating the floral variety in both subfamilies. For Mapanioideae, based on a comparative study on the development of the spicoids, we showed that developmental patterns appear to be consistent within tribe. Features such as the sequence of organ appearance, the size and shape of the spicoid primordium are the main developmental variations among the species, influencing the spicoid's morphological variation in

167

Mapanioideae. Furthermore, developmental features such as the sequence of bracts emerging alternate with the stamens and the presence of two lateral bracts arising as separate structures, allowed us to argue in favour of interpreting the spicoids of *Diplasia* as reduced inflorescences.

At last, we emphasized the value of understanding the morphological variation of spicoids in Mapanioideae as a source of informative features to a subfamily with notable inconclusive phylogenetic relations. The morphological features of the spicoids were considered informative at generic level since some genera with more than one species share a stable spicoid architecture. However, similarities in the spicoid morphology of different genera were observed, reinforcing the need of reinvestigate their phylogenetic boundaries combining molecular and morphological data. The ancestral character reconstruction allowed us to infer that the hypothetical ancestral morphology of the spicoid is composed of two lateral keeled bracts, two lateral stamens and a dimerous pistil, which resembles the morphology of *Hypolytrum* spicoids. Moreover, based on the most recurrent changes for each variable morphological character, we infer that evolutionary changes in the subfamily were mostly driven by the acquisition of structures.

Such results highlight the importance of morphological studies in elucidating gaps throughout the evolutionary story of Cyperaceae genera, especially concerning the wide range of morphological variation of the reproductive units found in the family. Therefore, further developmental, anatomical and evolutionary studies are crucial to push forward the knowledge towards such a rich and extraordinary family.