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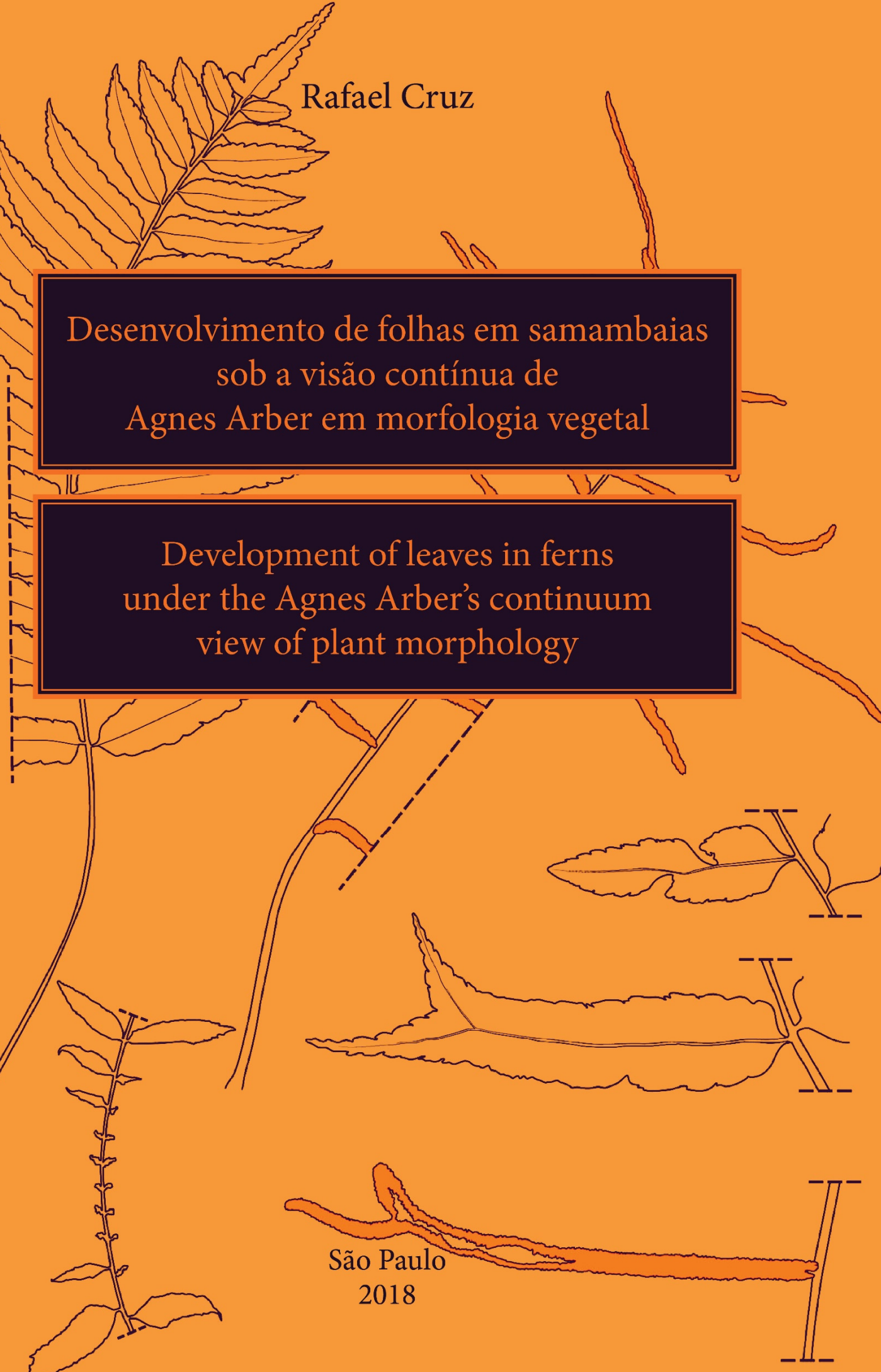
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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo para a obtenção do título de Doutor em Ciências Biológicas, na área de Botânica.

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

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Profa. Dra. Gladys Flávia A. Melo de Pinna
Orientadora

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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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.

Contents

General Introduction

General Introduction

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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Final Considerations

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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.