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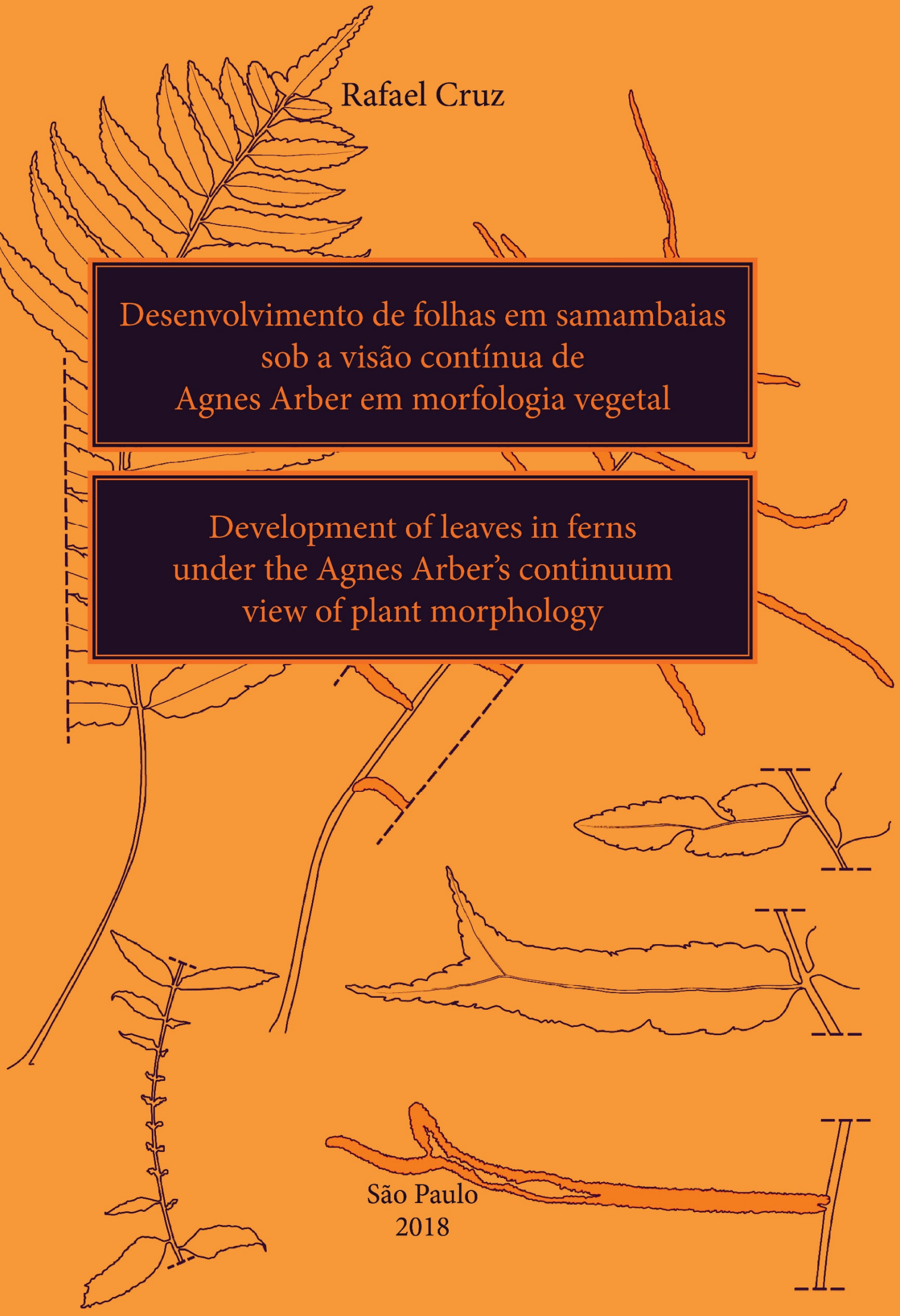
Development of leaves in ferns under the Agnes Arber's continuum view of plant morphology



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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo para a obtenção do título de Doutor em Ciências Biológicas, na área de Botânica.

Orientação: Profa. Dra. Gladys Flávia de Albuquerque Melo de Pinna

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Aos meus queridos amigos.

"The different branches [of biology] should not, indeed, be regarded as so many fragments which, pieced together, make up a mosaic called biology, but as so many microcosms, each of which, in its own individual way, reflects the macrocosm of the whole subject."

Agnes Robertson Arber
The Natural Philosophy of Plant Form (1950)

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Muito obrigado!

Resumo

A Morfologia Clássica em Botânica requer uma visão tipológica dos órgãos vegetais. Isso geralmente implica na classificação de caule, folha e raiz como unidades básicas e bem definidas. Samambaias são o grupo mais diverso de plantas sem flores e ocupam uma posição-chave na filogenia das plantas terrestres. Suas folhas geralmente são entendidas como homólogas às de espermatófitas. Ainda assim, possuem características intrigantes, como um meristema apical foliar com uma célula apical distinta, e podem ser muitas vezes divididas, lembrando a atividade de um sistema caulinar. Apresentamos um estudo do desenvolvimento foliar em algumas samambaias leptosporangiadas de diferentes morfologias para entender melhor como essas estruturas podem ter evoluído e as possíveis homologias entre seus processos ontogênicos.

A expressão dos genes de Classe I KNOX foi analisada na samambaia heteroblástica *Mickelia scandens*, uma vez que estão relacionados à determinação de órgãos em angiospermas. As duas cópias de Classe I KNOX são expressas mesmo em estruturas determinadas, como pinas. Mas uma redução da quantidade de transcritos está relacionada ao desenvolvimento da forma menos determinada da fronde que ocorre em indivíduos terrestres.

Usando ferramentas anatômicas clássicas, estudamos o desenvolvimento de folhas em samambaias relacionadas a *Mickelia scandens* que apresentam diferentes morfologias. Além disso, observamos mutantes de ocorrência natural em uma coleção. A estrutura básica das células apicais é essencialmente bem conservada em todo o grupo. Células marginais, classicamente apontadas como parte do meristema marginal, podem repetir em certo grau a atividade da célula apical da folha. Mudanças na estrutura e atividade dessas estruturas podem ser a razão pela qual a samambaia de folhas simples do gênero *Elaphoglossum* não fazem folhas compostas e porque a morfologia de uma folha normal pode ser alterada, produzindo estruturas anômalas.

Discutimos esses dados com base em conceitos de Agnes Arber de sistema caulinar-parcial identidade-em-paralelo, propondo uma interpretação da folha de samambaia não como um órgão bem definido, mas como um produto de processos de ontogênese, alguns deles típicos do sistema caulinar.

Abstract

Classical Morphology in Plant Sciences requires a typological view of plant organs. This usually implies in classifying stem, leaf and root as basic and well-defined unities. Ferns are the most diverse group of non-flowering plants and occupy a key position in the land plants phylogeny. Their leaves are usually understood as homologous to those of seed-plants. Still, they bear intriguing features, like a leaf apical meristem bearing a distinct apical cell, and that may be many times divided, resembling the activity of a whole shoot. We present a study about the leaf development in some leptosporangiate ferns of different morphologies to better understand how these structures may have evolved and the possible homologies between their ontogenetic processes.

Class I KNOX genes expression was analyzed in the heteroblastic fern *Mickelia scandens*, as they are related to organ determinacy in angiosperms. The two copies of Class I KNOX are expressed even in determined structures, like pinnae. But a reduction of the quantity of transcript is related to the development of the less determinate frond form that occurs in terrestrial individuals.

Using classic anatomical tools, we studied the development of leaves in ferns related to *Mickelia scandens* that present different morphologies. In addition, we observed natural occurring mutants in a collection. The basic structure of apical cells is essentially well conserved in all the group. Marginal cells, classically pointed as part of the marginal meristem, may repeat in some degree the activity of the leaf apical cell. Changes in the structure and activity of these structures may be the reason why simple-leaved ferns of the genus *Elaphoglossum* do not make compound leaves and why usual leaf morphology may change, producing anomalous structures.

We discuss this data based on Agnes Arber concepts of partial-shoot and identity-in-parallel, proposing an interpretation of the fern leaf not as a well-defined organ, but a product of ontogenetic processes, some of them typical of the shoot.

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General Introduction

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Leaves are usually defined as lateral appendages that occur in vascular plants sporophytes and should present vascularization, determinate growth, dorsiventral symmetry with flattening in the transverse plane and phyllotaxis, i.e. definite arrangement around the axis where they emerge from (Dengler & Tsukaya, 2001; Tomescu, 2009).

The advance of fossil discoveries and available morphological data have created a remarkable dichotomy between two, supposedly non-homologous, types of leaves: (*i*) microphylls, small structures with a single vein connected to the stem protostele; and (*ii*) megaphylls, bigger structures with complex venation, connected to the vascular system in the stem by leaf traces that are associated with parenchymatic tissue in the stele (Tomescu, 2009). These features have been pointed as morphological supports for molecular phylogenies that show two extant monophyletic groups of vascular plants: Lycophytes (plants with microphylls) and Euphyllophytes (plants with megaphylls) (Tomescu, 2009; Vasco *et al.*, 2013).

Although it seems that there is a clear distinction between those two leaf types, there is a lot of misleading examples, pointed by Tomescu (2009), that show the big complexity of the theme. Some euphyllophyte plants bear complex leaves associated to a protostele, as the extant genera of ferns *Lygodium* and *Gleichenia*, as in some fossil species, as *Elkinsia*, one of the first seed plants. Neither the size or the vascularization has been adequate defining criteria for this distinction, since euphylophytes of the genus *Equisetum*, many extant leaves of seed plants and many other fossils, bear much-reduced leaves, supplied by a single vein. Also, there are lycophytes species with big leaves (up to 1 m in Lepidodendrales and up 50 cm in living species of *Isoetes*) and complex

venation patterns (in some species of *Selaginella*). By using definitions based on classical morphology we have been creating more confusion and overlap between the concepts of microphylls and megaphylls, instead of clarifying them.

The Form Science, in Botany, had it beginning with the German writer and thinker Johann Wolfgang von Goethe, one of the most versatile men ever living. In 1790 he created the term "Morphology" and its comparative methodology in "*Vershuch die Metamorphose der Pflanzen zu erklären*" ("An attempt of explaining the metamorphosis in plants") (Kaplan, 2001a). The metamorphosis, term that he had taken from the Greek language and from Ouvid's mythology, presents the leaf as the basic and repetitive unity from the vascular plant, and its morphological change would give rise to organs like cotyledons, bracts, floral parts and fruits (Claßen-Bockhoff, 2001).

Wilhelm Troll emerged as a big defender of the idea of archetypal organs, defining the root, the stem and the leaf as basic, straight and well-defined units. He used the principle of variable proportions (already pointed by Goethe) to explain deviations of the morphology from the "type" (equivalent to the archetypal and idealistic unit) (Kaplan, 2001a; Claßen-Bockhoff, 2001). Organ morphology would modify into other structures simply by changing its proportions, but still maintaining its identity. As example, the leaf margin can undergo a deep lobation developing subunits known as leaflets, producing a compound leaf during its development. Still, it would be a leaf, completely distinct from the stem. This idea had a strong adhesion in the Botany, with notable support of Donald Kaplan (Kaplan, 2001a,b) and it is still present as the classical morphological school.

Walter Zimmermann appears as one of the first scientists to look for purely objective proceedings to study morphology, in contraposition to the idealistic vision of

Goethe and Troll (Claßen-Bockhoff, 2001). His most significant contributions are in considering a strictly phylogenetic in the Plant Form. The extant units would be forms found in ancestral plants, that undergoing a transformation (change of the character state, caused by mutations followed by natural selection), would give rise to the extant diversity in their descendants. About the organs of extant vascular plants, they would be originated from an ancestral (and not ideal, as proposed by Goethe) basic unit, the telome, that is present in plants similar to the extinct *Rhynia* (Zimmermann, 1976). According to this theory, the telome would be a radially symmetrical organ, with dichotomic branching, and a protostele; and in adult plants, they could have been sterile (phylloid) or fertile (sporangia).

The fusion of axial telomes, according to the author, can give rise to the extant stem, and the confluence of many protostelic unities, would had given rise to the medullated siphonostele. Zimmerman also applied the telome theory to explain the morphology of other unities of the plants, like the root, the inflorescence and stamens. In these organs, however, the theory was replaced by a series of studies and better theories. The telome theory is also strongly applied to explain the megaphyll origin, though.

According to the telome theory about megaphylls, at first, it would have had happened the **sobreposition** i.e. the dominance of a telome related to others. Then after, **planation** i.e. groups of telomes would have had taken a bidimensional position. Afterwards, it would have had occurred the **fusion** of telomes, by appearing tissues connecting these axis, creating the leaf of ferns and seed plants as we know today. Fossil records strongly supported the ideas of Zimmermann and the telome theory is, so far, the better explanation for the megaphyll origin. Known molecular mechanisms of development points the former two processes as possible and likely, but are limited when

explaining the fusion process that would be better replaced by **lateral outgrowth** of individual branches (Beerling & Fleming, 2007).

The third morphologist that based her ideas in Goethe was Agnes Arber, the first prominent woman in Botany. The most remarkable aspects of her view about plant morphology is that it is dynamic, and all the antithesis present there merge into a synthesis (Claßen-Bockhoff, 2001). Her most important theory was the **partial-shoot theory**. According to it, the leaf is not only an appendage in the stem, but part of the shoot and bears many features there present. Because of the lateral origin, the leaf does not have a radial symmetry, and even presenting some apical growth, it ceases very soon, determining its growth (Arber, 1950; Rutishauser & Isler, 2001). However, several aspects make it similar to a shoot: *(i)* leaf primordium present meristematic regions in its laterals that may originate stipules and leaflets; *(ii)* unifacial leaves present some degree of radial symmetry; *(iii)* the leaf presents a series of axial elements similar to the stem, as the petiole, the midrib and the rachis; *(iv)* the presence of venation patterns similar to branching patterns; *(v)* production of ectopic proliferous buds, as in *Kalanchoe daigreontiana* (Crassulaceae) (Claßen-Bockhoff, 2001). More than a modification, stem and leaf are adaptive peaks to the terrestrial life, but correlative concepts inside a whole: the shoot.

In the beginning of the last decade, the name **Fuzzy Arberian Morphology** was proposed to the continuous view of Plant Morphology, integrating studies published so far based on the Agnes Arber proposals (Rutishauser & Isler, 2001). This view proposes that land plant organs should not be seen as well-defined unities, but a group of gradual features: from radiality to dorsiventrality, from indetermination to determination. Transition zones that bear intermediate features (as the presents between the root and the

shoot and the nodal region) acquire a big importance because they are not only connection points, but an evidence that ontogenetic processes should be considered ingredients to produce different forms. Some organs usually taken as morphological misfits, like the indeterminate leaves of *Guarea* and *Chisocheton* (Meliaceae), would be better understood if we do not interpret them as entire leaves that became divided, but leaves with a reduced determination of growth, acting as a partial-shoot (Steingraeber & Fisher, 1986; Lacroix & Sattler, 1994).

Schneider (2013) points that it is notable that Fuzzy Arberian Morphology representatives did not discuss morphological aspects of ferns in big detail yet. This school advices a holistic view of the plant organs, that share a series of ontogenetic processes, and this is very evident in ferns.

As in the shoot apex, that bears the **shoot apical meristem** (SAM), the leaf apex of most of the extant ferns bears the **leaf apical meristem** (LAM). However, while in angiosperms the meristem bears many initial cells, the equivalent region in ferns is said to be unicellular, with usually only two (in leaves) or three (in roots and shoots) dividing faces, generating daughter cells that give rise to distinct merophytes (Imaichi, 2008). The single apical cell presents higher plasmodesmata density when compared with the promeristem of flowering plants or lycophytes (except in Selaginellaceae that have a similar structure of fern SAMs), possibly allowing a more efficient intercellular connection with reduced number of cells (Imaichi, 2008).

Nevertheless, fern SAMs, sometimes pointed as unicellular, have been focus of big discussion. Ambrose & Vasco (2016), present a quick review about fern meristems with new experimental data, showing that *Elaphoglossum peltatum* (Dryopteridaceae)

SAM, even presenting an evident apical cell, have a big number of peripheral cells that express Class I KNOX genes, related to the maintenance of the indetermination in meristems. According to them, fern SAM is complex and presents a characteristic zonation, with one apical initial, subapical initials and a cup-shaped zone. This apical initial has reduced mitotic activity when compared with their derivatives and may be equivalent to the angiosperm promeristem.

The development of the megaphyll in ferns starts in the SAM flanks, from a group of surface prismatic cells and subsuperface cells. One of the prismatic cells becomes bigger and oblique divisions turn it into a single apical cell, prominent over the others (Vasco *et al.*, 2013). The activity of the LAM is responsible for the apical growth. The primordium becomes coiled, protecting its younger parts and is known as fiddleheads or croziers. The lamina is produced by marginal meristems that originated from marginal initials derived from the apical cell derivatives and fractioning of this marginal meristems is pointed as responsible for the formation of pinnae and pinnules (Vasco *et al.*, 2013).

Gifford & Forster (1988) describe that the presence of marginal initials in the marginal meristems. However, few details are available about this marginal initials and possible resemblances to the single leaf apical cell. More recent studies, in the fern *Ceratopteris richardii* (Pteridaceae), that has been used as a new model-plant, points a different structure for pinnae apices: paired apical cells, with a different organization from the frond that have a single apical cell (Hill, 2001; Plackett *et al.*, 2015). Another notable sample in ferns is *Lygodium japonicum* (Lygodiaceae), where the activity of the LAM is permanent, creating a pinnate frond that may be many meters long (Imaichi, 2008).

The idea that fractioning of marginal meristems is responsible for pinnae formation conflicts Agnes Arber's ideas that present a view of **identity in parallel**. Instead of a division of the leaf, each pinna is homologous to a leaf, and not to a fraction of it. The compound leaf replicates a development of a whole shoot in some degree, producing lateral structures. In this sense, marginal initials are probably a reiteration of the leaf development in compound leaves.

Comparative studies with *Anogramma chaerophylla* (Pteridaceae), *Ceratopteris richardii* (Pteridaceae) and *Osmunda regalis* (Osmundaceae) leaves showed that there is Class I KNOX genes expression in these structures, what is related to a reduced determination of the leaf development (Bharathan *et al.*, 2002; Sano *et al.*, 2005; Vasco *et al.*, 2013). These genes, in many angiosperms, normally are expressed only in the SAM and not in leaf primordia, except in compound leaves, what made Champagne & Sinha (2004) consider compound leaves as partially homologous to shoots, as defended by Arber (1950). Another evidence of homology is that in *Osmunda cinnamomea* (Osmundaceae) leaf primordia, when excised and put in culture, mostly become shoots, while only a few of them become leaves (Steeves, 1993).

Ferns have an important role in the formulation of theories about vascular plant evolution and development studies can be helpful in the formulation of general concepts in plant morphology (White & Turner, 1995). This role occurs not because they are primitive, but because they have: (*i*) an important phylogenetic position (sister-group to seed plants), and (*ii*) an organizational versatility that may reveals basic principles of plant organogenesis.

This proposal represents the intention of filling up some lacunae in the studies about leaf development in ferns, by analyzing a group with remarkable morphological diversity, trying to comprise the homology of the ontogenetic processes and structures among the studied species and other land plants.

Dryopteridaceae has 40-45 genera, with 1700 mostly pantropical species. They are terrestrial, epipetric, hemiepiphytic, or epiphytic. Their rhizomes may be creeping, ascending or erect, sometimes scandent or climbing. Their petioles have numerous round vascular bundles arranged in a ring and the leaves are usually monomorphic, but sometimes dimorphic. Other features are pinnate or forking veins, that may be free or variously anastomosing; absent, round-reniform or peltate indusia (that may be lost in several lineages); round sori or acrostichoid in some groups; and other reproductive features, as described by Smith *et al.* (2006).

Leaf dimorphism between fertile and sterile leaves in ferns is present in some genera of this family and refers to a syndrome of many anatomical and morphological characters that maximize the dispersion of spores and minimize the metabolic cost during the construction of fertile leaves (Moran, 1987; Vasco *et al.*, 2013). According to Moran (1987), the genus *Polybotrya* have a fertile leaf that looks like a skeleton of a sterile one, with lamina reduced to narrow wings along the main veins. They are ephemerous (1-3 months) and have more parenchyma when compared with sterile leaves that have a rigid support of collenchyma and may be more than one year old. Some bizarre intermediate forms between these two types of leaves may be found with a variable degree of lamina reduction and, in some cases, with the basal portion of pinnae fertile and the apical part sterile with broader and green lamina.

Within Dryopteridaceae, there is also the bolbitidoid clade, composed by six genera characterized by an elongated ventral meristele (associated to root production), absence of trichomes in the leaves dimorphism between sterile and fertile leaves, formerly placed within Lomariopsidaceae because they share all the above cited characters with *Lomariopsis* (Smith *et al.*, 2006; Moran *et al.*, 2010a,b).

With the expansion of studies about the plant architecture, Gay (1993) and Hebant-Mauri & Gay (1993) present detailed descriptions of the bolbitidoid fern *Lomagramma guianensis* architecture, former name for *Mickelia guianensis* (Moran *et al.*, 2010a), that have dimorphic rhizome and trimorphic fronds. The young rhizome is terrestrial, slender, sinuous, with first leaves almost entire or lobed, and subsequently fronds become subpinnate with winged rachis. When it finds a tree, it starts to climb it passing by a drastic morphologic change: the rhizome becomes stouter and slightily flattened, producing large pinnate fronds that are three times larger than the terrestrial form. The connection with the younger form then is lost by rotting of the terrestrial form. The fertile leaf also is different, with very reduced lamina, almost restrict to the main veins, as in *Polybotrya*.

Moran *et al.* (2010b) present optimizations of a big number of characters in a molecular phylogeny of bolbitidoid ferns. It is possible to notice in the results a transition between many-times divided leaves in the species that they used as outgroups to a simple leaf condition in the epiphytic crown group *Elaphoglossum*, that is the sister group of *Mickelia* (Moran *et al.*, 2010a). The outgroup, composed by *Polybotrya*, *Megalastrum*, *Rumohra,* and *Lastreopsis*, have completely terrestrial forms with monomorphic fronds.

Studies about the leaf ontogenesis in the group are almost inexistent and would be of a big addition to the literature about the theme.

Thus, we highlight the following questions:

- Which development processes are related to the construction of a simple leaf or a compound leaf with many pinnae?
- Is the compound leaf an entire leaf that fractioned its margins, or is it a leaf that is reiterating the development of simple leaves in its laterals?
- Different leaf morphologies are associated with anatomical changes in the SAM and/or in the LAM? Are these structures formed by single, paired or more apical cells?
- What mechanisms are involved in the evolution of simple leaves to evolve from a compound condition in ferns?

We intend to clarify these questions based on Agnes Arber's morphological approach. By identifying processes of development, that may partially occur in shoots, we will observe the growth regions of leaves of diverse forms, better understanding what mechanisms control the transition from indetermination to determination and from the radiality to dorsiventrality.

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Chapter 1

Class I KNOX genes expression during the leaf development of the heteroblastic fern *Mickelia scandens*

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Abstract

Class I KNOTTED-LIKE HOMEOBOX (KNOX) genes are responsible for the indeterminacy maintenance in shoot apical meristems of seed plants. Fern leaves have a very distinctive apical meristem in the leaves, what may be related to a reduced determinacy of this structure. We analyzed the expression of Class I KNOX genes in the heteroblastic fern *Mickelia scandens*, during the leaf development. We did anatomical analyses, degenerate PCR amplification, in situ hybridization and qRT-PCR experiments. We found two copies of Class I KNOX for *Mickelia scandens*, that are both expressed in the shoot apical meristem, leaf apical meristem and determinate pinnae primordia. Cell divisions occur in the most apical regions of the analyzed structures and are not restricted to apical cells. Class I KNOX is downregulated in the developing leaves of the younger form that produce fewer pinnae when compared with primordia of the older form, an evidence that this class of genes is also related to determinacy control in ferns. As conclusions, fern fronds bear multicellular meristems that may act as shoots in some aspects, suggesting a homology between shoots and leaves.

Introduction

Vascular plant organs are classically defined based on their position, on their tissue organization (symmetry axes and vascular tissue) and on the presence, position, and activity of meristems (Dengler & Tsukaya, 2001; Kaplan, 2001). Within these criteria, leaves are lateral determinate organs and these features seem to apply well in most of the cases in seed plants. On the other hand, shoots are characterized by indeterminacy, detected by the expression of Class I KNOTTED-LIKE HOMEOBOX (KNOX) genes in the shoot apical meristem (SAM, Frangedakis *et al.*, 2016). Downregulation of Class I KNOX is one of the first signals of the origin of a determinate leaf primordium (Bharathan *et al.*, 2002) and plants with defective Class I KNOX genes may be unable to maintain the SAM, as the mutants *shoot meristemless* in *Arabidopsis thaliana* (Barton & Poethig, 1993; Endrizzi *et al.*, 1996) and recessive *knotted1* in maize (Kerstetter *et al.*, 1997).

Ferns are tricky in the sense that most of them produce leaves called fronds that are usually compound with lateral pinnae and a leaf apical meristem (LAM). The LAM and SAM of ferns have a distinctive prominent apical cell, that have been pointed as equivalent to a whole apical meristem by some authors (Sano *et al.*, 2005; Plackett *et al.*, 2015; Banks, 2015) or only the initial cells of a multicellular meristem by other authors (White & Turner, 1995; Ambrose & Vasco, 2016). Details about the expression of two Class I KNOX genes in *Elaphoglossum peltatum* characterize it as having a multicellular shoot apical meristem with an apical initial and actively dividing surrounding cells (Ambrose & Vasco, 2016). Class I KNOX transcripts were also detected in leaf primordia and in the multicellular apex of *Anogramma chaerophyla* (Bharathan *et al.*, 2002) and *Ceratopteris richardii* (Sano *et al.*, 2005). Proteins coded by this class of genes were

detected in the same regions in *Osmunda regalis* (Harrison *et al.*, 2005). Few details are available about the expression in the pinnae primordia or in the LAM, but the expression in the leaf primordium may be the cause of the delayed determinacy of fern fronds (Harrison *et al.*, 2005).

Organ determinacy seems to be the key character to understand the evolution and development of fronds. They show intriguing resemblances with indeterminate shoots (e.g. apical meristems presence, lateral organs production, and reduced or absent determinacy as in *Lygodium*) and do not fit on classic morphology concepts of leaves (Arber, 1950; Rutishauser & Isler, 2001; Rutishauser *et al.*, 2008). There is evidence that Class I KNOX genes are directly associated with the indeterminacy and are required to make compound leaves in most of the cases, representing a partial homology with the shoot (Champagne & Sinha, 2004). Class I KNOX genes seem to be the most important labeling of meristematic activity in fern shoots since WUSCHEL related genes that specify stem cells in seed plants apical meristems were found only in *Ceratopteris* roots so far (Nardmann & Werr, 2012).

In order to gather more information concerning fronds and pinnae development and LAM and SAM organization, we studied the expression of Class I KNOX in *Mickelia scandens*, a leptosporangiate fern with pinnate fronds that bears small fronds in the younger terrestrial form and longer fronds with more pinnae in the older climbing form (Fig. 1; Gay, 1993; Moran *et al.*, 2010).

Methodology

Plant material Shoot apices (usually containing small frond primordia covered by scales) and developing fronds of the terrestrial and climbing forms of *Mickelia*

scandens sporophytes were collected from specimens that occurs in a dense population in Fontes do Ipiranga State Park (São Paulo, Brazil). A *voucher* specimen is deposited in the SP Herbarium (Prado & Cruz 2332). Part of the material was stored in RNAlater® for RNA extraction and part were fixed in formalin-acetic acid-ethanol 50% (FAA) for *in situ* hybridization (ISH) experiments and anatomy.

RNA extraction and cDNA synthesis Total RNA were extracted with QUIAGEN RNeasy mini kit (Qiagen, Hilden, Germany) and cDNA synthesis was made by using Superscript III (Invitrogen, Carlsbad, CA, USA), following the manufacturers' protocols for these procedures. For quantitative real-time PCR experiments, cDNA was obtained with SuperScript IV VILO Master Mix (Invitrogen, Carlsbad, CA, USA).

Genes isolation and phylogenetic analyses Degenerate primers were used for KNOX (Forward: 5'-CCBGARCTBGACMABTTYATGG-3', Reverse: 5'-CCAGTGSCKYTTCCKYTGRTTDATRAACC-3') as in Ambrose & Vasco (2016) and for H4 gene (Forward: 5'-ATGTCWGGMMGRGGWAAGGGAGG, Reverse: 5'-CCRAADCCRTARAGVGTHCKKCC; used as positive control and cell division marker). Obtained sequences (S2) were then analyzed in the NCBI Conserved Domain Search to detect the presence of KNOX and H4 domains. In order to identify the Class of the KNOX genes, conserved parts were aligned with other known KNOX genes (sequences referenced in Ambrose & Vasco, 2016 and Frangedakis *et al.*, 2016) with Geneious version 10.1.2 (Kearse *et al.*, 2012). A phylogenetic tree was obtained with RAxML version 8 (Stamatakis, 2014) partitioned by codon position with GTR+Γ+I model as recommended in a PartitionFinder2 analysis (Lanfear *et al.*, 2012).

Anatomy and *in situ* hybridization (ISH) experiments Fixed material was embedded in paraplast (Fisher) and sectioned in a rotary microtome. For histological analysis, sections were stained with Safranin O 1% in ethanol, Crystal Violet 1% aqueous and Orange G 1% in clove oil (Flemming triple stain, Johansen, 1940). For ISH experiments, we followed the procedures previously described in Ambrose *et al.* (2000) and Vasco *et al.* (2016) using specific probes for Class I KNOX and H4 (as a positive control and cell division marker) generated with primers designed for them (S1).

Quantitative real-time PCR Transcript abundance was assessed by qRT-PCR analysis using a 7500 Real-Time PCR system (Applied Biosystems® by Life Technologies, NY, USA). A β-actin specific sequence was accessed with PCR reactions with primers (Forward: 5'-GATGGATCCTCCAATCCAGACACTGTA-3' and Reverse: 5'-GTATTGTGTTGGACTCTGGTGATGGTGT-3') and was used as housekeeping gene. The PCR reactions were performed with 5 µl of cDNA, 12.5 µl SYBR Green Master Mix (Applied Biosystems), 10 pmol/µl concentration for primers (specifically designed for qRT-PCR analysis, S1), and the following cycling conditions: 95ºC for 10 min, 44 cycles of 95ºC for 15 s, 55ºC for 30 s and 72ºC for 1 min. All reactions were performed in three technical replicates. Folding change was calculated with $\Delta\Delta C_T$ method (as in Cantero *et al.*, 2006) and statistical significance ($p \leq 0.5$) was determined with ΔC_T values by using One-Way ANOVA test followed by Tukey's pairwise comparison.

Results

We registered in the field one full developed frond from the epiphytic form with an anomalous pinna containing a basiscopic pinnule (Fig. 1k).

We found three different partial sequences for KNOX genes with our degenerate primers, with the conserved domains (S3). Two of them, *MsC1KNOX1* and *MsC1KNOX2*, are closely related to known fern Class I KNOX genes (Fig. 2). *MsC2KNOX1* is from Class II KNOX gene, a class functionally different that diverged from Class I prior to the ancestor of land plants (Furumizu *et al.*, 2015). We also successfully cloned one gene that codes for Histone H4.

There is a superposition between the expression of *MsC1KNOX1* and *MsC1KNOX2* in ISH experiments, although *MsC1KNOX2* probes resulted in detection not so strong and dark as in the sections labeled with *MsC1KNOX1* probes.

Not only the apical cell but some of the surrounding prismatic cells and the peripheral zone show Class I KNOX and H4 expression and are evidence of a multicellular meristem (Fig. 3). Procambial cells also show expression of these genes and are continuous with the SAM, showing that they still bear dividing cells (Fig. 3a). Boundaries between the leaf primordium and shoot apex do not express Class I KNOX genes (Fig. 3b) and the continuity of the SAM with the leaf primordium is restricted to procambial strands (Figs. 3c-d). Part of our experiments shows a clear expression of Class I KNOX genes in the SAM peripheral zone, but no expression in the apical cell and in some of the surrounding prismatic cells (Fig. 3d). The apical cell can be displaced laterally in the shoot apex and is bigger than derivative cells that gradually reduces in size in the apical meristem (Fig. 3e). H4 is expressed in the shoot apex, but the expression in the vascular system reveals dividing cells during its initial differentiation (Fig. 3f).

Both fronds types have similar expression pattern concerning Class I KNOX and H4 genes on the analyzed sections. The frond primordium has a distinct apical cell with

a lenticular distal face of the wall and two cutting faces (Fig. 4a). Derivative cells derivatives undergo divisions and inner cells are responsible for the establishment of the procambium (Fig. 4a). In the leaf apex, the apical and peripheral cells always express Class I KNOX genes during the development (Figs. 4b-c) and they are expressed continually in more cells of the apical region of the frond, procambium and pinnae primordia (Figs. 4d-e). H4 is expressed in the apical, immediate derivative cells and in some procambial cells (Fig. 4f).

Pinnae primordium emerges from the lateral of the frond (Fig. 5a). The pinna primordium has dividing parallel cells on its apex, but not a distinguishable single apical cell (Fig. 5b). The central region, where the vasculature of the costa will develop, also shows evident expression of H4 (Fig. 5c). Expression of Class I KNOX is detected in all the regions of the primordium at the beginning of their development (Figs. 5d-f), but become weakened in the adaxial region in older developing primordia (Figs. 5g-h). Expression of H4 is also weakened in the same case, revealing an early development of adaxial region while the abaxial region still bear dividing cells (Fig. 5i). In older pinnae primordium, cell divisions are concentrated in marginal cells and in the vascular system at the apex (Fig. 5j). Pinna primordium marginal cells are bigger and pyramidal with an outer lenticular face and are similar to leaf apical cells in transversal sections, with some radial divisions in the central vascular system (Fig. 5k). Marginal cells have four dividing plans, two of them responsible for marginal growth and two for proximodistal growth (Fig. 5l).

Although fold change values reveal smaller values for expression of Class I KNOX in apices when compared with developing leaves of the climbing form, the differences are statistically insignificant in our analysis (Fig. 6). However, the difference

is significant when we compare developing leaves from the climbing form with those of the terrestrial form (p=0.005 for *MsC1KNOX1* and p=0.0243 for *MsC1KNOX2*). In fact, the folding change of developing leaves of the terrestrial forms is 0.352 for *MsC1KNOX1* and 0.235 for *MsC1KNOX2* using the climbing leaf as a parameter. The difference is also significant between the shoot apices of all forms with terrestrial leaves, revealing that both copies of Class I KNOX are downregulated in these leaves.

Discussion

Our data reinforce that Class I KNOX is responsible for the maintenance of indeterminacy, this time supported by the decrease of Class I KNOX transcripts in developing fronds of the terrestrial form when compared with other structures (indeterminate shoot apices and less determinate fronds from the climbing form). It is likely that the simple decrease and not necessarily the absence of Class I KNOX expression is related to the determination of an organ.

This phenomenon may be the result of downregulation of these genes. In seed plants downregulation of Class I KNOX is usually done by ARP genes, although it was already shown in ferns the co-occurrence of both classes of proteins in shoot apices and frond primordia (Harrison *et al.*, 2005; Tomescu, 2009). This aspect made these authors suggest that these genes do not act antagonistically in ferns. This absence of antagonism could only be affirmed with quantitative experiments focusing on ARP genes and interaction experiments, though.

Frangedakis *et al*. (2017) observed by complementation essays, that *Ceratopteris richardii* Class I KNOX genes only partially complements the loss of function in the *Arabidopsis* mutant Class I KNOX *BP*, even with high levels of transgene

transcripts detected. According to the authors, because Class I KNOX proteins act together with the other class of TALE proteins BELL to target the nucleus, probably *Ceratopteris* copies could not interact with different occurs due to different BELL proteins in *Arabidopsis*. This reinforces that new experiments that reveal the interactions of other genes and proteins with Class I KNOX genes in ferns are necessary to better clarify their function and mechanisms of action. It is possible that other genes downregulate BELL during the development of ferns determinate organs, making Class I KNOX still important in the indeterminacy control in an ARP independent mechanism.

It is unlikely that the meristems, that are a group of cells with indeterminate cell fate, would be reduced to a single cell in ferns, that has strong indeterminate features. In fact, several authors (e.g. Ogura, 1972; McAlpin & White, 1974; Stevenson, 1976, 1978) proposed cytohistological zonation schemes for a multicellular structure, as reviewed by Ambrose & Vasco (2016). Based on Class I KNOX expression data in *Elaphoglossum peltatum*, and previous studies, these authors proposed a more simplified structure for shoot apical meristem of ferns: a single apical cell that rarely divides (may not express Class I KNOX genes in some apices) and a peripheral zone with rapidly dividing cells. In fact, our data concerning *Mickelia scandens* also show a similar pattern of expression. The reference to a unicellular meristem by some authors may be the result of many textbooks that describe in detail the single apical cell while lacking further information about the other meristematic cells (e.g. Esau, 1953; Fahn, 1982; Evert, 2006). The addition of two adjectives to the proposed zonation by Ambrose & Vasco (2016) may describe better this structure: a *quiescent* apical cell and a *proliferous* peripheral zone.

Although they are only part of the apical meristem, it is not possible to deny that quiescent apical cells have specific features, as demonstrated by transcriptomic data with

laser dissection in the fern *Equisetum arvense* (Frank *et al.*, 2015). These experiments showed more upregulated and downregulated genes in the "core domain" of the meristem than in the apical cell when compared with whole plants transcriptomes. The authors did not specify which concept they used for "core domain", but their figures showed the region surrounding the apical cell, that is in big part equivalent to the peripheral zone. It is also important to replicate these experiments in other fern models, due to unique morphological aspects of *Equisetum*, that bears connate and reduced leaves and a dominant stem.

Concerning the leaf primordia, Class I KNOX in all the region from the apical cell to the first pinnae primordium is another evidence of a multicellular meristem. The expression in pinnae primordium, even they are being the terminal unity, may represent some degree of indeterminacy, reinforced by the observed anomalous frond (Fig. 1) that resembles other species of the genus, *Mickelia furcata* (Moran *et al.*, 2010). Also, we show cell divisions at the pinna apex that together with Class I KNOX expression suggest a meristematic activity in this region. Early adaxial development in leaves is well known in flowering plants (Beck, 2010), and in ferns they may be responsible for the formation of fiddleheads, thus protecting the young parts of the frond and pinna primordia.

Fern meristems should be interpreted as a complex and highly organized interconnected network of cells with indeterminate fate, specialized zones (quiescent apical cell vs. proliferous peripheral cells) and capacity of producing new organs (fronds or pinnae). The presence of such structures in developing fronds that also bear the expression of genes related to indetermination is a strong evidence that Agnes Arber's Partial Shoot Theory (Arber, 1950) is correct. Arber said that "*the leaf is a partial-shoot, arising laterally from a parent whole-shoot*", based mainly on the presence of lateral

structures arising from axial elements in the leaf, as well in shoots. According to her, the shoot has a gradient of determination between stems and leaves, and compound leaves present the same gradient. Her theory should be strongly discussed now that new molecular evidence, as our results, has been shedding light on these aspects.

New studies in ferns with genes related to apical meristems and its regulation will certainly increase our understanding and can even detail better the zonation and functions of different cell niches. And the future is promising, as new sequences are available in transcriptome projects like oneKP (Matasci *et al.*, 2014) and the first fern genomes will be released (Sessa *et al.*, 2014).

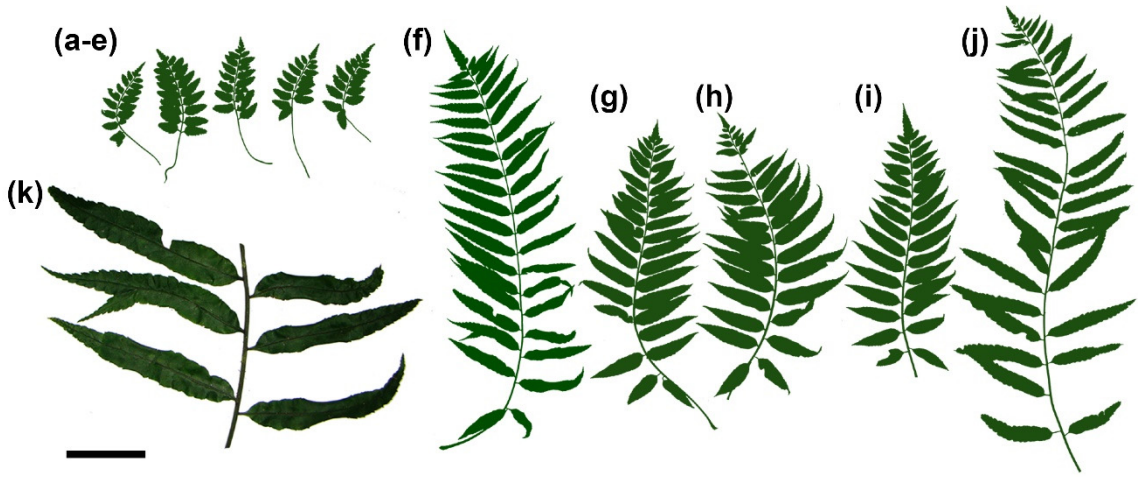
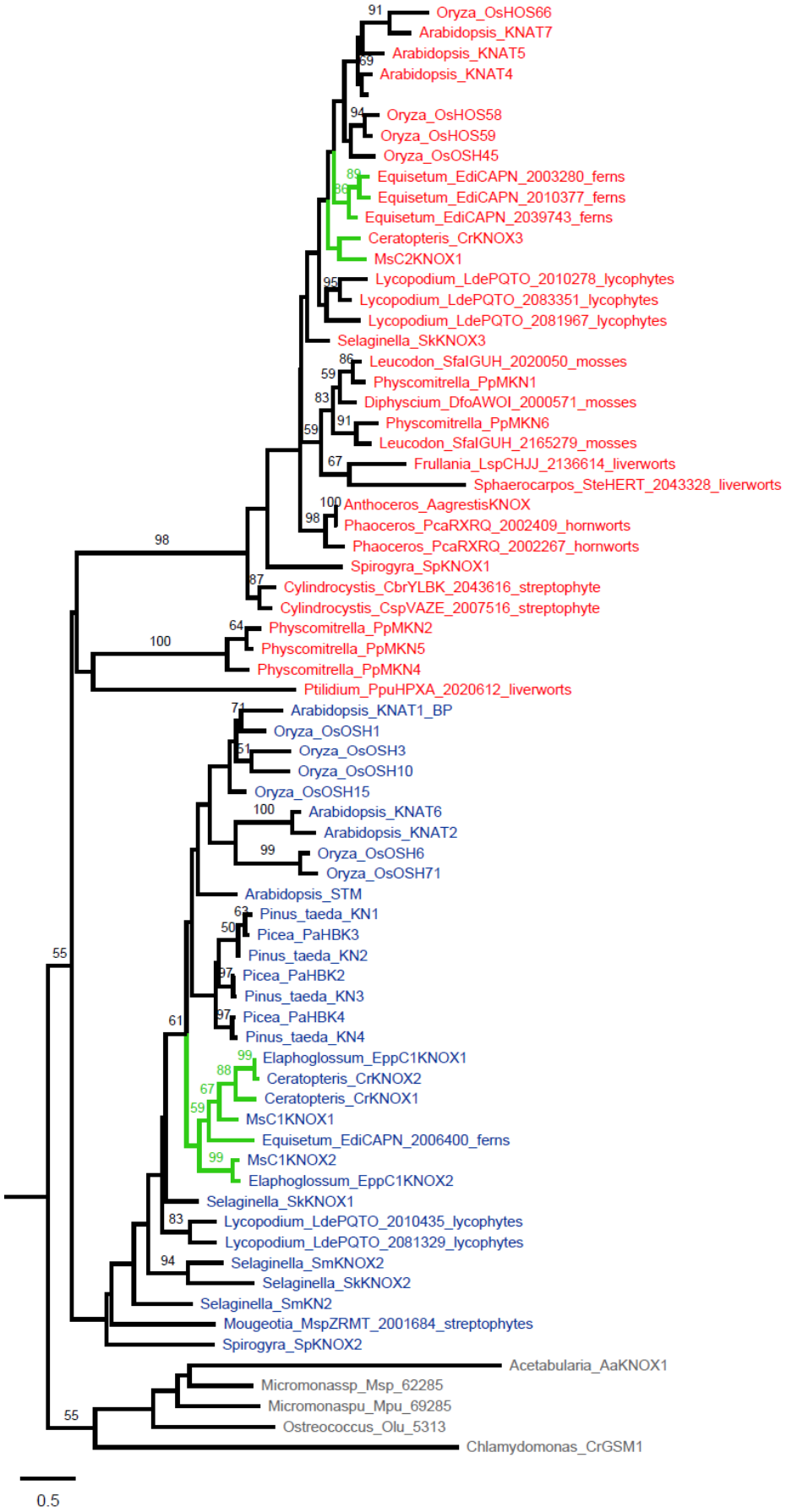


Fig. 1. Dimorphic fronds in *Mickelia scandens*. (a-e) Samples of the terrestrial form, and (f-j) from the climbing form, at the same scale. (k) Detail of the frond of the Fig. 1f, with a pinna bearing a basiscopic anomalous pinnule. Bar: (a-j) 10 cm; (k) 4 cm.


Oryza_OsHOS66
Arabidopsis_KNAT7
Arabidopsis_KNAT5
Arabidopsis_KNAT4
Oryza_OsHOS58
Oryza_OsHOS59
Oryza_OsOSH45
Equisetum_EdiCAPN_2003280_ferns
Equisetum_EdiCAPN_2010377_ferns
Equisetum_EdiCAPN_2039743_ferns
Ceratopteris_CrKNOX3
MsC2KNOX1
Lycopodium_LdePQTO_2010278_lycophytes
Lycopodium_LdePQTO_2083351_lycophytes
Lycopodium_LdePQTO_2081967_lycophytes
Selaginella_SkKNOX3
Leucodon_SfaIGUH_2020050_mosses
Physcomitrella_PpMKN1
Diphyscium_DfoAWOI_2000571_mosses
Physcomitrella_PpMKN6
Leucodon_SfaIGUH_2165279_mosses
Frullania_LspCHJJ_2136614_liverworts
Sphaerocarpos_SteHERT_2043328_liverworts
Anthoceros_AagrestisKNOX
Phaoceros_PcaRXRQ_2002409_hornworts
Phaoceros_PcaRXRQ_2002267_hornworts
Spirogyra_SpKNOX1
Cylindrocystis_CbrYLBK_2043616_streptophyte
Cylindrocystis_CspVAZE_2007516_streptophyte
Physcomitrella_PpMKN2
Physcomitrella_PpMKN5
Physcomitrella_PpMKN4
Ptilidium_PpuHPXA_2020612_liverworts
Arabidopsis_KNAT1_BP
Oryza_OsOSH1
Oryza_OsOSH3
Oryza_OsOSH10
Oryza_OsOSH15
Arabidopsis_KNAT6
Arabidopsis_KNAT2
Oryza_OsOSH6
Oryza_OsOSH71
Arabidopsis_STM
Pinus_taeda_KN1
Picea_PaHBK3
Pinus_taeda_KN2
Picea_PaHBK2
Pinus_taeda_KN3
Picea_PaHBK4
Pinus_taeda_KN4
Elaphoglossum_EppC1KNOX1
Ceratopteris_CrKNOX2
Ceratopteris_CrKNOX1
MsC1KNOX1
Equisetum_EdiCAPN_2006400_ferns
MsC1KNOX2
Elaphoglossum_EppC1KNOX2
Selaginella_SkKNOX1
Lycopodium_LdePQTO_2010435_lycophytes
Lycopodium_LdePQTO_2081329_lycophytes
Selaginella_SmKNOX2
Selaginella_SkKNOX2
Selaginella_SmKN2
Mougeotia_MspZRMT_2001684_streptophytes
Spirogyra_SpKNOX2
Acetabularia_AaKNOX1
Micromonassp_Msp_62285
Micromonaspu_Mpu_69285
Ostreococcus_Olu_5313
Chlamydomonas_CrGSM1
0.5

⪡**Fig. 2.** Phylogenetic relationships of KNOX genes. Class I KNOX genes names are blue and Class II KNOX genes names are red. Fern branches are green. Bootstrap percentage values below 50 are not shown.

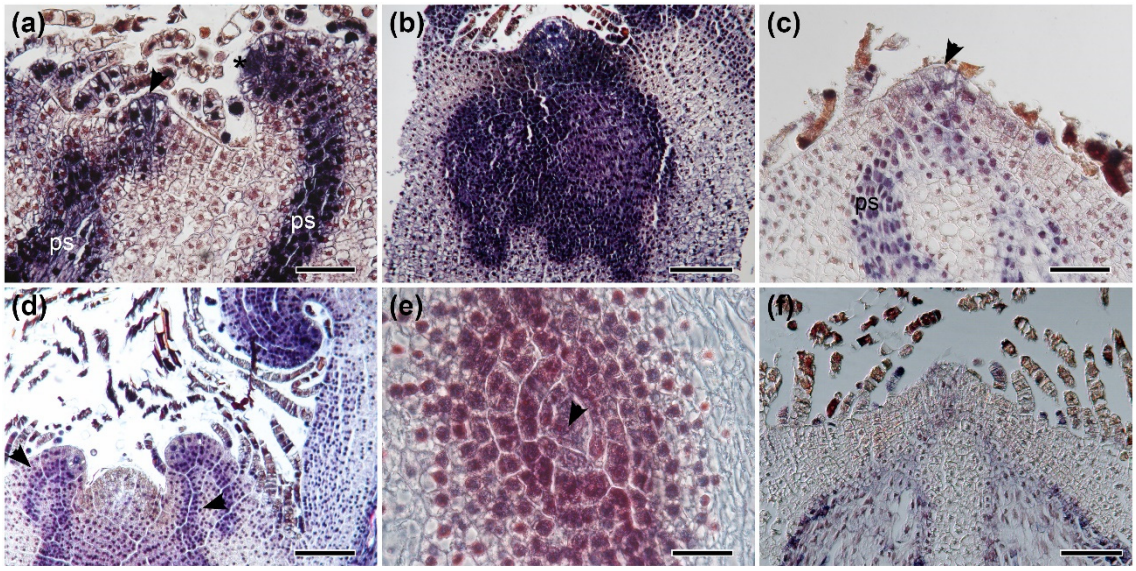


Fig. 3. Shoot apices. (a) Expression of *MsC1KNOX1* in the shoot apical cell (arrowhead), leaf apical cell (*), derivative cells and procambial strands (ps) (b) *MsC1KNOX1* expression in a big peripherical zone. (c) *MsC1KNOX2* expressed in shoot apical cell (arrowhead), derivative cells and procambial strands (ps). (d) In this apex, *MsC1KNOX2* is not expressed in the shoot apical cell. There are procambial strands (arrowheads) connecting the SAM and the leaf primordia. (e) *MsC1KNOX2* expressed in a bigger apical cell (arrowhead) and in the surrounding derivative cells. (f) H4 is expressed in the SAM and in the developing vascular system. Bars: (a) 100 µm; (b) 200 µm; (c) 75 µm; (d) 200 µm; (e) 50 µm; (f) 125 µm.

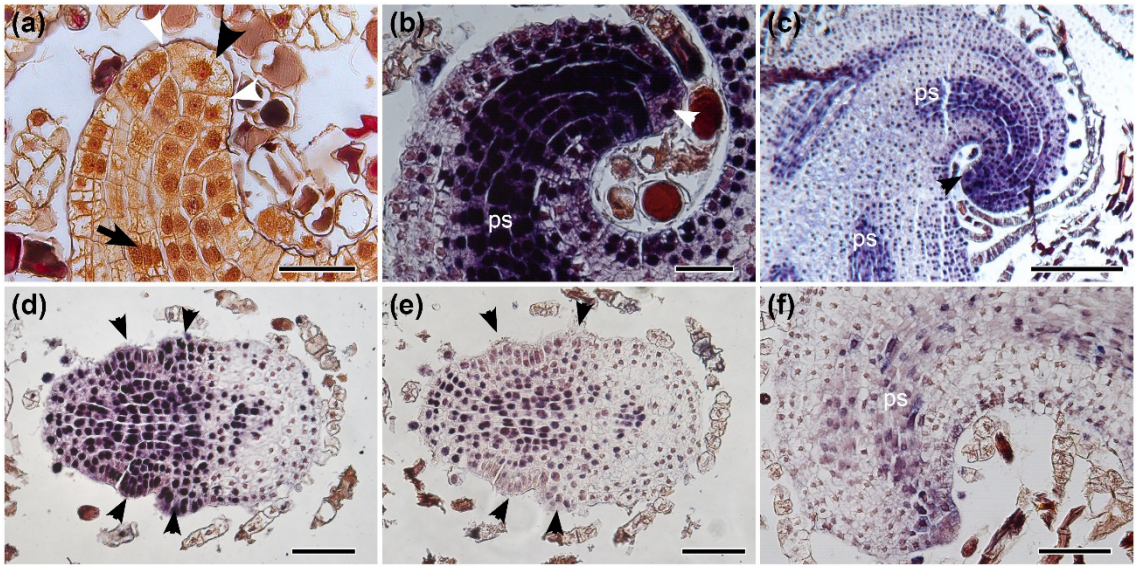
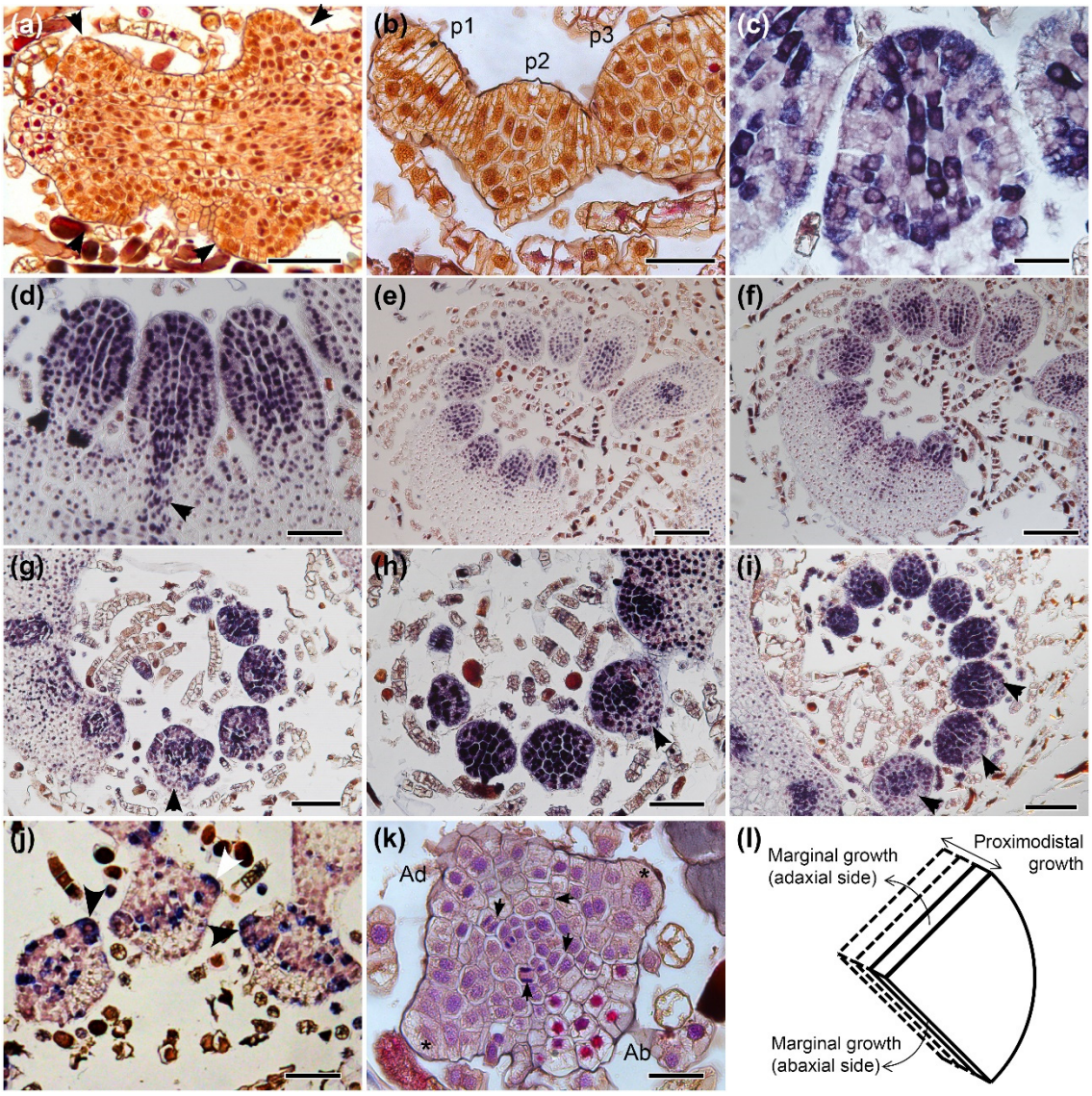


Fig. 4. Fronds development. (a) Apical cell with distal lenticular face (black arrowhead) and two dividing proximal cutting faces (their limits pointed by white arrowheads). Inner derivated cells form the procambium (arrow). (b) *MsC1KNOX1* and (c) *MsC1KNOX2* are expressed in apical cell (arrow head), derivatives cells and in procambial strands (ps). (d) *MsC1KNOX1* and (e) *MsC1KNOX2* expressed in young pinnae primordia (arrow heads). (f) H4 expression shows division in multiple cells in the apical region and procambial strands (ps). Bars: (a-b) 50 µm; (c) 200 µm; (d-f) 100 µm.


(a)
(b)
p1
p2
p3
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
Ad
Ab
(l)
Marginal growth
(adaxial side)
Proximodistal
growth
Marginal growth
(abaxial side)

⋖**Fig. 5.** Pinnae development. (a) Primordia (arrowheads) emerges from the margins of the frond. (b) Transversal section of the youngest pinna (p1) presents grouped cells on its apex with evident periclinal divisions. The base present divisions in multiple plans, visible in older primordia (p2 and p3). (c) H4 expression reveals division in multiple pairing cells on the apex of the primordium and in the central axis, where the vasculature will develop. (d) As the pinnae primordium increases in size, developing vascular traces expresses KNOX1 genes, exemplified by *MsC1KNOX1*. (e) *MsC1KNOX1* and (f) *MsC1KNOX2* are expressed in the entire young pinnae primordia. (g) H4, (h) *MsC1KNOX1* and (i) *MsC1KNOX2* are gradually downregulated in the abaxial side of the pinnae. (j) Cell divisions detected by H4 expression in marginal cells, some of them pointed by arrows. (k) Leaf primordium has marginal cells (*) with outer lenticular faces of the wall and radial divisions in the center (arrows). Adaxial side (Ad) has a denser cytoplasm when compared with the Abaxial side (Ab), revealing a late development of this region. (l) Marginal cells have four dividing plans, contributing to proximo-distal and marginal growth. Bars: (a,d,h,j) 100 µm; (b-c,k) 50 µm; (e-f) 200 µm; (g,i) 150 µm.

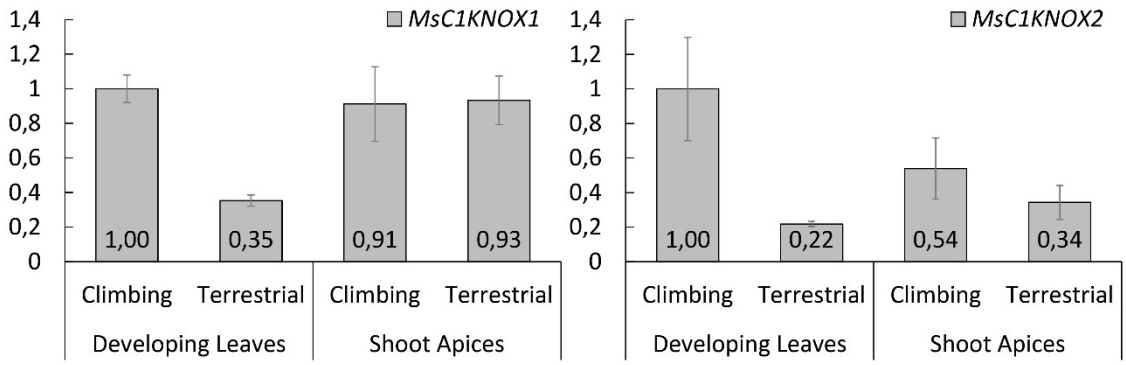


Fig. 6. Fold change values for *MsC1KNOX1* and *MsC1KNOX2* in apices and developing leaves of the terrestrial and climbing forms. In developing leaves of the terrestrial form, these genes have significant values for downregulation when compared with apices of both forms and developing leaves of the climbing form.

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Supplementary Material

S1. Primer sequences (5'→3')

Primers designed for probes labeling:

MsC1KNOX1ProbeF	GAAGTACCATGACGAGCTGATGC
MsC1KNOX1ProbeR	CCGCTATACTTGCGAAGCAG
t7_MsC1KNOX1ProbeR	CTAATACGACTCACTATAGGGCCGCTATACTTGCGAAGCAG
MsC1KNOX1ProbeF	CAAACCCTTCAACGAAGCCATA
MsC1KNOX1ProbeR	GTTTGAGGGTAGTGATGTAGC
t7_MsC1KNOX2ProbeR	CTAATACGACTCACTATAGGGGTTTGAGGGTAGTGATGTAGC
MscandensH4ProbeF	ATGTCTGGCCGGGGAAAGGGAGG
MscandensH4ProbeR	CCGAAGCCATAGAGGGTACTTCC
t7_MscandensH4ProbeR	CTAATACGACTCACTATAGGGCCGAAGCCATAGAGGGTACTTCC

Primers designed for qRT-PCR experiments:

MsC1KNOX1qRTF	GTGATGAGAAAGGGGAGCTAG
MsC1KNOX1qRTR	GCCTCCTCCTCCACAGATGA
MsC1KNOX2qRTF	GACACAGAGCAAGACATCGA
MsC1KNOX2qRTR	TTCAGTTTGAGGGTCCATG
MsActinqRTF	CGTCTGGATCTTGCTGGC
MsActinqRTR	CTCGCTCAGCAGTGGTT

S2. Obtained partial sequences

MsC1KNOX1
>GAGTTGGACCACTTTATGGAGACATATTGCAACGTGCTGCAGAAGTACCATGACGAGCTGATGCAGCCA
TACAAGGAGGCCATGGCCTTCTTTAGAAAGATAGAGCTTCAACTAAATGCTCTGAGCAAGGGGAGCTTAA
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MsC1KNOX2
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MsC2KNOX
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GACCCTCAAACTGAAGACAAGCAATTGAAAGAGCAGCTTCTTCGAAAGTATAGGGGCTACATCACTACCC
TCAAACATGAATTTATGAAGAAGAAGAAGAAAGGCAAGCTTCCCAAGGATGCTCGGCAGCAACTCCTGGA
CTGGTGGAATGACCACTACAAGTGGCCCTACCCGACTGAAGCAGAGAAGGGAACGCTAGCAGAGACGACG
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MscandensBActin
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CTGATGGTCAGGTGATCACCATT

MscandensH4
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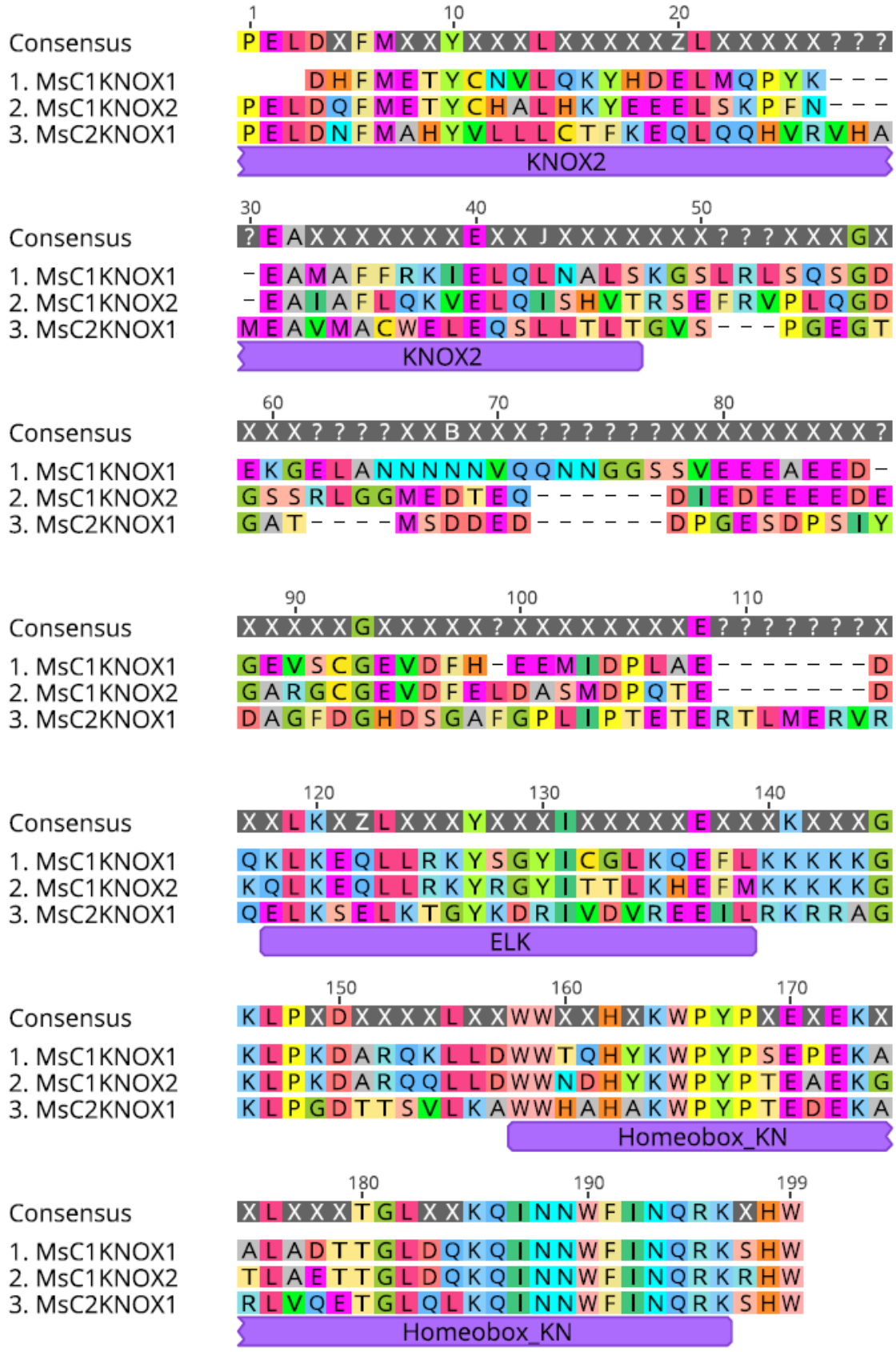
S3. Alignment of partial KNOX proteins sequences and conserved domains



Chapter 2

Reiterative role of apical and marginal cells in the development of fern leaves

Rafael Cruz, Jefferson Prado & Gladys F. A. Melo-de-Pinna

Abstract

Development mechanism has been advanced in flowering plants. However, technical barriers limit these studies in ferns, an important group for the understanding about leaf and shoot evolution in vascular plants. We studied the anatomical development of leaves in the species, *Elaphoglossum vagans*, *Lastreopsis amplissima*, *Lomariopsis marginata*, *Megalastrum connexum*, *Polybotrya cylindrica* and *Rumohra adiantiformis* by using classical anatomical technics of sectioning and staining. In addition, we observed specimens in the SP herbarium of those species, *Elaphoglossum lingua*, and *Mickelia scandens*, searching for unusual morphologies that may occur in some leaves.

As main results, we found that the shoot apical meristem of these species bears the typical tetrahedral cell with three dividing faces and the leaf apical meristem have the typical two-faced cell. The apex of the frond form distinct marginal cells, each of them bearing strong similarities to the leaf apical cell, except in the simple-leaved fern *Elaphoglossum vagans*. Pinnae and pinnule primordia start their apical growth with grouped cells that keep the original form of marginal cells, also producing their own marginal cells. In more divided leaves, one of these grouped apical cells in pinnae and pinnules may be prominent and become an exact copy of the leaf apical cell. The observed plants with unusual morphologies include plants with mixed reproductive identity, differential marginal growth, compromised apical growth and reduced determination.

Apical and marginal cells of ferns may be homologous evolutionally and ontogenetically. Their characteristic geometry may allow specific cell divisions and their interconvertibility may drive the balance between apical and marginal growth. An unusual activity of them or changes in transcription may generate the observed abnormalities. At the same time, a reiterative development in ferns leaves supports the homology with shoots and the identity-in-parallel of their divisions.

Introduction

Leptosporangiate ferns comprise 80% of the diversity of the non-flowering plants (Schuettpelz & Pryer, 2007) and, together with other ferns, occupy a key position in the land plants phylogeny, either as an outgroup for seed plants or when used for comparisons between early diverging embryophytes and seed plants (Plackett *et al.*, 2015). Their leaves are usually compound and known as fronds (Vasco *et al.*, 2013). Fronds are of particular importance in the evolution of leaves since studies pointed these structures as homologous to those ones in seed plants, based mainly in the Zimmerman's Telome Theory, thus being called megaphylls (Zimmermann, 1952; Beerling & Fleming, 2007; Tomescu, 2009; Vasco *et al.*, 2016). According to this theory, megaphylls evolved from leafless dichotomous axes of the ancestral sporophyte – the telomes – after three processes: (*i*) elongation of a central telome causing the lateralization of others (overtopping), (*ii*) two-dimensional disposal of these lateral telomes (planation), and (*iii*) fusion of these telomes through the formation of connecting tissue (syngenesis). There are some critics to this theory, that propose more than a single origin for leaves in ferns and seed plants (Corvez *et al.*, 2012).

In the last decades, with the advance of molecular studies, a big number of development mechanisms have been revealed for leaves in flowering plants (Tomescu, 2009; Blein *et al.*, 2010; Bar & Ori, 2014). However, technical barriers, like the presence of many scales covering young parts (Vasco *et al.*, 2013), usually very large and non-completely sequenced genomes (Sessa *et al.*, 2014; Wolf *et al.*, 2015) and frequent polyploidy (Wolf *et al.*, 2015) in ferns, have restricted most of the leaf development studies made in the group to its anatomical aspects. These studies have always highlighted the presence of a distinct single apical cell at the apical meristem of the shoot apex and

developing fronds as a central and important piece of the meristem (Ogura, 1972; Gifford & Foster, 1988; Sanders *et al.*, 2011; Plackett *et al.*, 2015), suggesting a homology between shoots and fronds. Specific division plans are responsible for daughter cells that give rise to distinct merophytes i.e. packages of cells derived from a single daughter of the apical initial (Plackett *et al.*, 2015). Superficial cells of a merophyte close to the shoot apical cell are known as prismatic cells, and one of them can give rise to the leaf apical cell, initiating the development (Bierhorst, 1977; Imaichi, 2008). Leaf apical cells of ferns usually are three-sided, with one lenticular free surface that provides freedom of expansion during the growth together with two cutting faces that meet at one edge (Korn, 1993).

Frequently both pinnae and pinnules have been pointed as a product of a differential outgrowth of specific regions in the marginal meristem that is present are present in the lateral of the frond and pinnae, respectively (Saha, 1963; Wardlaw, 1963; White & Turner, 1995). Single apical cells sometimes are reported in apices of pinnae and pinnules primordia during the development (Saha, 1963), but also the apical structure of these structures have been pointed as adjacent paired cells with two cutting faces each (Hill, 2001; Plackett *et al.*, 2015), as in the apical meristem of *Selaginella* leaf and shoot. Sanders *et al.* (2011), based on computational sector analysis, created a predictive model for *Nephrolepis exaltata* (once-pinnate) pinnae development, showing that they are formed by the single initial apical cell and few marginal initials that, in a coordinated manner, generate the final morphology.

In our previous study for the once-pinnate species *Mickelia scandens*, we showed that cell divisions are rare in the margin of the pinna primordium and frequent in the group of cells in the apex during the apical growth, without an evident dominating single cell

responsible for it (Cruz et al. 2018[1]). In the same study, we also showed that Class I KNOX genes, usually related to indetermination of growth, is expressed through all the pinna primordium during this same initial stage, even in marginal cells. For one specimen found in the field with detected an anomalous pinnule arising in a pinna side, as an evidence of reduced determination.

This study presents a comparison between apical and marginal cells during the leaf development in some species of ferns with different leaf morphology and related phylogenetically to *Mickelia scandens*. Here we show that different apical organization of leaf meristems are present in the developing structures depending on how divided the leaf is, although they take several similarities between them. We also describe some of the naturally occurring mutants that we found in a herbarium collection, a good source of interpretation for possible development mechanisms and for creating some hypothesis about how they changed towards the evolution of ferns.

Methodology

With the intention to compare our development data with our previous study concerning *Mickelia scandens*, we have chosen some related species with different morphology based on the Dryopteridaceae phylogeny of Moran *et al.* (2010). In addition, we collected *Lomariopsis marginata*, a species of Lomariopsidaceae, formerly related to the studied species and morphologically similar to *Mickelia scandens*, as an outgroup (Fig. 1). Vouchers were deposited in the SP herbarium and are enlisted, together with the morphology of the studied plants, in Table I.

[1] The first chapter of this thesis.

Table I. Plants collected in the field at Alto da Serra de Paranapiacaba Reserve[1], Fontes do Ipiranga State Park[2] and University of São Paulo[3].

Species	***Vouchers* (SP Herbarium)**	**Frond Morphology**
Elaphoglossum vagans (Mett.) Hieron[1]	Hirai et al. 754	Dimorphic simple
Lastreopsis amplissima (C. Presl) Tindale[2]	Prado & Cruz 2331	Monomorphic multi-pinnate
Lomariopsis marginata (Schrad.) Kuhn[2]	Prado & Cruz 2333	Dimorphic once-pinnate
Megalastrum connexum (Kaulf.) A.R. Sm. & R.C. Moran[2]	Prado & Cruz 2330	Monomorphic multi-pinnate
Polybotrya cylindrica Kaulf[2]	Hirai et al. 750, Prado & Cruz 2329	Dimorphic multi-pinnate
Rumohra adiantiformis (G. Forst.) Ching[3]	Cruz 30	Monomorphic multi-pinnate

Shoot apices and developing leaves (fiddleheads) were collected and fixed in formalin-acetic acid-ethanol 50% (FAA) for 48 hours and stored in 70% ethanol. The material was submitted to ethanol/tert-butanol gradient dehydration and embedded in paraffin (Johansen, 1940). Serial sections were obtained with a rotary microtome and stained with Safranin and Astra Blue (Bukatsch, 1972) or with the Flemming's Triple Stain (Johansen, 1940) and mounted on slides with synthetic resin.

We also searched in the collection of the SP herbarium for plants of the analyzed species with anomalous morphologies, illustrating the structures in order to discuss them based on our developmental data. For this analysis, we included *Mickelia scandens* (Aubl.) R.C. Moran et al. and *Elaphoglossum lingua* (C. Presl) Brack.

Results

The shoot apical meristem (SAM), protected by large multicellular scales in all analyzed species, contains a distinct shoot apical cell (SAC, Figs. 2a-d), with three dividing faces (Fig. 2a) and one external face (Figs. 2b-d). Its surrounding daughter cells give rise to merophytes that gradually increase their densely stained content (probably phenolic compounds) forming a peripheral zone at the surface (Figs. 2a,c). Some small cells formed beneath the apex do not accumulate these phenolic contents and gradually become more elongated developing into procambial strands (Fig. 2b). Leaf primordium arises at the flanks of the SAM, initiating its development and are very distinct due to its apical cells with the external face usually rounder than the SAC (Fig. 2d).

In all our analyzed samples we detected two division plans in these cells, forming two mediolateral oriented merophytes, classifying them as wedge-shaped apical cells (Figs. 3a-c). The two daughter cells may undergo periclinal divisions immediately,

resulting in more than two cells in contact with the leaf apical cell (Fig. 3a,c), or divide anticlinally, and then, the resulting merophytes cells divide periclinally (Figs. 3b,c). More divisions contribute to the primordium radial increasing, and as the primordium grows, its typical circinate vernation starts to become evident (Fig. 3c-d). The primordium, as in the shoot, gradually increase their phenolic content at the apex, except in procambial cells (Figs. 3a-e), making it evident how early the vascular organization is defined in a longitudinal sagittal section. In a lateralized sagittal plane, it is possible to detect a series of anticlinal divisions of the apex, corresponding to the marginal cells (Fig. 3f).

The marginal cells are reduced in *Elaphoglossum vagans* (Fig. 4a) when compared with other species, where different processes take place. In the other species, some marginal cells undergo more periclinal divisions, initiating the development of pinnae (Fig. 4b). The pinna primordium is recognizable by a group of apical cells, with very evident anticlinal divisions (Fig. 4c) and narrow in a paradermal section of the structure. As they look very similar at this stage, it is not possible to detect if these cells are derived from a single marginal initial or a group of already formed marginal cells that coordinately started a development of a lateral pinna. As the primordia grow, their own rows of marginal narrow cells are formed in their laterals. Some anticlinal divisions are also in the apex, generating central cells that will form the pinna midrib. The same structure is evident in pinnules primordia, at least at their initial stages (Figs. 4d-e). In a sagittal section, this structure is surprisingly similar to the leaf apical cell in a sagittal section of the frond, although they are actually part of a row of similar cells (Fig. 4f).

In some more developed pinna primordia, in more divided leaves (not in *Lomariopsis marginata*), we can observe that one single cell may become prominent over the others and replicate the exact development of the initial frond, with a single apical

cell (Fig. 5a), what requires to carefully observe the sequence of sections allowing us to determine the apical cell as single. *Lastreopsis amplissima*, *Lomariopsis marginata,* and *Rumohra adiantiformis* have a notable accumulation of densely stained content during the development of dermal and cortical tissues. This feature helped us to conclude that cells at the abaxial region develop first in any of the analyzed structures (Figs. 5b-e). It is also helpful to identify provascular tissues, as they are not densely stained. The earlier development of the abaxial region generates the typical curvature in a fiddlehead structure, allowing the protection of younger structures and causing the circinate vernation during the development (Fig. 5c).

Marginal cells are evident in all analyzed species, except in *Elaphoglossum vagans*, that bears very reduced ones. In any transversal sections of pinnae or pinnules primordia, marginal cells will resemble a leaf apical cell, revealing distinct two cutting faces (abaxial and adaxial) and one outer lenticular face that does not divide (Figs. 5d-e). But because they are truly organized in rows of similar cells, they have additional two triangular cutting faces between the parallel marginal cells (Fig. 5f). They are present even in *Lomariopsis marginata* pinnae, that are determinate structures, what is an evidence that they have another function besides forming pinnule primordia. We detected some marginal growth by adaxial and abaxial divisions of these marginal cells during the initial stages of lamina development (Fig. 5g), although they are not persistent and other cells seem to contribute to the lamina growth after some time. But paradermal sections of a pinna primordium of *Lomariopsis marginata* reveals that marginal initials generate rows of well-defined mesophyll or procambial cells in the pinnae margins (Fig. 5h). Except for the frond *Elaphoglossum vagans*, we could not detect any single apical cell in the apical structure of terminal divisions.

From the morphological analysis, we found out anomalous developed leaves in the two analyzed *Elaphoglossum* species (Figs. 6-7), in *Lomaripsis marginata* (Fig. 8), in *Mickelia scandens* (Fig. 9), and in *Polybotria cylindrica* (Fig. 10). Curiously, all of these species normally bear holodimorphic leaves, with broader laminar tissue in the sterile leaves, and short margins in the lamina of fertile leaves, that bear acrostichoid sori. For *Elaphoglossum lingua* we found a hemidimorphic leaf (Fig. 6c), with its apical portion bearing sori in the margin of the lamina, although a sterile portion is present besides de midvein. For both species of *Elaphoglossum*, we detected leaves with retuse apices (Figs. 6f,7c) and leaves with differential marginal growth (Figs. 6d-e,7d-g). In some of these samples, we could not detect, macroscopically any kind of marginal growth in the affected sector (Figs. 6e,7d). For *Mickelia scandens*, we also found some differential marginal growth in a pinna. More interesting are two samples, one sterile and one fertile, that can be interpreted either as bifurcated pinnae, or pinnae bearing one acroscopic pinnule each. For this species, we also found a series of atrophied pinnae in the frond. *Lomariopsis marginata* pinnae also presented differential development of the margin (Figs. 8c-d) and a hemidimorphic pinna (Fig. 8e). In this case, different of *Elaphoglossum lingua* anomalous frond, the basal part of the pinna is fertile, and the apex is sterile, with a broader margin. *Mickelia scandens* presents a frond with an entire median sector with atrophied pinnae (Fig. 9c), with basal and apical bearing regular pinnae. The analyzed samples also presented a pinna with differential marginal growth (Fig. 9d), and both sterile (Fig. 9e) and fertile (Fig. 9f) divided pinnae. For *Polybotrya cylindrica*, we also found intermediate fertile-sterile structures. One first order pinnule present reduced laminar tissue in its apex (Fig. 10c), as typical of fertile fronds, although it does not bear

sori. One second order pinnule present almost the same situation with the apical part fertile, but with sori (Fig. 10d).

Discussion

Apical cells in ferns

Apical cells in ferns have been a matter of discussion in many development studies (White & Turner, 1995; Imaichi, 2008; Vasco *et al.*, 2013; Plackett *et al.*, 2015). Plackett *et al.* (2015) present a review about shoot development in land plants and affirms that the apical cell is equivalent to a high organized multicellular SAM of seed plants. It is important to understand that, although the apical cells may act as initials and have a specialized function, it is only part of a multicellular meristem, possibly equivalent to angiosperms SAM central zone (Nardmann & Werr, 2013; Frank *et al.*, 2015; Ambrose & Vasco, 2016). Our results are consonant with the usual geometry found for most ferns where shoot apical cells have three dividing faces and the leaf apical cells have two dividing faces, what is said to be directly related to the three-dimensional and planar form of these organs, respectively (Imaichi, 2008).

Merophyte initials have elongated anticlinal cell walls, that are parallel to the dividing faces where they came from. Even with some occurring periclinal divisions, the anticlinal divisions of these cells are predominant in the younger merophyte cells in both SAM and LAM. It is also notable the occurrence, in both structures, of small cells that develop into procambial strands right beneath the apical cells and merophyte initials. The basic differences between these structures at this specific stage seem to be connected only to the geometry of the apical cell. Although many studies describe a reduced mitotic activity of these cells (White & Turner, 1995; Ambrose & Vasco, 2016), the maintenance

of their geometry is essential for the form maintenance. We could detect, in our previous study, that both SAM and LAM of *Mickelia scandens* present mitotic activity in the apical and derivative cells (Cruz *et al.*, 2018).

Interestingly, studies with eusporangiate ferns *Botrychium* (Ophioglossales) and *Angiopteris* (Marattiales), and with the leptosporangiate fern *Osmunda* (from the basal order Osmundales) reveal that their leaf apical cells have three cutting faces, instead of the two normally found in the other ferns (Bierhorst, 1977; Imaichi & Nishida, 1986; Imaichi, 2008), and any bilateral symmetry in this group is secondary to the apical development. It is possible that the ancestral condition for leaf apical cells in ferns ancestral is an exact repetition of the geometry of a shoot apical cell. And even the leaf apical cell is fundamentally a modified shoot apical cell. However, it is really hard to track down this evolution, as other groups of eusporangiate ferns are Psilotales and Equisetales, groups that bear much modified or reduced leaves (PPG I, 2016). Even the sister group of Osmundales (the remaining leptosporangiate ferns) have as earlier diverging branch the filmy ferns (Hymenophyllaceae), that have the leaf lamina one cell layer thick between the veins (Vasco *et al.*, 2013; PPG I, 2016).

The role of marginal cells in pinnae and pinnule development

Marginal meristem fractionation for the formation of pinnae was described in some detail by Hagemann (1984) for the formation of pinnae. Curiously, the same author proposed an alternative terminology for this meristem, “marginal blastozone” (from Greek: budding zone), based mainly in an argumentation of the organismic theory that form growth and organogenesis are independent of cell divisions (Kaplan & Hagemann,

1991; Hagemann & Gleissberg, 1996; Hagemann, 1999). He advocates that plant cells have a static nature and growth of organ form occur before histogenesis and cell differentiation, so an interpretation based on organogenesis would fit better for these structures.

We do not intend to provide a long discussion on the meristems concepts for seed plants, but there is some misinterpretation in this view that would be enlightened by some more recent data, ours included. Marginal cells of the laminar structures in the species studied by us, depending on their position in the primordium, have well-defined divisions plans. Pinnae and pinnule arise by an increment of anticlinal divisions of these cells. Our previous data show that during apical growth, most marginal cells are formed in the apical region of a pinna primordium by anticlinal divisions of grouped cells and central divisions are responsible for axial growth (Cruz *et al.*, 2018). In all our pinnae and pinnule primordium, we see these grouped cells anatomically organized in a similar way. Some authors observed similar structures, with different interpretation describing them either as paired cells in *Ceratopteris richardii* (Hill, 2001; Plackett *et al.*, 2015) or derivate from a single initial in *Nephrolepis exaltata* (Sanders *et al.*, 2011), by doing computational or conventional anatomical analysis. We cannot confirm that these grouped cells came from a single marginal initial or a group of marginal initials, but the dominance of a cell over the others seems to be the origin of the single apical cell present in the pinnae and pinnules primordia of more divided leaves.

The interesting geometry of these grouped apical cells, found in most of our analyzed species, is an evidence that they retain some features of the marginal cells from where they came from. Similar cells were previously described for *Nephrolepis exaltata* frond primordia and *Mickelia scandens* pinnae primordia (Sanders *et al.*, 2011; Cruz *et*

al., 2018). It is notable how similar they are when compared to the leaf apical cells, due to their two wedge-shaped cutting faces, whose laterals seems to divide specifically abaxial and adaxial domains. The interconversion from a four-sided marginal-type cell to a single two-sided apical type cell probably happens easily with a geometric reorganization. In this case, one central cell from the grouped ones lose the abaxial and adaxial cutting faces that are already reduced during the initial development of the primordium.

Groups of marginal cells that start the apical organization of pinnae primordia probably correspond to what Saha (1963) considered as loci of sustaining meristematic activity when studying three species of leptosporangiate ferns. For the former *Dryopteris aristata* (Dryopteridaceae), now classified in *Arachniodes* in the same family, he affirmed that the central cell of this group becomes the apical cell, reiterating the activity, very similar to what we have found for most of our analyzed species. But he concludes that at some time the two-sided apical cell is transformed back into the original prismatic cells, affirmation made without supporting images. Comparing with our data, he observed for this species the same two different types of apical organizations in the divisions of a frond: one is the grouped cells in the beginning of development of pinnae and pinnules and other, derived from it, that is a repetition of the previously developed axis, like pinnae repeating the development of the frond. We did not find any evidence that the determination of a terminal primordium involves the reorganization of grouped apical cells, as observed by Saha (1963).

The concept of blastozones, besides the usual concept problems in Biology, is devoid of the necessary linkage to genic regulation and new development data as proposed by Townsley & Sinha (2012). The presence of a dichotomy in the development

into a cellular perspective (division drives growth/meristems) or the organismal perspective (division merely accompanies growth/blastozones) is too restrictive and not real. Evidence shows that cells are not autonomous and there is a positional cooperation on their behavior, making them unities of morphogenesis controlled by factors that govern the development of the organ. These factors may derive from individual cells or cell populations (Beemster *et al.*, 2003; Tsukaya, 2003). The apical and marginal cells observed in our study seems to have a refined geometric control, positioning populations of cells of specific geometries in specific places, making them competent for a development process of an organ.

Simplifying

In *Elaphoglossum vagans*, the observed marginal cells do not seem to have the same organization of the other species and this may be related to it simple morphology. Contrary to what usually happens to angiosperms, simple leaves in leptosporangiate ferns are derived from ancestors with more divided leaf (Moran *et al.*, 2010c; Vasco *et al.*, 2013, 2015). As these big marginal cells seem to be necessary for the formation of a pinna primordium, possibly *Elaphoglossum vagans* cannot undergo the growth process of a lateral segment.

It is interesting that even terminal structures that do not produce lateral structures, as *Lomariopsis marginata* pinnae may present these marginal cells, very similar to previous data for *Mickelia scandens* (Cruz *et al*., 2018). Cell divisions in the abaxial and adaxial cutting faces of these structures occur contributing to the beginning of margin formation. But how can *Elaphoglossum* construct its entire lamina without such cells?

Possibly lamina formation is independent of them at some level. In fact, in plants where they occur, we did not find any evidence that their activity is persistent during all the margin formation and secondary processes may act expanding the margins of a laminar unity.

If this is true, there are two hypotheses on how the simple-leaved *Elaphoglossum* evolved: *(i) Elaphoglossum* ancestor suddenly lost the big wedge-shaped marginal cells, abruptly losing the capacity to generate pinnae, but keeping secondary processes of margin expansion; and (*ii*) *Elaphoglossum* ancestor already had an entire leaf, and the big marginal cells were gradually lost for not being necessary anymore.

Identity-in-parallel

The mathematician Michael Barnsley developed an iterated function system (i.e. a function that is repeatedly executed using the output from one iteration as the input of the next) capable of creating graphically a fractal image very similar to a *Asplenium adiantum-nigrum* frond or to other species if the function is slighted altered (Barnsley, 2000). Although development biologists that completely understand[2] how these complex fractal functions works are still rare, the similarity to a real fern frond may impress any botanist. Reiteration is well known in Botany and described as the repetition of the same architectural unit within a plant (Hallé, 1999). This process have been described even for some ferns (Gay, 1993; Tomescu *et al.*, 2008; Sanders *et al.*, 2011).

We mentioned before the possible homology between leaf and shoot apical cells. Based on their ontogenetic origin and the shape of the two larger cutting faces, the

[2] We do not.

resemblance between leaf apical cells, grouped cells of pinnae and pinnula apices and marginal cells is remarkable. This aspect should be interpreted as evidence of homology between them. If this assumption is correct, apical cells ultimately generate axes with new homologous apical cells that may repeat similar programs of development many times within itself in a reiterative process. The frond itself would repeat a development of the shoot in some degree, and the pinnae replicate the development of the frond.

In fact, all the divisions, from the shoot to terminal pinnules starts with apical growth, with ultimate segments undergoing laminar growth. It is evident in our results that even laminar organs start with a predominantly radial increase, generating an axial primordium. This interpretation would generate problem to interpret the fern leaf as a well-differentiated category from axial organs, like the stem. The use of the "Fuzzy Arberian Morphology" is recommended by Schneider (2013) for ferns as it provides the interpretation of structures based in the fact that leaves, shoot and roots are linked by shared developmental processes. This school of morphology is based in a series of morphological and philosophical studies of the British botanist Agnes Arber (1879-1960) that consider different organs as having overlapping developmental pathways and features instead of a rigid classification between root, stem, and leaf (Sattler, 1996; Kirchoff, 2001; Rutishauser & Isler, 2001).

Agnes Arber pointed that an essential element in Zimmermann's telome theory is that leaves are primarily conceived as a radial and branched structure and a secondary development generates the flattening and dorsiventrality. Her most prominent hypothesis is based in a series of classic studies of important morphologists (e.g. Casimir de Candolle, Johann von Goethe, Walter Zimmerman) and her own data, that she expressed in the sentence: "the leaf is a partial-shoot, arising laterally from a parent whole-shoot".

This affirmation is mainly evident in leaves with many leaflets. She used the concept of identity-in-parallel for a hypothesis that the leaflet is repeating a developmental pathway of the whole leaf. Applied to our plants, the pinnules are homologous to pinnae, that are homologous to fronds and homologous to the shoot in some degree, sharing development processes. This hypothesis is reinforced by the expression of Class I KNOX genes in angiosperms, that are expressed in indeterminate organs meristems, but usually not expressed in a determinate leaf primordium, except in compound leaves, possibly acting reducing the determination of the primordium (Champagne & Sinha, 2004). *Mickelia scandens* quantitative data of Class I KNOX expression corroborate that these genes may have some of the same function in ferns (Cruz *et al*., 2018). If the self-replication of development programs really occurs in ferns, at some degree, all the segments in a frond share Arber's identity-in-parallel and are components of biological fractals like the Barnsley's fern.

Abnormalities in morphology

One of the hypotheses that we proposed for *Elaphoglossum vagans* morphology involves the loss of the big marginal cells due to a developmental error. About errors in the development, Arber (1950) recognize plant form as a limitation, and the removal of suppressors and inhibitors factors may act as a releasing agent, allowing the form to reveal its completeness. According to her, abnormalities, although seem with distrust by many botanists, show incontestably what the plant *can* do. Also, it is undeniable that some mutants have similarities with the ordinary morphology in other species, and by understanding what got wrong in one species, we can understand what get right in other species, as in many natural occurring hemidimorphic species of ferns. Even

morphologists that were diametrically opposed to Arber's holistic view of the shoot recognized the importance of studying mutants to deduce gene functions and development programs. However, for them, these studies should not make any kind of reinterpretation that could hurt the typological view of a basic organ. As an example, we have Donald Kaplan, that carried a typological view based on the German botanist Wilhelm Troll (Kaplan, 2001a) and completely denied the interpretation of a compound leaf as an intermediate feature between shoots and leaves (Kaplan, 2001b), as proposed by molecular studies (Sinha, 1999).

One *Mickelia scandens* ramified pinna was described in our previous study (Cruz et al., 2018) and this time we present other two ramified pinnae, one of them fertile. This situation can, again, be explained by a reiterative process. As *Mickelia scandens* bears their big marginal cells (Cruz *et al*., 2018), it has the group of cells necessary for the pinnule development, repeating again the development of pinnae. Super expression of Class I KNOX in angiosperms can make very ramified leaves with reduced determination (Hareven *et al.*, 1996), and our previous data showed evidence that these genes also are related to indetermination in ferns and are expressed in pinnae primordia (Cruz *et al*., 2018).

The series of atrophied pinnae found in *Mickelia scandens* is probably the most complex defect found, but a great evidence of the acropetal growth and how the normal program can be retaken during the development of the frond. The pinnae or fronds with affected marginal growth probably follow the same principle. At some time, marginal growth is affected but can be retaken at variable degree, generating these unusual lobed leaves. It is also possible that all the affected sector is originated from damages of a single initial cell, like one merophyte initial, that is replaced by divisions of the leaf apical cell.

Retuse apices represent damaged apical growth, possibly by losing the activity of the LAM.

It is notable that all the mutants that we have found are from species that bear dimorphic fronds. The basic differences between the two types of leaves are how broader they are (marginal growth) or how tall they are (apical growth). Probably these plants normally have a very refined control of development for transiting from one situation to another, and any factor may easily affect one of the steps. Describing the "bizarre, intermediate" forms of some *Polybotrya* leaves, Moran (1987) illustrated samples with basal fertile portions and apical sterile portions, the opposite of what we have found for *Polybotrya cylindric*a. According to him, this situation suggests a single macromutational switch that can produce all the modification between the two types of fronds. He concludes that this hypothesis is improbable since dimorphy is an ensemble of many characters, thus controlled by many mechanisms. At that time, most scientists were unaware of important mechanisms of transcription, that took more time to substantially advance (as in the studies led by Roger D. Kornberg, that was awarded by the Nobel Prize in Chemistry of 2006; Nobel Media AB 2014), and their role in the plant development. The elucidation of the ABC system, for example, showed how single mutations in transcription factors (TFs) are sufficient to change completely a morphology of an organ (Causier *et al.*, 2010). This happens mainly because many TFs are necessary to start the transcription of more than a single development-related gene, some of them other TFs. The change (activation or inactivation) of a single point of this cascade by genetic, hormonal or environmental factors can drastically change the development of an organ (Gonzalez, 2015), likely acting as the macromutational switch suggested by Moran (1987).

LFY transcription factors, for example, play a role transforming inflorescence meristems of angiosperms in floral meristems. Curiously, one *Ceratopteris richardii LFY* homolog is expressed in developing reproductive fronds of this species (Himi *et al.*, 2001). If *LFY* or another TFs are involved in the reproductive transition of dimorphic fronds, they probably affect the apical and marginal growth and are regulated by an environmental or hormonal factor. If this factor presses the Moran's macromutational switch during the development of a leaf, we can have the mixed morphology.

Finally, some of these morphologies are unusual for these species but represent a morphology that may be normal for other species. Could these sudden changes in development be the origin of other morphologies? Although the gradualism is the prevalent idea concerning Darwinian evolution, there is evidence that profound mutant phenotypes, known as "hopeful monsters", may establish a new evolutionary lineage and may be the start point of adaptive radiations (Theißen, 2009).

The need for new models and interpretations for fern leaves

The key phylogenetic position of ferns in the land plants summed to all of the barriers described in the introduction make any obtained data related to evolution in this group very desirable. The proposition of the very distinct aquatic plants *Azolla* (with very reduced leaves) and *Ceratopteris* (with very divided leaves) as model plants and efforts to sequence their genomes (Sessa *et al.*, 2014) will certainly provide important information concerning the development and evolution of fern leaves. However, their unusual leaf morphologies may rise problems if we try to apply the same processes discovered in these models to other groups. A plural effort should be made to study in

different aspects ferns of different morphologies, as they may reveal the universal and specific mechanisms of development. There is evidence that a fern frond is a fractal system of reiterative development based on similar cells. Even though, there is some diversity in these cells and a big diversity in final morphology. More studies are welcomed to explore how these cells evolved within the ferns and which developmental processes govern them.

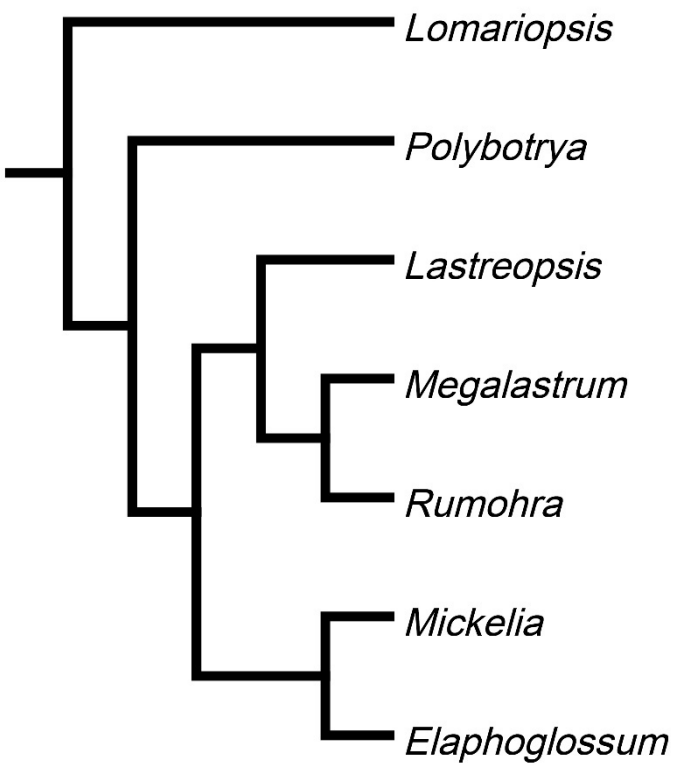


Fig. 1. Relations between the genera of the studied species based on previous phylogenies (Moran et al., 2010a,b; PPG I, 2016).

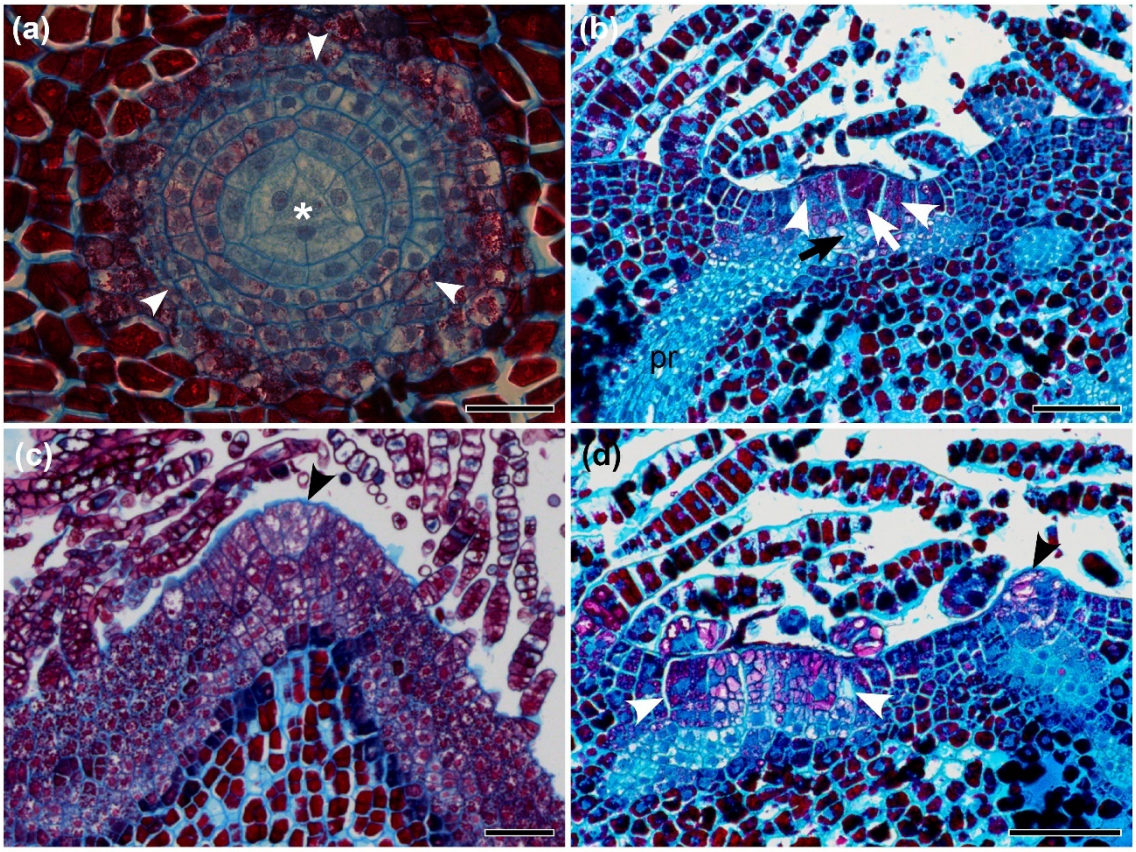
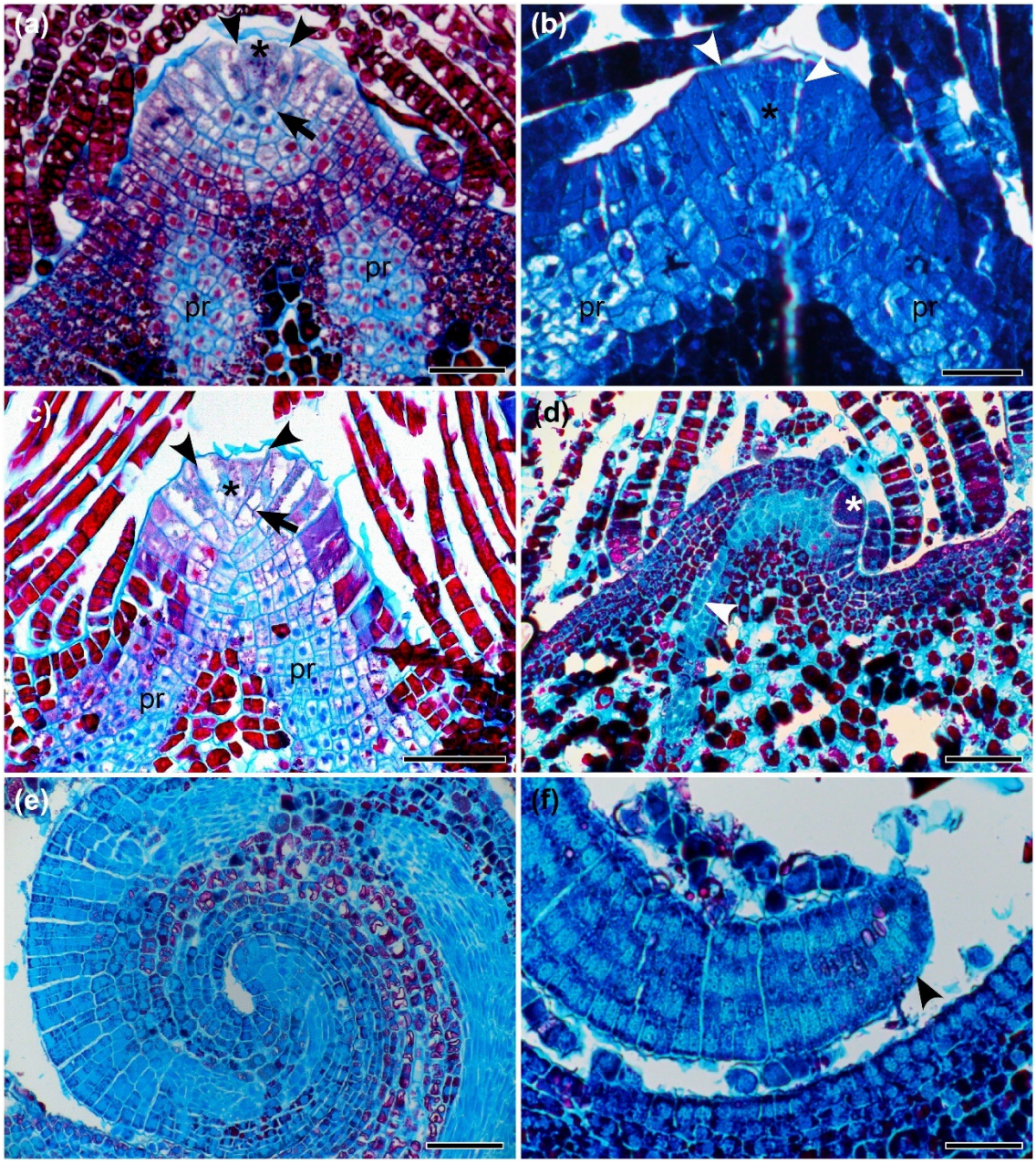


Fig. 2. Shoot apex. **(a)** *Lastreopsis amplissima*, transversal section. Tetrahedral apical cell (*), with three cutting faces, generating three merophytes, with boundaries between them pointed by arrowheads. **(b)** *Elaphoglossum vagans*, longitudinal section. Apical cell (white arrow), surrounded by prismatic cells (arrowheads). Beneath it, smaller meristematic cells (black arrow), associated with a procambial strand (pr). **(c)**. *Lomariopsis marginata*, longitudinal section. Apical cell (arrowhead) and more densely stained derived cells. **(d)** *E. vagans*, longitudinal section. A group of prismatic cells (between two white arrowheads) and a leaf apical cell with an outer lenticular face (black arrowhead). Beneath it, smaller cells with less dense content. **Bars:** a=50µm; b-d=100µm.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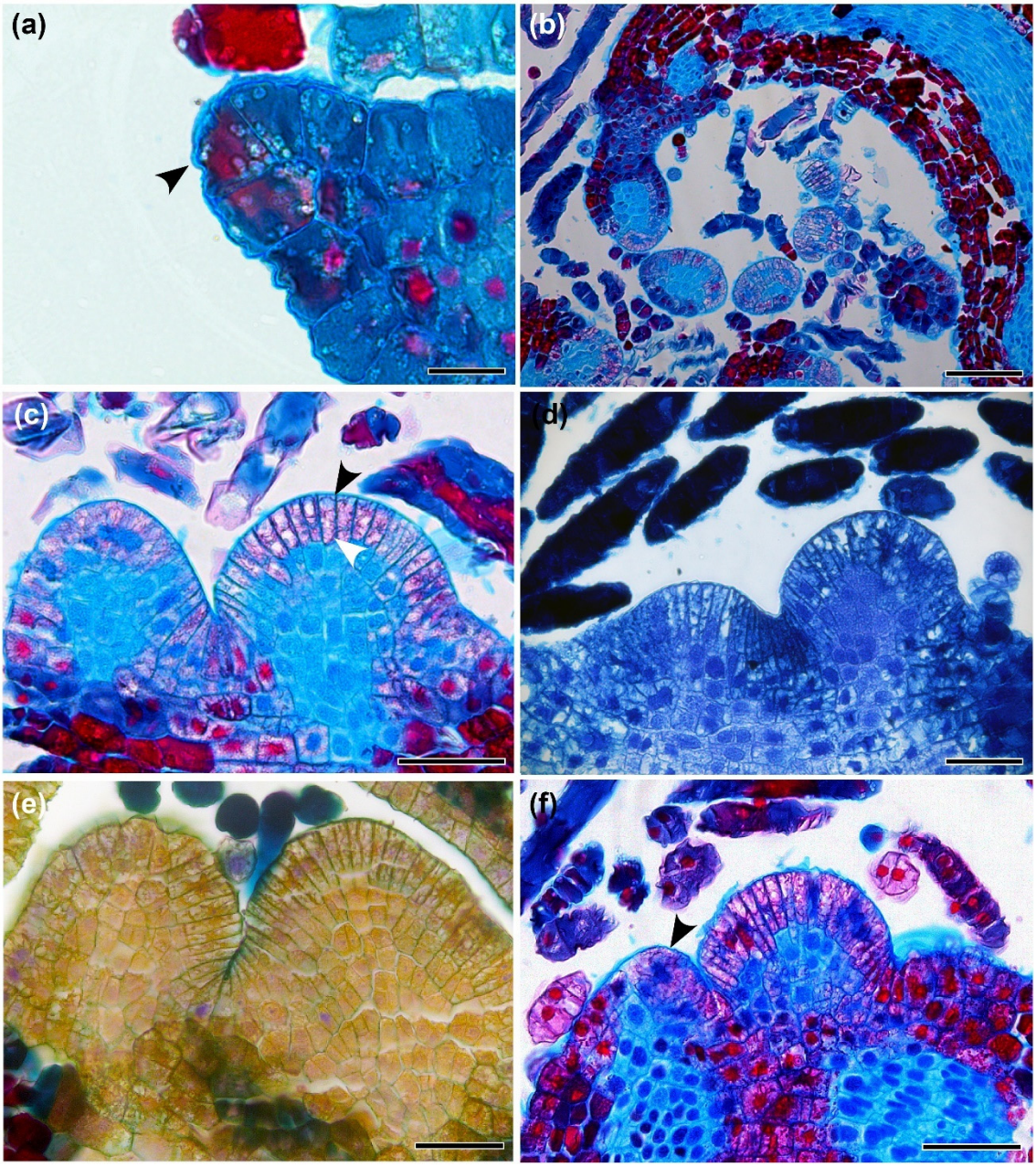
⪡**Fig. 3.** Leaf apex, longitudinal sections. **(a)** *Lomariopsis marginata*, **(b)** *Polybotrya cylindrica* and **(c)** *Lastreopsis amplissima* showing a single apical cell (*) with two cutting faces (arrowheads), forming daughters that are merophytes initials that divide anticlinally and may divide periclinally (arrows). Two procambial strands (pr) are visible in the laterals. **(d-f)** *Elaphoglossum vagans*, sagittal plane. **(d)** Primordium with a distinct apical cell (*) and a procambial strand (arrowhead). **(e)** Bigger primordium coils forming the fiddlehead, thus protecting its apex. **(f)** In a more lateralized plane, in the apical region (arrowhead) anticlinal divisions are present, forming marginal cells. **Bars:** a,c-e=100µm. b,f=50µm.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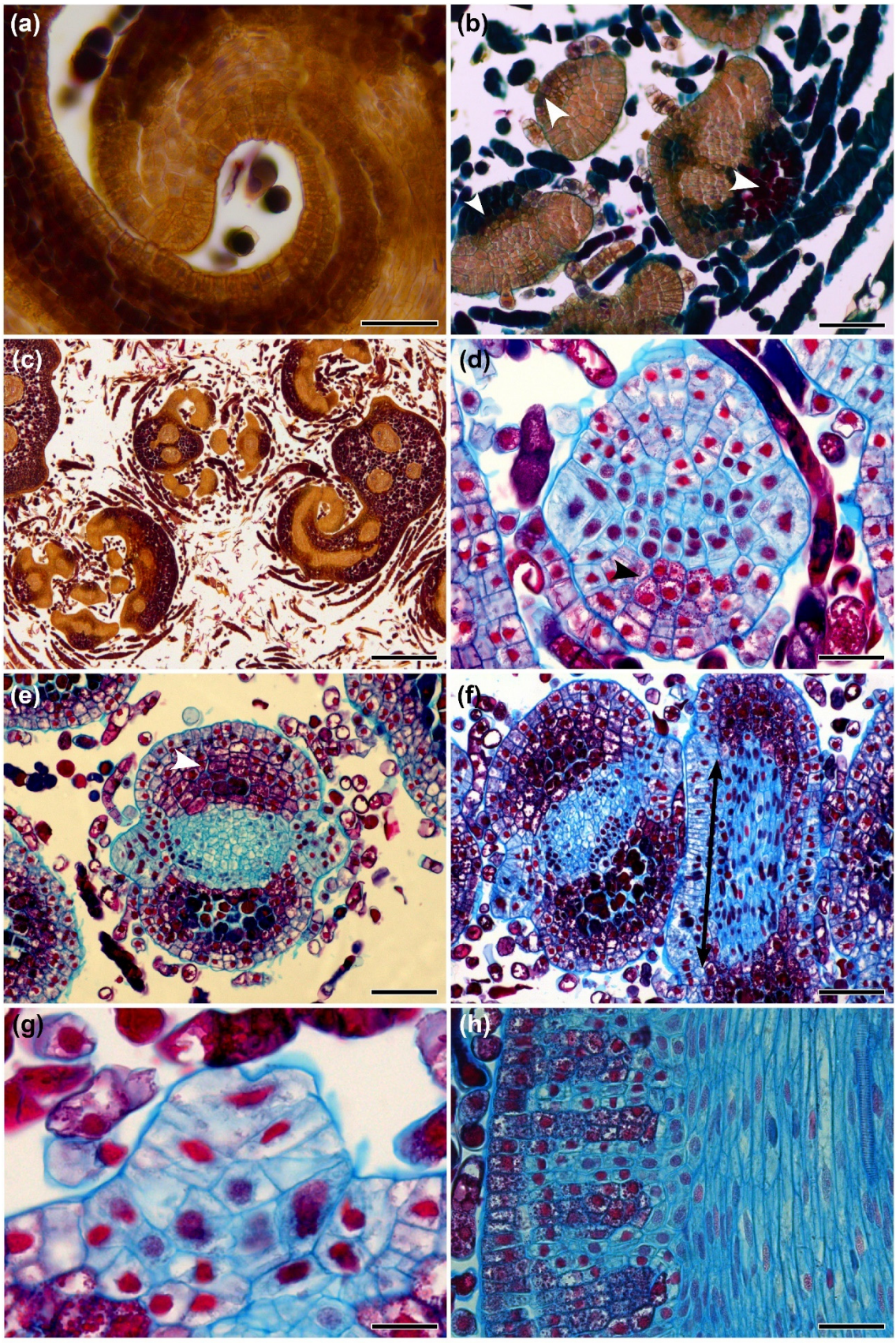
⪡**Fig. 4.** Lateral development. **(a)** *Elaphoglossum vagans*. Transversal section of the frond, showing reduced marginal cell, even in the apex of the frond. **(b-c)** *Lomariopsis marginata*. **(b)** Lateralized sagittal plane of the frond, showing the gradual development of pinnae. **(c)** Two pinnae primordia, in paradermal section, with grouped apical cells. We pointed with arrowheads one anticlinal (black) and one periclinal wall (white). **(d-f)** Similar structures for pinnules in **(d)** *Polybotrya cylindrica*, **(e)** *Rumohra adiantiformis* and **(f)** *Lastreopsis amplissima*. In (f), as the axis is curved, it is possible to obtain a sagittal view of the left pinnule primordium apex (arrowhead). **Bars:** a=20µm. b=100µm. c-f=50µm.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⪡**Fig. 5.** Lateral development. **(a-c)** *Rumohra adiantiformis*. **(a)** Longitudinal section of pinnule apex with a single apical cell. **(b)** Early development of the abaxial region of pinnules in transversal sections evident by the accumulation of phenolic compounds (arrowheads). **(c)** Curved structures, related to the early abaxial development and fiddlehead organization. **(d-h)** *Lomariopsis marginata* developing pinnae. **(d)** Accumulation of phenolic compounds in the abaxial region (black arrowhead) followed by **(e)** accumulation in the adaxial region (white arrowhead). **(f)** Two pinnae primordia. The right one is a paradermal section showing the row of marginal cells (parallel to the black line) in comparison with marginal cells transversally sectioned in the left primordium. **(g)** A detail of the marginal cell and its two cutting faces directed to the abaxial and adaxial regions. **(h)** Paradermal section showing lamina growth through rows of cells. Some of them do not accumulate phenolic compounds and will develop into vascular tissue. **Bars:** a,d,h=50µm. b,e,f=100µm. c=50µm. g=25µm.

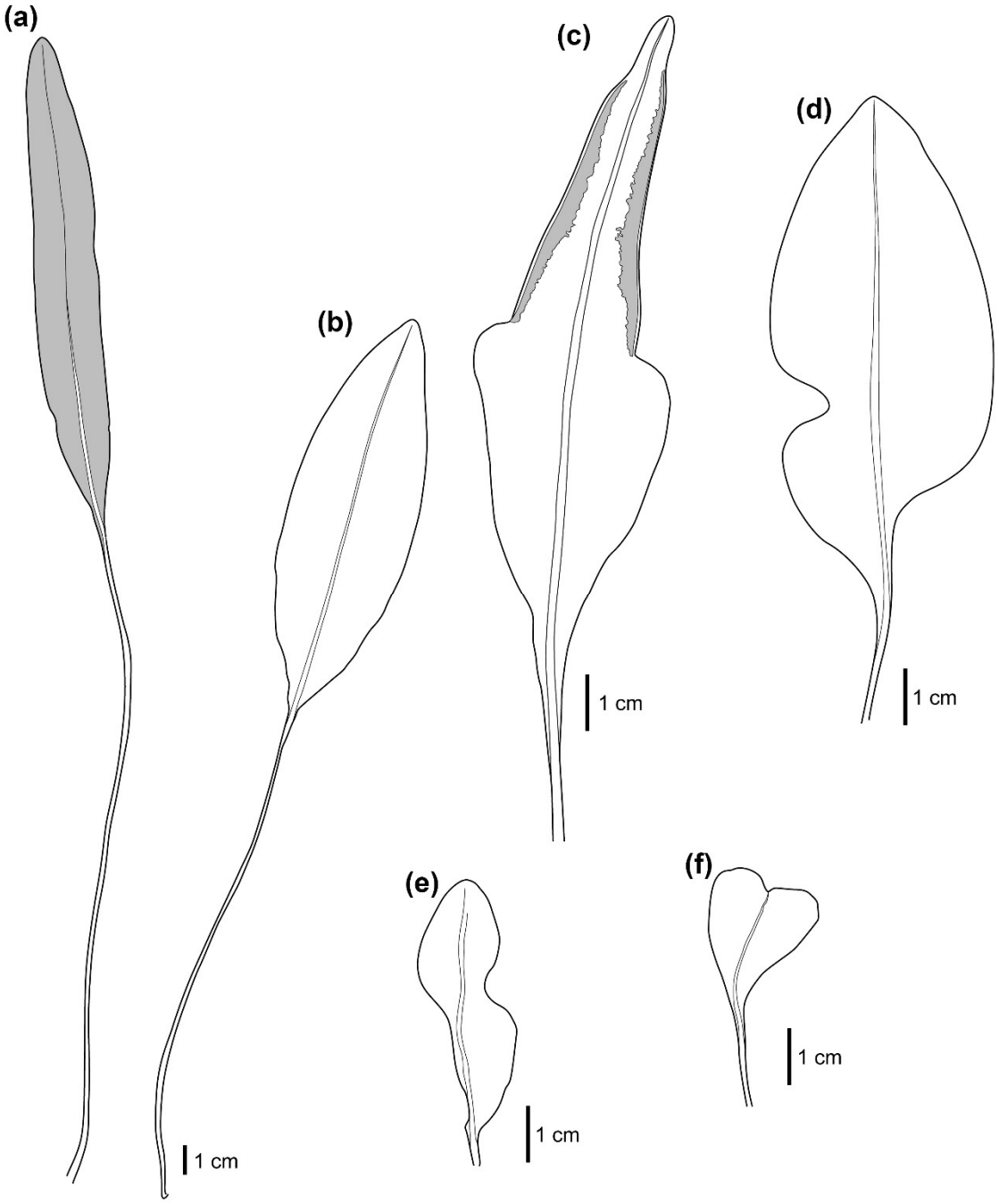


Fig. 6. *Elaphoglossum lingua*. **(a)** Fertile leaf with normal development. **(b)** Sterile leaf with normal development. **(c)** Hemidimorphic leaf with apical part showing fertile features. **(d-e)** Leaves with differential marginal growth. **(f)** Leaf with retuse apex. a-c: *O. Handro 531*; d: *H. Luederwaldt s.n*. (SP 21169); e: *F.C. Hoehne s.n*. (SP 1114); f: *M. Sugiyama 1121*. Illustration by Marcelo Kubo.

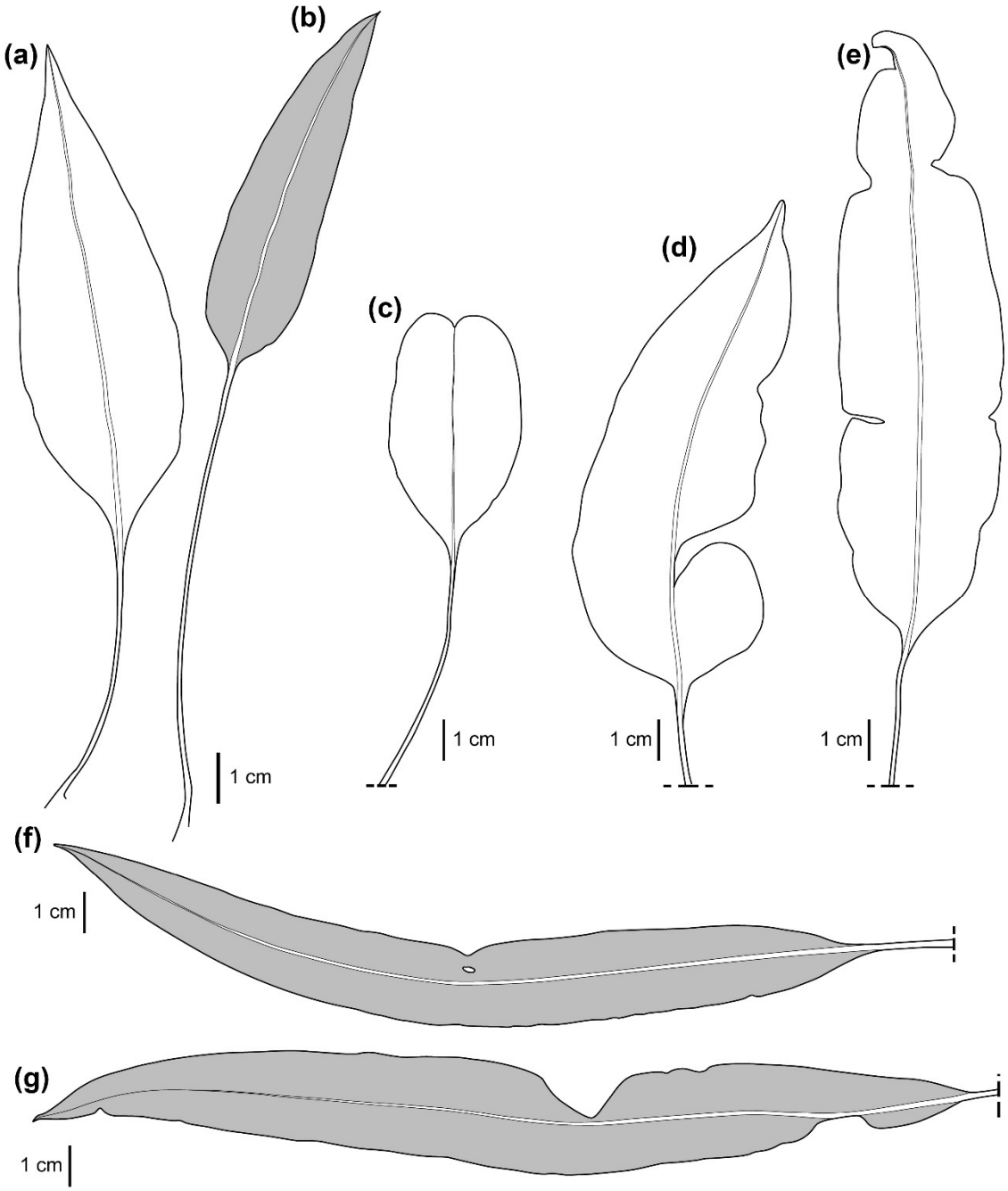
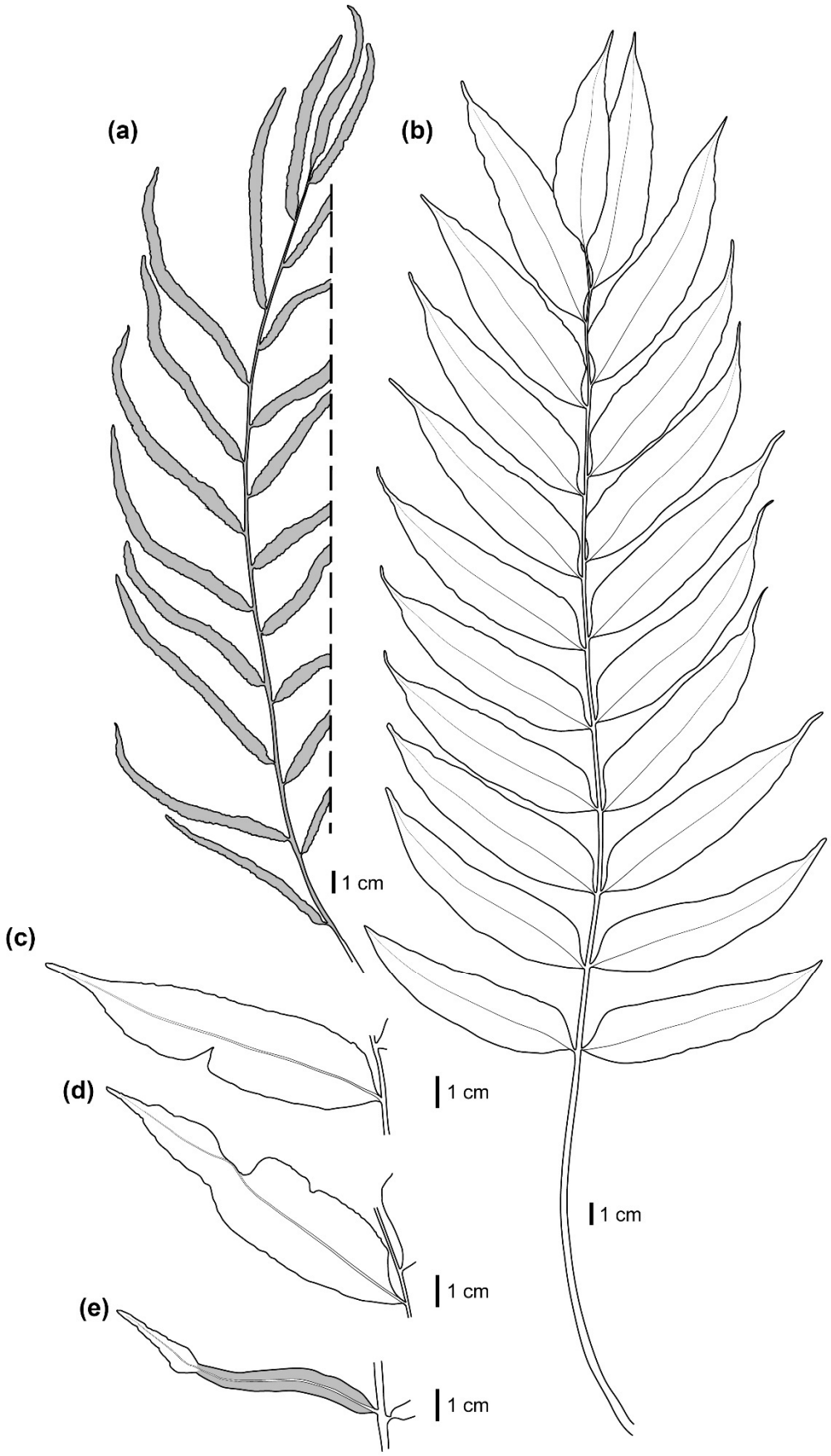


Fig. 7. *Elaphoglossum vagans*. **(a)** Sterile leaf with normal development. **(b)**. Fertile leaf with normal development. **(c)** Leaf with a retuse apex. **(d-e)** Sterile leaves with differential marginal growth. **(f-g)** Fertile leaves with differential marginal growth. a-c: *A. Tosta Silva 405*; d-e: *G.E. Valente 1434*; f: *R.Y. Hire 667;* g: *R.C. Forza 3947*. Illustration by Marcelo Kubo.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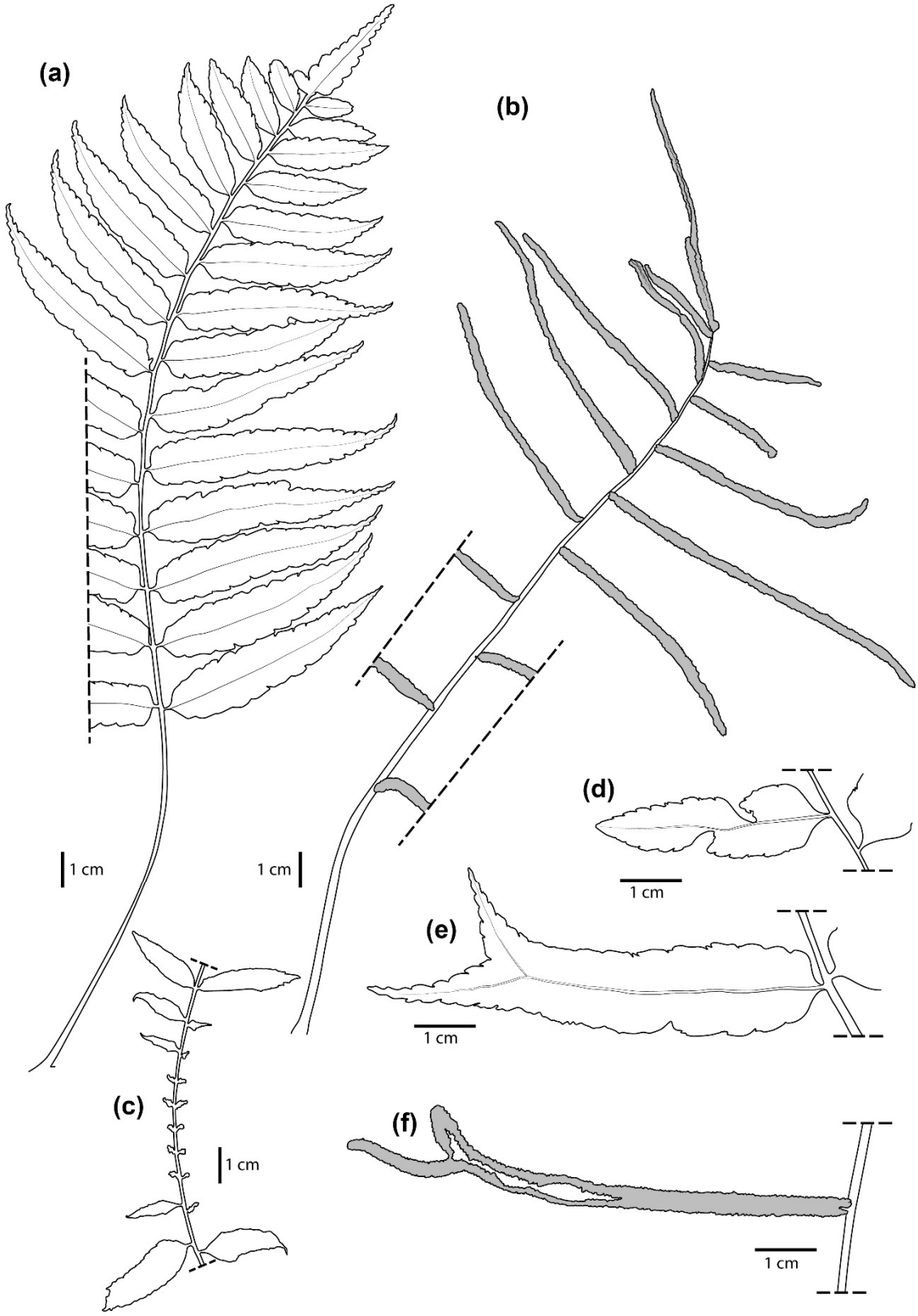
1 cm

⪡**Fig. 8.** *Lomariopsis marginata*. **(a)** Fertile leaf with normal development. **(b)** Sterile leaf with normal development. **(c-d)** Leaves with differential marginal growth. **(e)** Hemidimorphic pinna with a sterile apical part and a fertile basal part. a-b,d: *Prado 972*; c: *H. Luederwaldt s.n.* (SP 21163); e: *W. Hoehne 5606*. Illustration by Marcelo Kubo.


(a)
1 cm
(b)
1 cm
(c)
1 cm
(d)
1 cm
(e)
1 cm
(f)
1 cm

⪡**Fig. 9.** *Mickelia scandens*. **(a)** Sterile leaf with normal development. **(b)** Fertile leaf with normal development. **(c)** Serial atrophied pinnae in a sector of the leaf. **(d)** Pinna with a differential marginal growth. **(e)** Divided sterile pinna. **(f)** Divided fertile pinna. a,e: *M.J.M. Christenhusz 4913*; b,f: *C.A.A. de Freitas 695*; c: *J. Prado 1610*; d: *Schwartsburd* 317. Illustration by Marcelo Kubo.

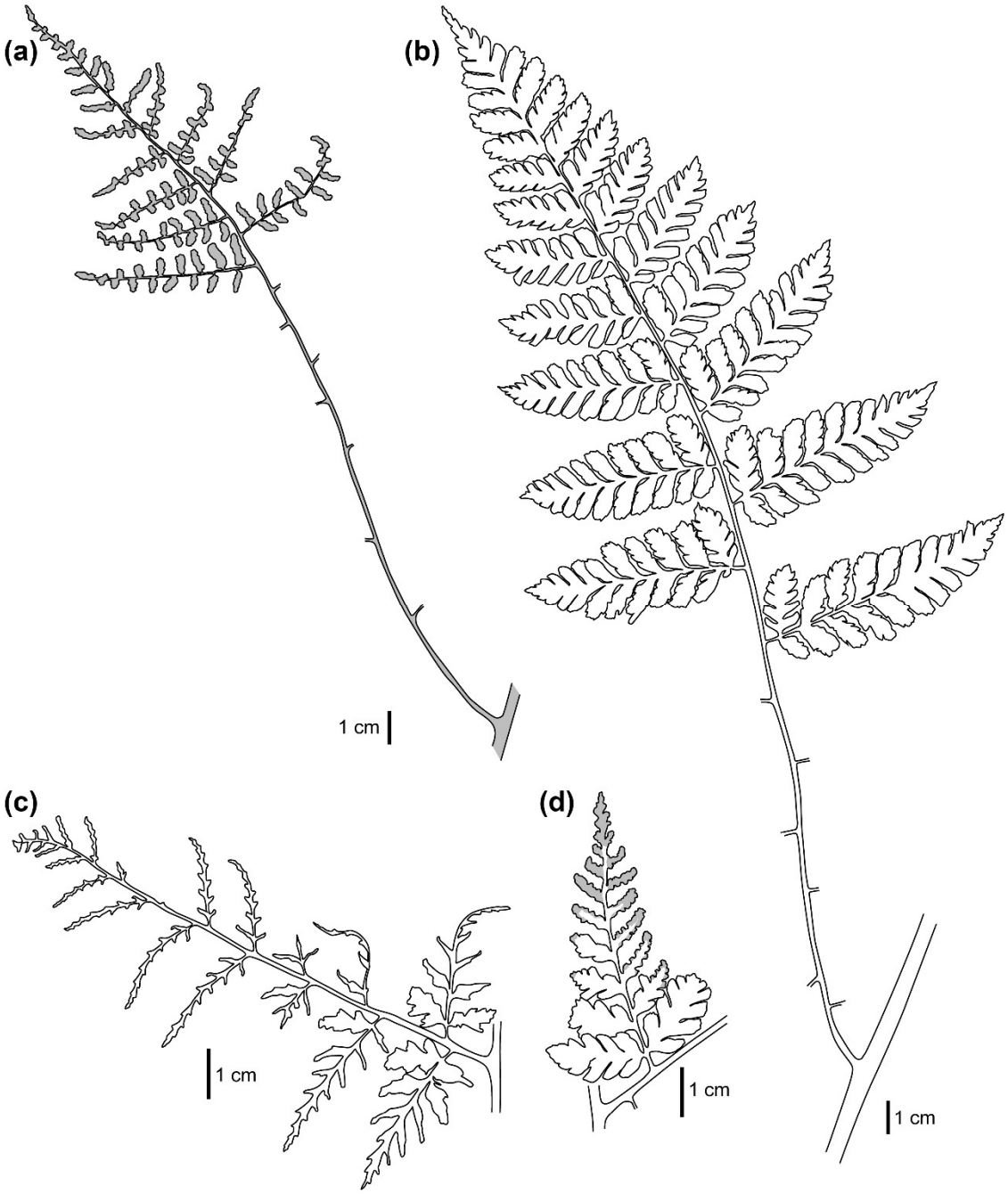


Fig. 10. *Polybotrya cylindrica*. **(a)** Fertile pinna with normal development. **(b)** Sterile pinna with normal development. **(c)** Pinnule with both fertile and sterile features. **(d)** Pinnule with both fertile and sterile features, although not presenting sori. a: *O. Yano 21029*; b: *S.B. Pimentel 5*; c: *H. Luederwaldt s.n.* (SP 21557); d: *M. Wacket s.n.* (SP 21556). Illustration by Marcelo Kubo.

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Final Considerations

Final Considerations

It is impressive how much the humanity, in our very recent and short history, advanced in the knowledge about vascular plants faced to *ca* 400 million years of evolution that culminated with *ca* 375000 extant species. Even though, Botany is an old science full of traditionalism and conservatism. New discoveries, tools, and technics should serve as a trigger for a better interpretation of a large number of morphological structures already described. It is not possible anymore to fit all this diversity into well-defined categories ignoring the existence of developmental processes overlap. Ferns always challenged classical interpretations, due to the presence of very complex leaves with an intriguing development.

The traditional view that shoots are indeterminate organs and leaves are not discussed in the first chapter. We present data about the expression of Class I KNOX genes that may control indeterminacy processes in a heteroblastic fern, showing that they are expressed in leaves and their pinnae. And decreased expression is related to a more determinate morphology. This is an evidence that in some level, fern leaves may be acting as a shoot, as proposed by Agnes Arber in her partial-shoot theory.

Apical and marginal cells were many times described in ferns developing shoots and leaves meristems. Based in our data about some Dryopteridaceae and previous studies, we present a discussion about the geometry and how they may be interconvertible evolutionarily and ontogenetically, being another evidence about the homology between shoots, leaves and their divisions. We also showed that the change in processes that involve these cells may affect the development, creating natural mutants. The fern leaf is a reiterative system, where processes of development may occur in an organ that was

generated by the same processes. Agnes Arber's identity-in-parallel concept is in agreement with this view.

Ferns, occupying a key phylogenetic position and having an intriguing morphology, are possibly the most important source of new information for the understanding of how vegetative structures evolved in land plants history and what are the basic mechanisms underlying their developments. These studies should not be tied to restrictive rules. Instead, we should use a view that consider plant form as product of integrative processes.