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FLORAL ANATOMY AND DEVELOPMENT OF SPECIES OF
PHYLLANTHACEAE, PICRODENDRACEAE, EUPHORBIACEAE AND
PANDACEAE

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Floral anatomy and development of species of Phyllanthaceae,
Picrodendraceae, Euphorbiaceae and Pandaceae

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Picrodendraceae, Euphorbiaceae e Pandaceae

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"In the end, we will conserve only what we love; we will love only what we understand; and we will understand only what we are taught."

(Baba Dioum, 1968.)

Dedico

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A Flor do Sonho

*A Flor do Sonho, alvíssima, divina,
Miraculosamente abriu em mim,
Como se uma magnólia de cetim
Fosse florir num muro todo em ruína.*

*Pende em meu seio a haste branda e fina
E não posso entender como é que, enfim,
Essa tão rara flor abriu assim! ...
Milagre ... fantasia ... ou, talvez, sina ...*

*Ó Flor que em mim nasceste sem abrolhos,
Que tem que sejam tristes os meus olhos
Se eles são tristes pelo amor de ti?! ...*

*Desde que em mim nasceste em noite calma,
Voou ao longe a asa da minha'alma
E nunca, nunca mais eu me entendi ...*

Florbela Espanca

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ABSTRACT

Euphorbiaceae *s.l.* are distributed in the most varied types of vegetation and habitat, being one of the biggest, most complex and diversified families in the angiosperms. Its classification was discussed during long time by many authors and with the phylogenetic analyses was proved its polyphyletic origin, bearing the dissolution in six distinct families: Phyllanthaceae, Picrodendraceae, Putranjivaceae, Pandaceae, Peraceae e Euphorbiaceae *s.s.* Considering the floral diversity of these families, four species were selected to this study, aiming to sample the different groups: *Phyllanthus urinaria* (Phyllanthaceae), *Piranhea trifoliata* (Picrodendraceae), *Alchornea sidifolia* (Euphorbiaceae *s.s.*) and *Microdesmis caseariifolia* (Pandaceae). There are few detailed literature about the floral structure of the representants from the allied families of Euphorbiaceae *s.l.*, which makes difficult the accurate usage of the floral characters in studies about systematics and evolution of these groups. Furthermore, information on the functional implications of these characteristics in the biology of the species are limited. In this context, the aim of this work was analyse the floral morphology, with an emphasis on the development patterns, vascularization and secretory structures. For this purpose, floral buds and flowers in different stages of development were fixed, dehydrated, embedded in Paraplast or historesin, sectioned and stained, besides the SEM and TEM analysis that helped on the investigation of the floral ontogenesis and the nectariferous tissue. As main results of the structural analysis, we found that *Phyllanthus urinaria* has sepals and petals, as well *Piranhea trifoliata*, both from the clade Phyllanthaceae + Picrodendraceae. Then, flowers from these groups should be studied from its origin and vascularization to have a better understanding of the evolutionary path of the perianth in these families. *Piranhea trifoliata* also have staminodes that may indicate a transitional process during the evolution of the group. In *Alchornea sidifolia* we found development patterns that explain the formation of characteristics of anemophilous plant, such as the production of more flowers in the male

inflorescences than the more compact and fewer flowered female inflorescences, the presence of unisexual flowers with reduced perianth, absence of nectaries, and female flowers with extensive stigmatic receptive surface. This species also has a peculiar characteristic regarding the number of carpels, which is generally three in the Euphorbiaceae and Malpighiales, even though *A. sidifolia* has two carpels. *Microdesmis caseariifolia* displayed variation in the number of stamens, shown a placental obturator and a pistilode, and all these features are novelties to the Pandaceae. This study adds to the floral knowledge of the different families, as well as enable to raise issues to be addressed in future studies about the structure and evolution of floral characters in Malpighiales.

Keywords: *Alchornea*, floral development, *Microdesmis*, nectaries, *Phyllanthus*, *Piranhea*, SEM, TEM, vascularization.

RESUMO

As Euphorbiaceae *s.l.* distribuem-se nos mais variados tipos de vegetação e habitat, sendo uma das maiores, mais complexas e diversificadas famílias das angiospermas. Sua classificação foi discutida durante muito tempo por diversos autores e, atualmente, as análises filogenéticas, comprovaram seu polifiletismo, dando suporte ao desmembramento em seis famílias distintas: Phyllanthaceae, Picrodendraceae, Putranjivaceae, Pandaceae, Peraceae e Euphorbiaceae *s.s.* Considerando a diversidade floral destas famílias, quatro espécies foram selecionadas para este trabalho, visando amostrar os diferentes grupos: *Phyllanthus urinaria* (Phyllanthaceae), *Piranhea trifoliata* (Picrodendraceae), *Alchornea sidifolia* (Euphorbiaceae *s.s.*) e *Microdesmis caseariifolia* (Pandaceae). Poucos são os estudos detalhados sobre a estrutura floral de representantes das famílias segregadas de Euphorbiaceae *s.l.*, o que dificulta o uso acurado dos caracteres florais em estudos sobre sistemática e evolução dos grupos em questão. Além disso, as informações sobre as implicações funcionais dessas características na biologia das espécies são limitadas. Nesse contexto, o objetivo desse estudo foi analisar a morfologia da floral, com ênfase nos padrões de desenvolvimento, vascularização e estruturas secretoras. Para isso, botões florais e flores em diversos estágios de desenvolvimento foram fixados, desidratados, incluídos em parafina ou historresina, seccionados e corados, além das análises em MEV e MET que auxiliaram na investigação da ontogênese floral e tecido nectarífero. Como principais resultados das análises estruturais, encontramos que *Phyllanthus urinaria* possui sépalas e pétalas, bem como *Piranhea trifoliata*, espécies pertencentes ao clado Phyllanthaceae + Picrodendraceae. Logo, flores desses grupos devem ser analisadas a partir de sua origem e vascularização para que haja um melhor entendimento da trajetória evolutiva do perianto nessas famílias. *Piranhea trifoliata*

também apresentou estaminódios que podem indicar um processo transicional durante a evolução do grupo. Em *Alchornea sidifolia* nós encontramos padrões de desenvolvimento que explicam a formação de características de plantas anemófilas, como a produção de mais flores nas inflorescências masculinas do que nas femininas, flores unissexuais com perianto reduzido, ausência de nectários e flores femininas com uma extensa região estigmática. Essa espécie também apresenta uma característica peculiar em relação ao número de carpelos, que geralmente são três nas Euphorbiaceae e na das Malpighiales, mas *A. sidifolia* apresenta dois carpelos. *Microdesmis caseariifolia* mostrou variação no número de estames, presença de obturador placentário e pistilódio, sendo todas essas características novidades para a família. Este estudo contribuiu com o conhecimento floral das diferentes famílias, bem como permitiu criar hipóteses a serem abordadas em futuros estudos sobre a estrutura e evolução de caracteres florais em Malpighiales.

Palavras-chave: *Alchornea*, desenvolvimento floral, MEV, MET, *Microdesmis*, nectários, *Phyllanthus*, *Piranhea*, vascularização

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General Introduction

1 – From the former Euphorbiaceae *sensu lato* to the new six families

The former Euphorbiaceae *sensu lato* went through several classifications and was studied by many researchers, such as Carolus Linnaeus, Antonii Laurentii De Jussieu, Grady Webster, Alan Radcliffe-Smith and many other researchers that contribute to know Euphorbiaceae today.

Carolus Linnaeus, in the XVIII century, observed for the first time the Tricoccae family, which had a few genera within the order Naturales (Bruyset 1787). This family years after was known as Euphorbiaceae. The name given to the family was in the classification Antonii Laurentii de Jussieu in 1789 in *Genera Plantarum*, Euphorbiaceae had about 30 genera divided in two big groups, one with flowers of an entire style and another with bifid style, and both groups have unisexual flowers and placental obturator.

In XIX, Euphorbiaceae was classified according to the system of Baillon (1858) and it was divided in seven series. Although, in the same century, appeared new systems of classification, since the description and contributions to classification were added to the family. These contributions were mainly in the monographies of the swiss botanist Jean Müller Argoviensis and in the *Prodomus Systematis Naturalis Regni Vegetalis* (De Candolle, AP) of 1866. In the work of Müller, he classified the family based in the Müllerian system, the first one to organize Euphorbiaceae in subfamilies, tribes and subtribes.

Years later, the efforts of Airy Shaw were influential, he recognized seven segregate families: Androstachydeae, Bischofiaceae, Hymenocardiaceae, Peraceae, Picrodendraceae, Stilaginaceae, and Uapacaceae (Airy Shaw, 1965, 1966).

However, the systematics of Euphorbiaceae *s.l.* was greatly advanced by the professor Grady Linder Webster who widely studied the family and gave countless contributions to the classifications of it, as well to the description of taxa in the family.

He assumed Euphorbiaceae had 300 genera and five subfamilies (Phyllanthoideae, Oldfieldioideae, Acalyphoideae, Crotonoideae and Euphorbioideae) spread through the neotropics (Webster 1975). The morphological characters were crucial in the arrangement of these subfamilies, the main ones were the number of ovules per locule, the presence of laticifers and the pollen grain morphology.

The heterogeneity of Euphorbiaceae *s.l.* is known for long time and it reflected in an extensive history of attempts to dismember the family in at least 20 different ones have been proposed (Webster 1987). Corner (1976) and Huber (1991) advocated a split of uniovulate Euphorbiaceae from the biovulate taxa based on seed coat characters.

Webster kept working with the family and in 1994 he published the paper with the synopsis of the genera and suprageneric taxa of Euphorbiaceae, where he states the presence of 317 genera organized in 49 tribes. In this paper, there are some detailed morphological descriptions that strengthened the classification of Euphorbiaceae *s.l.* as monophyletic, although other studies were already proposing another type of circumscription (Webster 1994 a,b).

The number of ovules per locule was one important character to the discussion about the detachment of Euphorbiaceae *s.l.* The exclusion of the subfamilies with biovulated carpels was supported to preserve the monophyly of Euphorbiaceae (Huber 1991, Meeuse 1990)

With the advent of the phylogenetic analyses, Euphorbiaceae *s.l.* were first shown to be potentially polyphyletic by Chase et al. (1993). Since then, various publications have corroborated and refined these results with more taxon sampling (Wurdack and Chase 1996; Fay et al. 1997; Litt & Chase 1999; Savolainen et al. 2000b; Chase et al. 2002, Wurdack *et al.* 2005, Tokuoka and Tobe 2006), so the separation of Euphorbiaceae *s.l.* into five families was accomplished. The subfamilies Phyllanthoideae and

Oldfieldoideae, both biovulated comprised the families Phyllanthaceae, Picrodendraceae and Putranjivaceae (part of Phyllanthoid in Euphorbiaceae *s.l.*), and the uniovulated genus were arranged within Pandaceae (tribe Galearieae in Euphorbiaceae *s.l.*) and Euphorbiaceae (Euphorbioideae, Crotonoideae and Acalyphoideae).

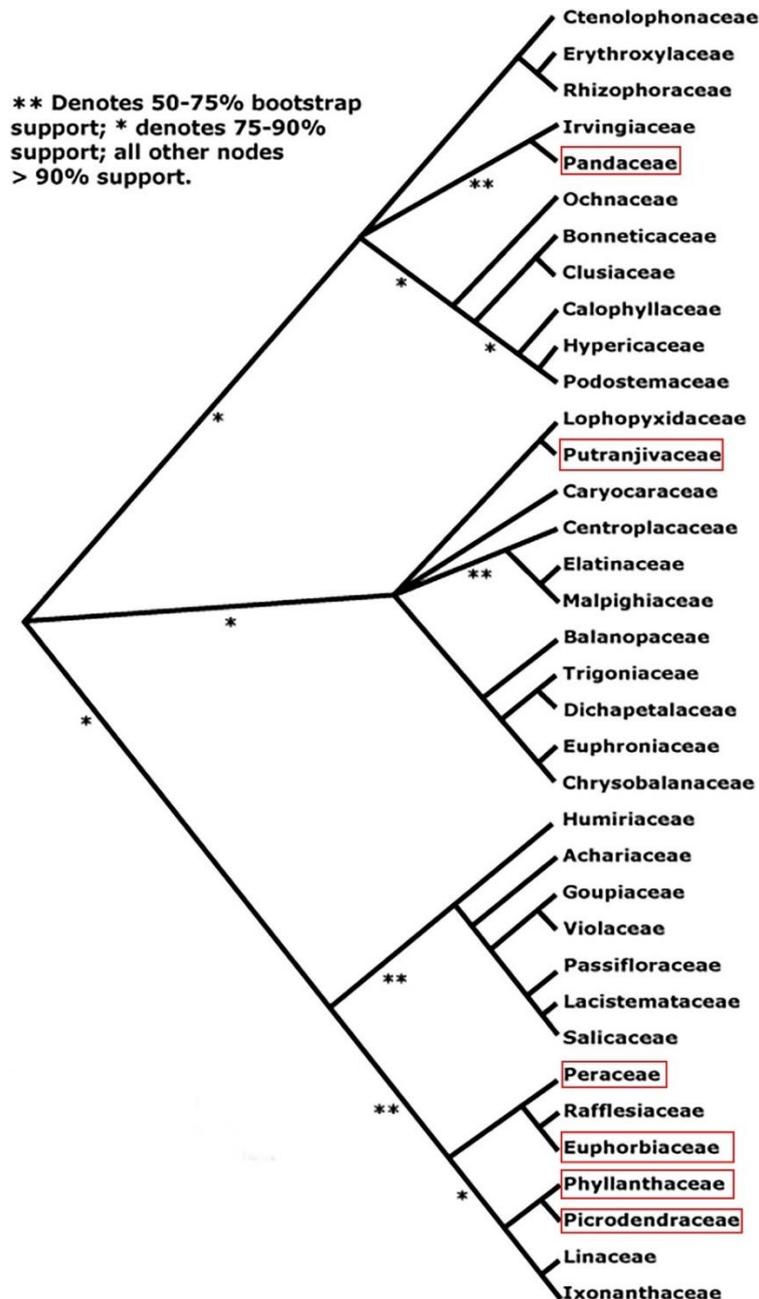


Figure 1: Updated phylogenetic tree of Malpighiales at APweb (Stevens, 2001)

Further studies supported the monophyly of these new families (Wurdack et al. 2005), despite the various questions about the Euphorbiaceae *s.s.* monophyly, which

demonstrate a great morphological variation between its species, such as the features of the ovules development that have an exotegmic palisade layer in species of the whole family, except for Peroideae (Tokuoka 2007). Wurdack and Davis (2009) in their phylogenetic studies have suggested the presence of other modifications beyond the ovules that elevated Peroideae to the family Peraceae. The most recent classification of angiosperms (APG IV 2016) recognize six lineages of Euphorbiaceae *sensu lato* (Figure 1).

Here we studied species of four different families, in order to analyze the floral variations and development pattern to observe the reason why they were together for so long time based in the morphology. However, studying the group we acknowledge that the common characteristics in the flowers of the segregated families also extend to other Malpighiales and some of them, such as Pandaceae and Putranjivaceae are not phylogenetically related to the former Euphorbiaceae *s.l.* anymore, not being possible the comparisons that would be done. Then, the objective of the work kept that was to do the floral development and anatomy of the flowers, but the approach focused in the currently family of each species.

One species of Phyllanthaceae, Picrodendraceae, Euphorbiaceae and Pandaceae were studied and below is presented the specific features for each of these families.

2 – Phyllanthaceae

Phyllanthaceae as previously said is from the subfamily Phyllanthoideae of Euphorbiaceae *s.l.*, excluding *Centroplacus Drypetes*, *Phyllanoa*, *Putranjiva*, *Sibangea*, but including *Croizatia*, *Dicoelia*, *Tacarcuna* sensu Webster (1994b) and Radcliffe-Smith (2001). The major reason why several authors encouraged splitting Euphorbiaceae *s.l.*

was the seed coat anatomy (Corner 1976, Meeuse 1990, Huber 1991) and in the recent studies by Tokuoka and Tobe (1999, 2001) the distinctiveness of the newly recognised APG families with regards to seed coat anatomy was confirmed, especially the differences between Phyllanthaceae, Putranjivaceae and Euphorbiaceae *s.s.*

The new family has approximately 2000 species in about 60 accepted genera. It is a pantropical family of trees, shrubs, semi-succulents, annual herbs, and even a free-floating aquatic species (Savolainen et al. 2000, Chase et al. 2002, Hoffman et al 2006, Stevens 2001). Most Phyllanthaceae are uniform with mainly alternate, simple, glandular leaves with pinnate venation, entire margins, and a simple indumentum and do not have latex.

The inflorescences are cymose, with diclinous, small and actinomorphic flowers, although having a great diversity in shape, size and number of floral organs (Hoffman et al. 2006). In general, the flowers have nectariferous disc, the gynoecium is 3-carpellary, rare 4-carpellary, it is 3(4)-locular with two ovules per locule, the fruits are capsules and the seeds do not have caruncule (Hoffman et al. 2006, Kathriarachch et al. 2006, Silva and Sales 2007). According to Webster (1994b) the ovules are anatropous with an obturator.

3 – Picrodendraceae

Picrodendraceae was the formely Oldfieldoideae in Euphorbiaceae *s.l.*, except for Croizatiaeae and Paradrypetes sensu Webster (1994b) and Radcliffe-Smith (2001) (APG II 2003). The family is mostly tropical and comprises trees or shrubs (Webster and Berry 1999, Radcliffe-Smith 2001, Stevens 2001). It has about 21 genera and 100 species. Picrodendraceae is poorly known morphologically and is one of the lesser investigated

between the segregated families of Euphorbiaceae *s.l.* (Merino Sutter *et al.* 2006, Stevens 2001). Phyllanthaceae and Picrodendraceae, the Phyllanthoids are strongly supported as a sister taxa, they share characters, such as the entire margins of the lamina; small flowers, anthers extrorse (introrse), bistomal micropyle and presence of obturator and nucellar beak (Stevens 2001). The related publications with Picrodendraceae are mainly concentrate on the structure or taxonomy of single species or genera (e.g. Berg 1975, Hakki 1985, Forster 1995, 1997a, b, Dahlgren and van Wyk 1988, Forster and van Welzen 1999).

Merino Sutter *et al.* (2006) observed the flowers are monoclamydeous with a trimerous perianth with two whorls, less often tetramerous with one or two whorls of sepals. The gynoecium is trimerous in 17 genera, dimerous in two genera, and more variable 2–3-, (3–4-), or (3–5-)merous in eight genera (Radcliffe-Smith 2001, Merino Sutter *et al.* 2006). The ovary is generally surrounded by a nectariferous disc or disc segments (Radcliffe-Smith 2001), has two ovules per carpel that are bitegmic, crassinucellar, anatropous or hemitropous, and epitropous (Webster 1994b, Radcliffe-Smith 2001, Merino Sutter *et al.* 2006).

4 – Euphorbiaceae

Euphorbiaceae *s.s.* is the largest component of Euphorbiaceae *s.l.* and remains as one of the largest plant families even in the recent circumscription (Wurdack *et al.* 2005). The phylogenetic studies of the family stand on the work of Webster (1975, 1994b) and coincidentally agreed with that of the uniovulate subfamilies Acalyphoideae, Crotonoideae, and Euphorbioideae (Wurdack *et al.* 2005). The family comprises Acalyphoideae (excluding Dicoeliae and Galearia), Crotonoideae and Euphorbioideae *sensu* Webster (1994) and Radcliffe-Smith (2001) and Cheilosoideae, that was elevated

to subfamily in the work of Wurdack *et al.* (2005). It has a pantropical distribution and has about 245 genera and 6300 species (Govaerts *et al.*, 2000; Radcliffe-Smith, 2001; Wurdack *et al.*, 2005). The species occur in the most variable type of vegetation in diverse growth form, such as trees, shrubs, subshrubs and herbs (Barroso *et al.*, 1991; Sousa and Lorenzi, 2006).

Among Malpighiales families, Euphorbiaceae *s.s.* are incomparable in species richness, morphological and phytochemical diversity, and economic importance. The distinguished economic products include cassava (*Manihot esculenta*), rubber (*Hevea brasiliensis*), castor (*Ricinus communis*) and tung oils (*Vernicia* spp.), candilla wax (*Euphorbia* spp.), and ornamental poinsettias (*Euphorbia pulcherrima*) (Wurdack *et al.* 2005) and allied to all this importance there are, consequently more studies.

This family is characterized by having terminal or axillary, basically cymose; flowers solitary or in glomerules, these often grouped into spiciform or capitate thyrses or sometimes very reduced (pseudanthia) (Webster 1994b). The flowers are generally not colorful, embraced by bracts, actinomorphic, achlamydeous, monochlamydeous and rare dichlamydeous; they are in monoecious or dioecious plants. The androecium has one or many stamens free or connate, anthers with rimose dehiscence, rare poricide and may present nectariferous disc. The gynoecium is tricarpellary, with uniovulated locules (Webster 1994b; Radcliffe-Smith 2001).

Such as the floral morphology, there is also a diversity of pollinators and pollination syndromes in Euphorbiaceae. Some species are pollinated by wind (Daumann 1972; Korbecka *et al.* 2011), other by insects (Fiala *et al.* 2011), bats (Steiner 1982) and even hummingbirds (Webster 1994a). Euphorbiaceae are mostly entomophilous (Webster 1994a), although the subfamily Acalyphoideae stands out for having a considerable number of wind-pollinated species.

5– Pandaceae

Pandaceae is the most peculiar family segregated from Euphorbiaceae *s.l.* and probably because of this, many researchers suggested it should be a separated family. According to Radcliffe-Smith (1987) it is one of the small taxa that has been changed to inside and outside the Euphorbiaceae *s.l.*, and the first one to realize that this group should be separated was Forman (1966), he observed that the genus *Panda* resembled *Microdesmis* and especially *Galearia*, and only the latter two were combined in the tribe Galearieae by Bentham (1880). Engler and Gilg (1912) even introduced the order Pandales with as sole family the Pandaceae. Takhtajan (1980) and Cronquist (1981) also recognized Pandaceae as a distinct family. However, it was generally stated as part of the Euphorbiaceae *s.l.* (Webster 1994; Radcliffe Smith 2001), and classified in the subfamily Acalyphoideae, tribe Galearieae. Webster (1975), for a while, maintained it as a family-level segregate within Euphorbiales, but he changed his opinion and kept it in the subfamily Acalyphoideae, tribe Galearieae (Webster 1987).

Currently, Pandaceae is not related to Euphorbiaceae and the other segregates families (Chase et al. 2002, APG II 2003), it is sister group of Irvingiaceae (Stevens 2001) and comprises 16 species shared in three genera, *Panda*, *Galearia* and *Microdesmis*. The monotypic *Panda* occurs in tropical West Africa, and *Galearia* and *Microdesmis* are found in South East Asia and West Africa (van Welzen 2011).

Pandaceae are generally difficult to identify, it lacks simple diagnostic characters and is identified via the generic level, since *Galearia* has long terminal or cauliflorous racemes and *Microdesmis* has petaloid flowers in fascicles are normally easily recognized (van Welzen 2011). This family that is the most different from Euphorbiaceae *s.l.* have

the perianth is pentamerous and petals are present, lacks floral nectariferous disc, the gynoecium may variate between 2–5 merous, orthotropous ovules and the vascular bundle not extend beyond the chalaza (Webster 1994b, Tokuoka and Tobe 2003, Merino Sutter *et al.* 2006).

6 – General structure of the thesis

In order to know more about the flower morphology of these different families, mainly the ones that are scarcely studied, such as Picrodendraceae and Pandaceae, this PhD dissertation was proposed.

The thesis is composed of this general introduction, followed by four chapters that are in format of manuscript, and final considerations. The layout of each chapter is in accordance to the journal that we intend to submit. In general terms, the chapters contain the following information:

In the **first chapter**, we carried out an analysis of the floral structure and ontogeny of *Phyllanthus urinaria* focusing on the perianth. With the data obtained we related the characters with the currently phylogeny for the group and stand out the presence of petals in *Phyllanthus*, due the direction of formation of the different whorls in the perianth, the origin of the nectary and the vasculature. This paper was published in the International Journal of Plant Sciences.

The **second chapter** consist in the study of the floral morphology of *Piranhea trifoliata*, a native species of Brazil. The aim of this study was to investigate the structure of staminate and pistillate flowers of *Piranhea trifoliata*, focusing on the perianth, presence of staminodes and appendages in the ovule, which generate a mistaken

description to the genera until now. We intend to submit this paper to the Botanical Journal of the Linnean Society.

In the **third chapter**, we investigated the ontogeny, anatomy and vasculature of flowers of *Alchornea sidifolia* Müll. Arg. in order to find structural features that allow wind pollination in the species, since the morphology of the inflorescence and flowers show characteristics of anemophilous syndrome and the subfamily Acalyphoideae have few species that have this type of pollination. This manuscript was submitted to the Botany.

The **fourth chapter** show for the first time the anatomy of staminate and pistillate flowers of Pandaceae. The species studied was *Microdesmis casearifolia* from Southeast Asia, that have interesting characters and variations, such as the eventually presence of nine stamens instead ten and a pistillode, this latter structure is not found in species from the currently clade of Pandaceae that comprises eight families, suggesting a discussion about this character evolution. We intend to submit this article to the Plant Systematics and Evolution.

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Chapter I

Floral structure and development reveal presence of petals in *Phyllanthus* L.

(Phyllanthaceae)

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ABSTRACT

Premise of the Research: *Phyllanthus* is a pantropical genus and the largest in Phyllanthaceae with a wide diversity of habits and floral morphologies. The flowers are considered monochlamydeous, but some divergences are found in the literature. The aim of this study was to investigate the floral structure of *Phyllanthus urinaria* based on ontogenetic, structural, and vascular analyses.

Methodology: Flowers and buds of *P. urinaria* were embedded in Paraplast and sectioned using a rotatory microtome for analysis under LM. SEM was performed for additional structural and ontogenetic data.

Pivotal Results: The primordia of the sepals are the first structures to be formed in a counterclockwise direction, followed by the petals, which arise in a clockwise direction. Nectaries are formed on the base of the corolla in the staminate and pistillate flowers; then, the two perianth whorls merge, fusing at the base of the adult flowers. The three stamens have filaments postgenitally fused and free anthers. The ovary has three carpels congenitally connate with two ovules hemitropous with one long nucellar beak per locule and styles partially fused.

Conclusions: Our results demonstrate for the first time the petaliferous nature of the inner whorl of the perianth in *Phyllanthus*, based on ontogenetic and anatomical evidence. The presence of a perianth with sepals and petals is a possible synapomorphy of the clade formed by *Phyllanthus* sect. *Embllica* + *Phyllanthus* sect. *Urinaria* and *Breynia*, *Glochidion*, and *Sauropus*, now under *Phyllanthus* in the current phylogenetic circumscription of the tribe Phyllantheae.

INTRODUCTION

Phyllanthaceae is a pantropical family of trees, shrubs, and herbs with ca. 60 genera and 2000 species (Hoffmann et al. 2006). This family was long considered a subfamily of Euphorbiaceae sensu lato (Webster 1987, 1994a, 1994b; Radcliffe-Smith 2001) but was elevated to family level after molecular data indicated that Euphorbiaceae sensu lato was not monophyletic (Wurdack et al. 2004; Samuel et al. 2005; Hoffmann et al. 2006; Kathriarachchi et al. 2006). This family is well known for its potential pharmacological and medicinal uses for several health problems, such as diarrhea, dysentery, urinogenital disorders, and kidney problems (Ramasamy et al. 2011; Kuttan and Harikumar 2012; Linhares et al. 2015).

Phyllanthaceae share a combination of floral characters with other families of Malpighiales (e.g., Picrodendraceae and Euphorbiaceae), such as monochlamydeous flowers, threecarpellary ovaries, and ovules with a nucellar beak (Merino Sutter et al. 2006). Within Phyllanthaceae, *Phyllanthus* L. is the largest genus of the family with a pantropical occurrence (Hoffmann et al. 2006; Kathriarachchi et al. 2006) and a wide diversity of growth form (Webster 1994a), floral and pollen morphology (Punt 1962; Bancilhon 1971; Webster 1994a), and chromosome number (Webster and Ellis 1962). Molecular analyses have been performed in Phyllanthaceae and tribe Phyllantheae (Wurdack et al. 2004; Kathriarachchi et al. 2006) and led to a recircumscription of *Phyllanthus*. Hoffman et al. (2006) included *Breyenia* J. R. & G. Forst., *Glochidion* J. R. & G. Forst., and *Sauropus* Blume under *Phyllanthus*.

Phyllanthus in its current circumscription is a genus with ca. 1270 species (Kathriarachchi et al. 2006), notable for having a diverse morphology of the perianth, which is regarded as monochlamydeous (Radcliff-Smith 2001). The only study of the floral development of *Phyllanthus* was done by Zhang et al. (2012); it focused on the

androecium and gynoecium of *Phyllanthus chekiangensis* Croizat & F.P. Metcalf, a species from China. Among other aspects, analyses of morphological and anatomical characters are essential to find possible synapomorphies in phylogenies based on molecular data, then based on the phylogeny of the tribe Phyllantheae (Kathriarachchi et al. 2006). We decided to study one of the species from the clade *Phyllanthus* sect. *Emblica* + *Phyllanthus* sect. *Urinaria* that includes species with six perianth parts.

Phyllanthus urinaria L. is in the phyllanthoid branching group recognized by Kathriarachchi et al. (2006) in their phylogeny. This weed species has a pantropical distribution (Webster 1957, 1997) and is part of the section *Urinaria* Webster. The species is characterized by having six organs in the perianth of the staminate and pistillate flowers, disk segmented in the staminate and patelliform in the pistillate, four-colporate pollen, three stamens with vertically dehiscent anthers, and spheroidal ovary (Webster 1957). The aim of this study was to investigate the floral structure of *P. urinaria* based on ontogenetic, structural, and vascular analyses focusing on the perianth.

MATERIAL AND METHODS

Inflorescences and flowers in several stages of development were collected at the Área de Proteção Ecológica do Rio Guamá (APEG) in Belém, Brazil, weedy population. Voucher specimens were provided, and exsiccates were deposited in the herbarium of Pará State University: *Phyllanthus urinaria* L. = T.S.S.Gama-6 (voucher: MFS 005833).

Flowers in different stages of development (i.e., meristems; flower buds; and preanthetic, anthetic, and postanthetic flowers) were isolated, fixed in FAA (formalin, acetic acid, 50% ethyl alcohol) for 24 h (Johansen 1940) and NBF (neutralbuffered formalin) for 48 h (Lillie 1965), and subsequently stored in 70% ethyl alcohol. For anatomical studies, the material was dehydrated in a tert-butyl alcohol series (Johansen

1940), embedded in Paraplast (Leica Microsystems, Heidelberg, Germany), and transversely and longitudinally sectioned with 12 μm of thickness by a Leica RM2145 rotary microtome (Leica Microsystems, Wetzlar, Germany). The sections were stained with 1% safranin in ethanol 50% and 1% astra blue (Gerlach 1984), and the slides with the sections were mounted in Permount resin (Fisher Scientific, Pittsburgh, PA). Photomicrographs were obtained using a Leica DMLB LM (Leica Microsystems) using the image-archiving software Scan System Images (IM50).

For the ontogenetic study, micromorphological analyses were also performed using material fixed in FAA. After the isolation of floral parts, this material was dehydrated in an increasing ethanol series up to 100%, critical-point dried in a Balzers CPD 030 critical-point dryer (Balzers, Liechtenstein, Germany), mounted on aluminum stubs, sputter-coated with gold using a Balzers SCD 050 sputter-coater, and examined using a Zeiss DSM 940 SEM (Zeiss, Oberkochen, Germany).

Photographs were processed using Adobe Photoshop and Illustrator software (Redwood City, CA). Vasculature drawings were prepared from digital photographs using Adobe Illustrator software.

RESULTS

Inflorescences

Phyllanthus urinaria L. presents phyllanthoid branching (Webster 1956) with cymose and glomerular inflorescences located on the axil of the leaves on the proximal or distal region of the branch. The flowers are diclinous and arranged in unisexual inflorescences.

The first inflorescences to develop are in the proximal region of the branch and the last ones in the distal region. Inflorescences may also occur in the terminal region of

the branch. Generally, the pistillate inflorescences are proximal and the staminate are distal; however, some variations may occur. For example, there may be pistillate inflorescences along the entire branch, or the pistillate inflorescences may be proximal and the staminate distal but the flower at the terminal region is pistillate. The staminate inflorescence is a cymule with two or three flowers (fig. 1*a*) subtended by one bract and two bracteoles, while pistillate inflorescences are uniflorous with one bract and two bracteoles (fig. 1*b*).

Flowers

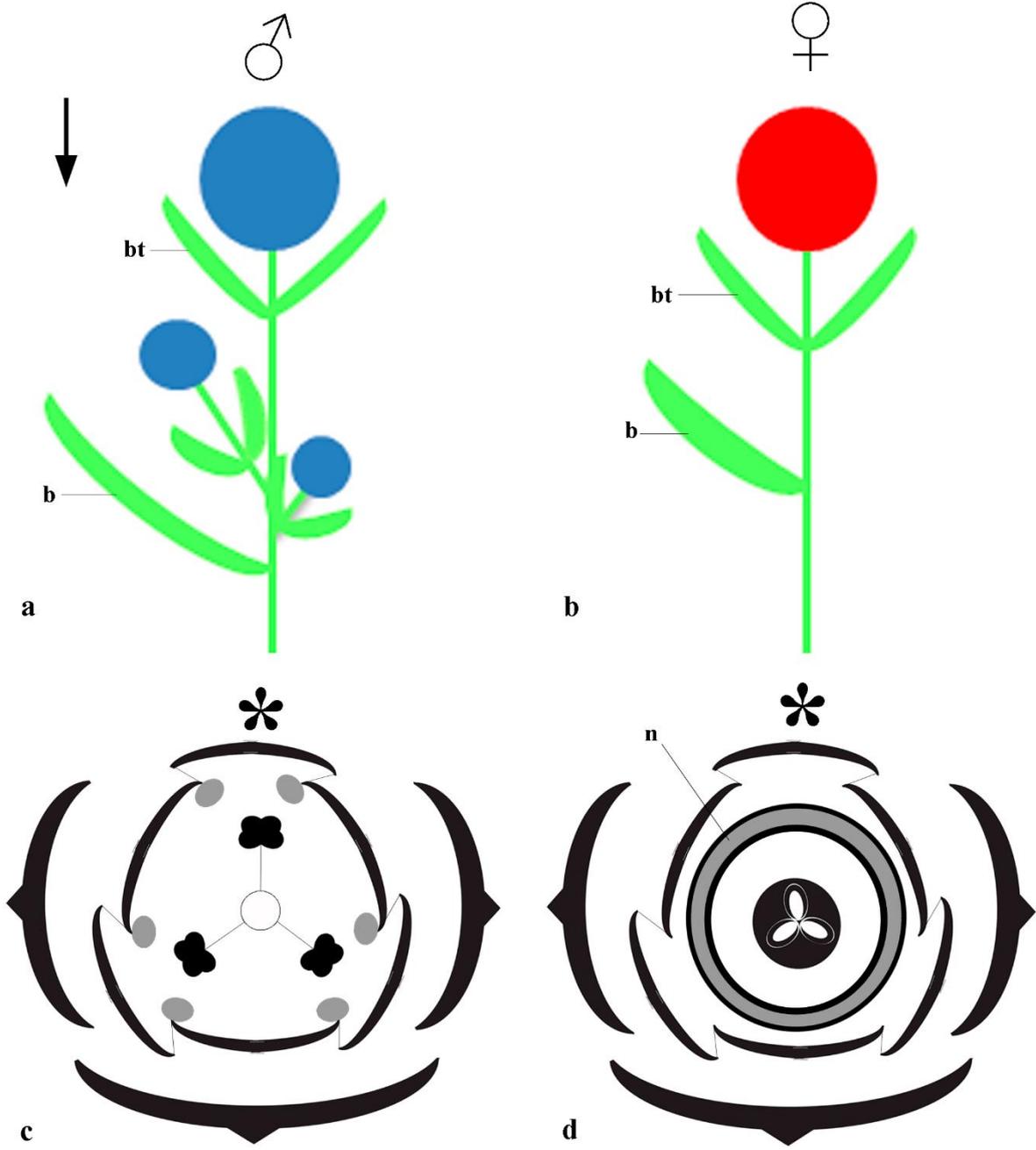
The pistillate and staminate flowers are tiny, measuring approximately 1–1.5 mm length. The flowers are trimerous, homochlamydeous, with two whorls, arranged in an imbricate aestivation (fig. 1*c*, 1*d*); the organs are similar in size and color and are recognized here as sepals in the outer whorl and petals in the inner whorl, which are slightly adnate at the base (fig. 2*a*, 2*b*).

Staminate flowers

Floral structure

The staminate flower has a fused perianth with a lobed nectary (Fig.*a*), and three monadelphous stamens (Fig. 2*c*), subtended by a short pedicel (Fig. 2*d*). All six perianth organs have the same size and are formed by a uniseriate epidermis, composed of small cells with thin walls; the mesophyll is homogeneous with two to three layers of parenchymatous cells and some idioblasts containing druses and colateral bundles (Fig. 2*b*).

Figure 1: Schematic view of *Phyllanthus urinaria* inflorescences and the floral diagrams of flowers. A, staminate cymule with three flowers. B, uniflorous pistillate cymule. C, staminate flower diagram. D, pistillate flower diagram. b, bract. bt, bracteole. n, nectary.



The staminate flower has six small extrastaminal nectaries (fig. 2e), which are located in the lateral basal region of the petals, with two nectaries per petal (fig. 2a); the nectaries are slightly fleshy with a thickened, irregular shape. The nectaries are nonvascularized and have only secretory parenchyma, releasing the nectar through stomata located on the gland apex.

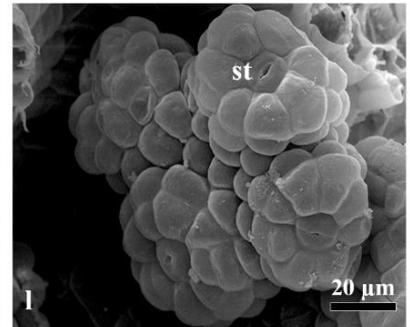
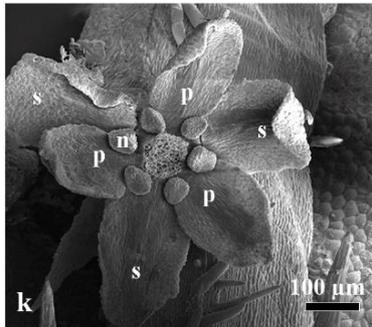
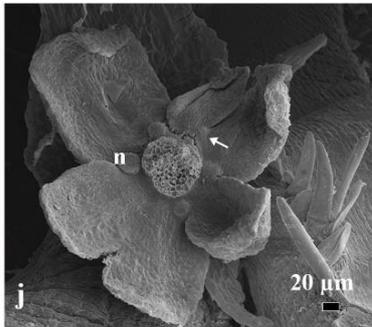
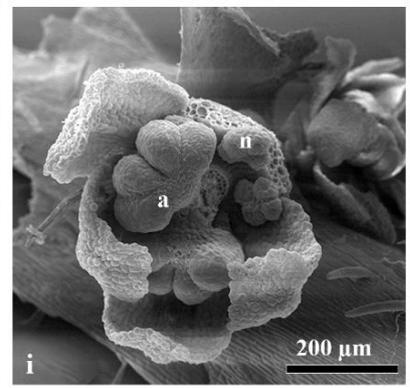
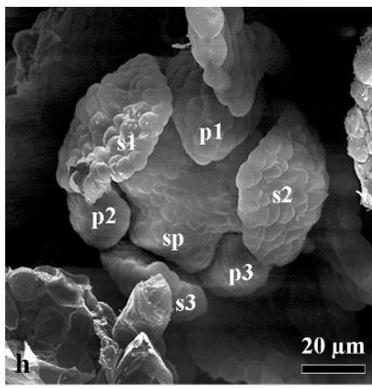
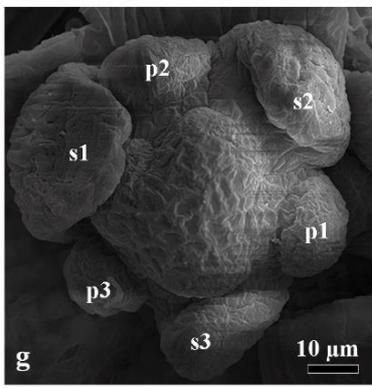
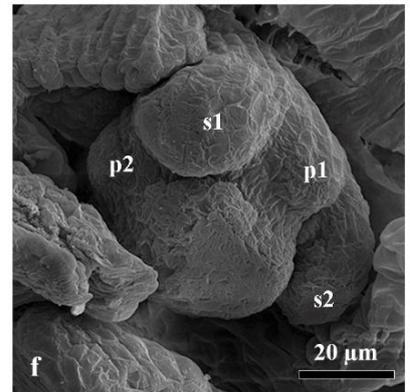
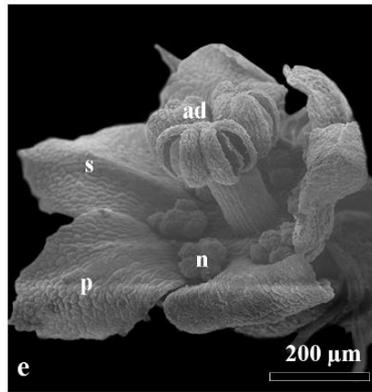
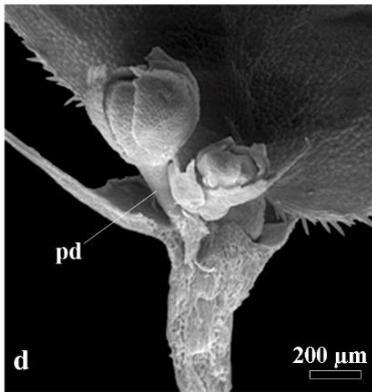
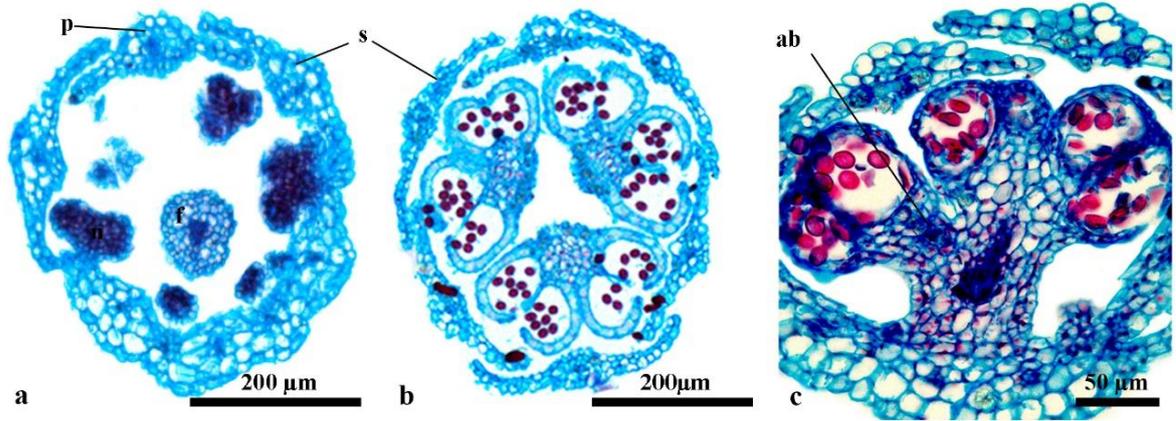
The three stamens are monadelphous, and the filament column shares only one concentric vascular bundle (fig. 2a). The anthers are bithecal and tetrasporangiate, free at their major extent and united at the base (figs. 2b, 2c, 3g). The mature anther wall is formed by epidermal cells tangentially elongated and an endothecium composed of palisade cells with lignified secondary thickening forming trabeculae. The anthers are extrorse and dehisce by a longitudinal slit (fig. 2e).

Ontogenesis

In the staminate flowers, the sepals are the first structures to be formed, beginning as three outer protuberances at the floral meristem and developing in a clockwise direction (fig. 2f–2h). Then, the three protuberances of the inner whorl begin their formation in a counterclockwise direction (fig. 2g, 2h). After the sepals and petals differentiate and elongation begins, the organs from the outer and inner whorls become fused at the base by postgenital fusion. Toward the inside of the petal primordia, the floral dome develops a triangular shape (fig. 3h), and the androecium initiates in the lateral region of this dome, alternating with the inner whorl.

During anther development (fig. 2i), the filament column begins to elongate as a single structure. The nectary appears at the base of the adaxial face of the petal margins close to the point of union of the perianth (fig. 2j, 2k), generating six nectaries that have a small, rounded shape in the initial stages and become a slightly fleshy group of cells when mature (fig. 2l), at which point the nectaries are fully differentiated and secretory.

Figure 2: Structure and ontogenesis of staminate flowers of *Phyllanthus urinaria*. A, adnate perianth whorls on the base of the flower with a lobed nectary. B, bithecal and tetrasporangiate anthers. C, monadelphous stamens. D, cymule with flowers short-pediceled. E, extrorse anthers with a longitudinal slit. F-G, initial phases of the perianth development. Note sepals growing in a clockwise direction and petals in a counterclockwise direction. H, beginning of the androecium formation, the two whorls closing and the floral apex with a triangle shape. I, maturation of the anthers. J, nectary initiation on the adaxial face of petals margins. K, immature nectaries showing a fleshy appearance. L, stomata on a mature nectary. a, anther. ab, anther bundle. ad, androecium. n, nectary. p, petal. pd, pedicel. s, sepal. sp, stamen projection. st, stoma.



Vasculature

The floral stele is a eustele composed of six vascular bundles observed at the pedicel level (fig. 3*a*). In the receptacle at the level of the first whorl (calyx), three traces diverge from the stele and vascularize the sepals (fig. 3*b*, 3*c*). Each trace reaches the sepal base without ramification and does not leave a gap in the floral stele, which is replaced by another vascular bundle. This sole bundle of the sepal remains unbranched along the whole sepal.

In the next whorl (corolla), the remaining three fundamental bundles of the stele diverge to vascularize the three petals (fig. 3*b*, 3*c*). During the divergence of the petal traces, narrow branches of each trace shift to the center of the floral axis, forming a concentric bundle, which will vascularize the androecium (fig. 3*c*–3*e*). Unlike the calyx vascularization, each corolla trace divides into three before reaching the petal base with these two new lateral traces formed only by phloem (fig. 3*b*, 3*c*). These phloem strands extend to the petal margins reaching the base of the nectary without entering it (fig. 3*c*–3*e*). The sole bundle of the petal remains unbranched along the whole petal (fig. 3*e*–3*h*).

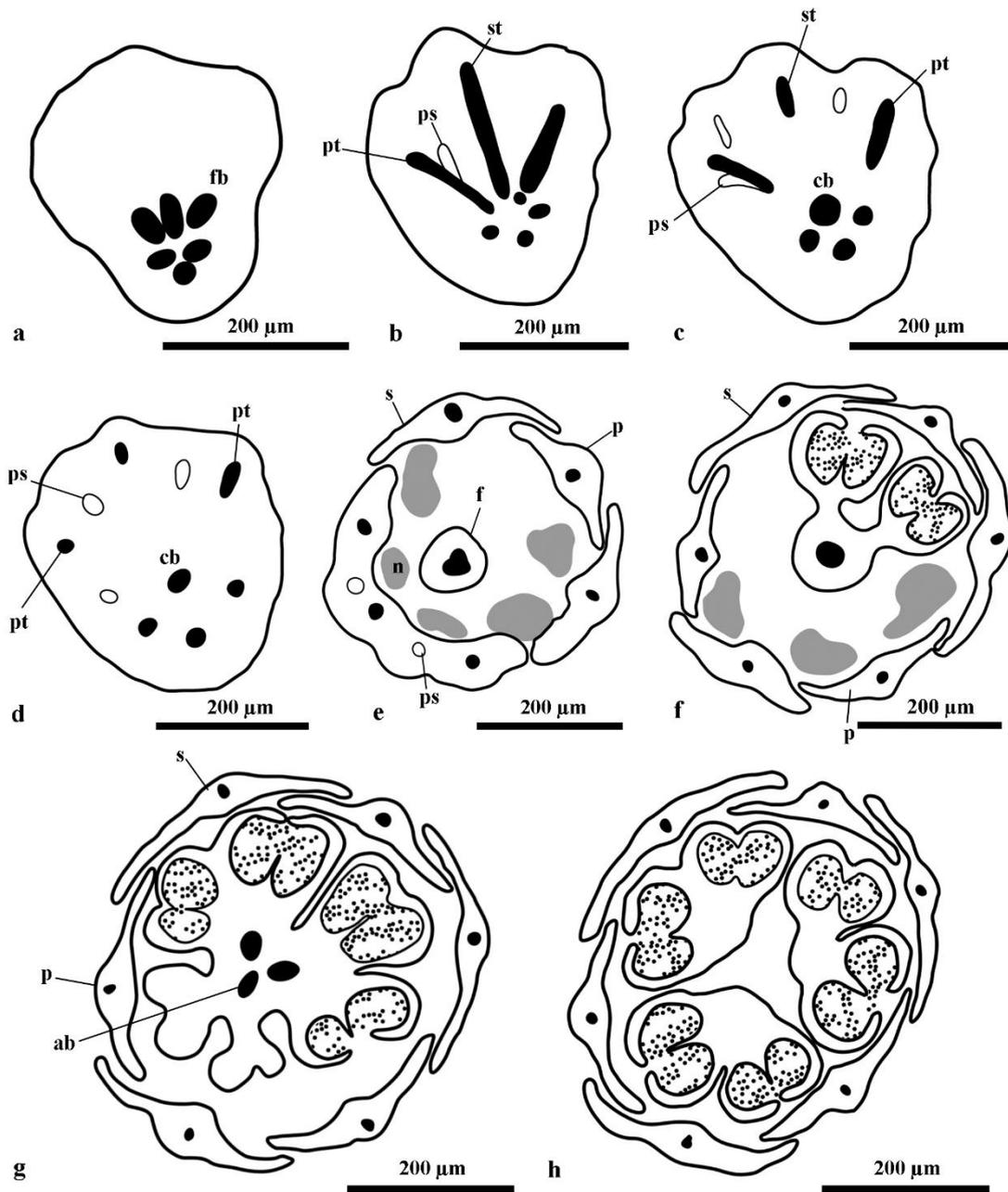
The filament column is vascularized by one concentric amphicribal bundle (fig. 3*e*, 3*f*), which corresponds to the remnant of the floral stele. At the level of the anthers, it is possible to see the single bundle divide into three to vascularize the united base of the anthers (fig. 3*g*), which are free and nonvascularized in the median region (fig. 3*h*).

Pistillate flowers

Floral Structure

The pistillate flowers are composed of a fused perianth with a patelliform nectary (fig. 4*a*) and a tricarpellate, trilocular syncarpous gynoecium with two ovules per locule

Figure 3: Vasculature of staminate flowers of *Phyllanthus urinaria*. A, fundamental bundles at the pedicel level. B, sepal and petal traces divergence in the receptacle. The trace of sepals does not branch, the trace of the petals divides in three, one central and two laterals formed only by phloem. C, Petals traces divergence and formation of the central bundle. D, phloem strand located on the base of the nectary and between the sepal and petal traces. E, nectary lobes formed on the margin of petals, filament column is vascularized by one concentric amphicribal bundle. F, the bundle of the filament column remains undivided until the base of the anthers. G, the filament bundle ramifies in three to vascularize the united base of the anthers. H, median region nonvascularized of the anthers. ab, anther bundle. cb, central bundle. f, filament fb, fundamental bundles. n, nectary. p, petal. ps, phloem strand. pt, petal trace. s, sepal. st, sepal trace.



(fig. 4*b*). The structure of the calyx is very similar to that of the staminate flower, which is also fused but only next to the receptacle (fig. 4*a*). Each sepal has one vascular bundle with the largest region of the mesophyll containing three or four layers of parenchyma cells.

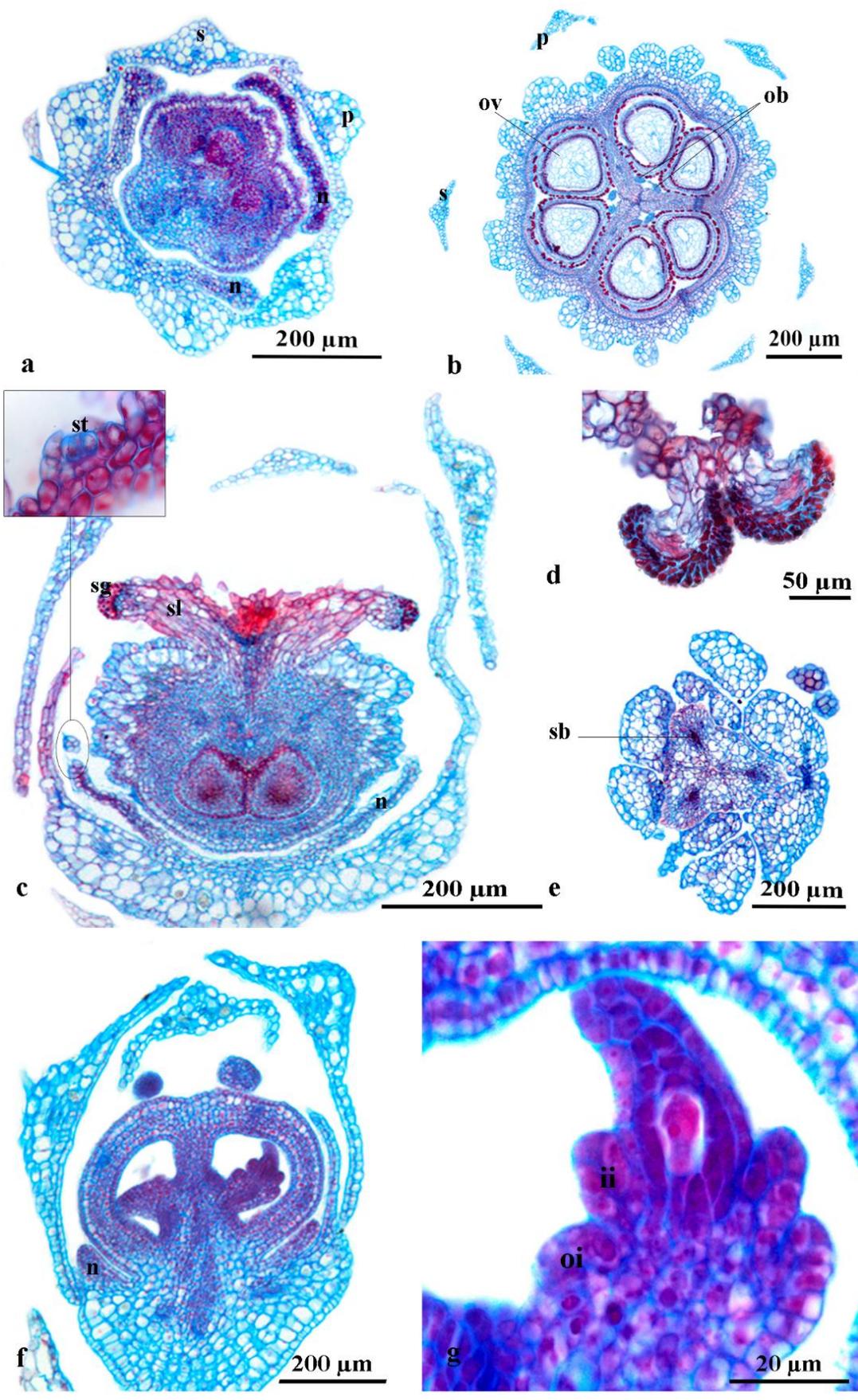
Three nectaries originate from the base of the adaxial face of the corolla. The three coralline nectaries extend laterally and merge becoming patelliform (fig. 4*c*) and six lobed distally, generally thin with the parenchyma vascularized only by phloem strands. This nectary has stomata on the abaxial surface of the epidermis in the distal region of the nectary (fig. 4*c*).

The ovary has three bifid styles, which are fused at the base (fig. 4*d*, 4*e*). The adaxial surface of the epidermis of the style is formed by elongated cells, while the cells on the abaxial surface are smaller and slightly papillose (fig. 4*c*).

The ovary has a tuberculate surface, formed by wall projections (fig. 4*c*). The outer epidermis of the ovary is uniseriate with cells slightly elongated radially. The mesophyll is homogeneous and consists of one or several layers of parenchyma cells, where the vascular bundles occur. The inner epidermis of the ovary is uniseriate with small cells.

The ovules are bitegmic, hemitropous with axile placentation (fig. 4*f*, 4*g*). The outer integument is formed by two layers of cells, while the inner portion has two or three layers of cells at anthesis. The inner integument has a distinctive endothelium stained in red. The micropyle is formed only by the inner integument. The nucellar beak is long, spreading throughout the micropyle (fig. 4*g*). The placentary obturator is small, composed of a group of cells in palisade, located just above the micropyle.

Figure 4: Floral structure of pistillate flowers of *Phyllanthus urinaria*. A, fused perianth at the base with a patelliform nectary. B, tricarpellary gynoecium with two ovules per locule. C, longitudinal view showing the corolline nectary with a stoma (inset) in the apical portion. D, bifid stigma. E, short fused portion of the style. F, longitudinal view showing the initial development of the hemitropous bitegmic ovules with axile placentation. G, detail of the ovule with a long nucellar beak. ii, inner integument. n, nectary. ob, obturator. oi, outer integument. ov, ovule. p, petal. s, sepal. sb, style bundle. sg, stigma. st, stoma.



Ontogenesis

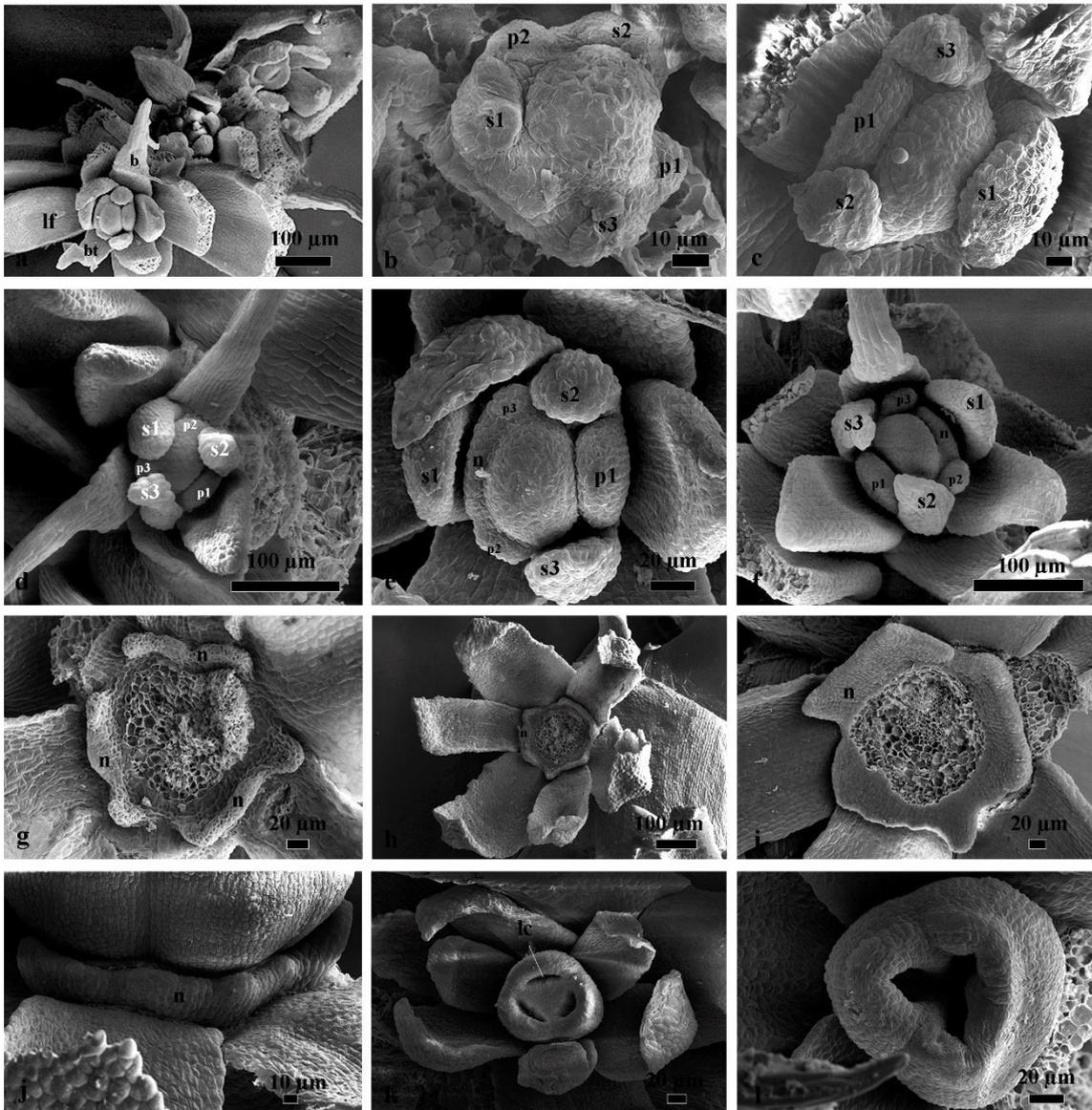
The flower ontogenesis begins with the formation of the perianth. Three protuberances on the flower dome arise more or less simultaneously in a whorl in a counterclockwise direction (fig. 5*a–5f*). The flower development is followed by the whorl of petals that appear to arise in a different direction from the first whorl, in this case, in a clockwise direction. From the adaxial surface of the petals, the nectary originates early (fig. 5*g*) and expands upward and laterally, merging and becoming patelliform (fig. 5*h–5j*). After differentiation of the sepals and petals and at the beginning of their elongation, they become fused at the base by postgenital fusion.

The floral dome remains hemispherical until it is consumed by the formation of the gynoecium, at which point it appears as a flat triangle, which will subsequently form the three carpels that alternate with the three inner perianth organs. The gynoecium is differentiated into a trilocular ovary (fig. 5*k, 5l*). The carpels are congenitally united with septa visible in the early stages of the development in the synascidiate region. In each carpel, two ovules arise when they are already closed, so the ovules are no longer visible from the outside (fig. 5*l*). The dorsal region of the carpels begins to elongate, generating the styles. The gynoecium is completely connate up to the stigma. The styles elongate and become slender and bifid.

Vasculature

The floral stele is a eustele formed by nine vascular bundles in the receptacle (fig. 6*a*). At the level of calyx (fig. 6*b*), three unbranched traces diverge from the stele to the sepals, and the gaps left by them are filled (fig. 6*c, 6d*). The bundle of the sepal remains unbranched along the whole sepal.

Figure 5: Floral development of pistillate flowers of *Phyllanthus urinaria*. A. young terminal uniflorous inflorescence. B, beginning of the flower development. C, corolla initiation. D, sepals growing in a clockwise direction and petals in a counterclockwise direction. E-F, perianth organs development and the emergence of the nectary. G-H, immature patelliform nectary on the adaxial surface of the petals. I-J, mature nectary in frontal (I) and lateral view (J). K, ovary formation of the syncarpous tricarpeillary gynoecium. L, dorsal part of the carpels beginning to elongate, to form the styles b, bract. bt, bracteole. lc, locule. lf, leaf. n, nectary. p, petal. s, sepal.



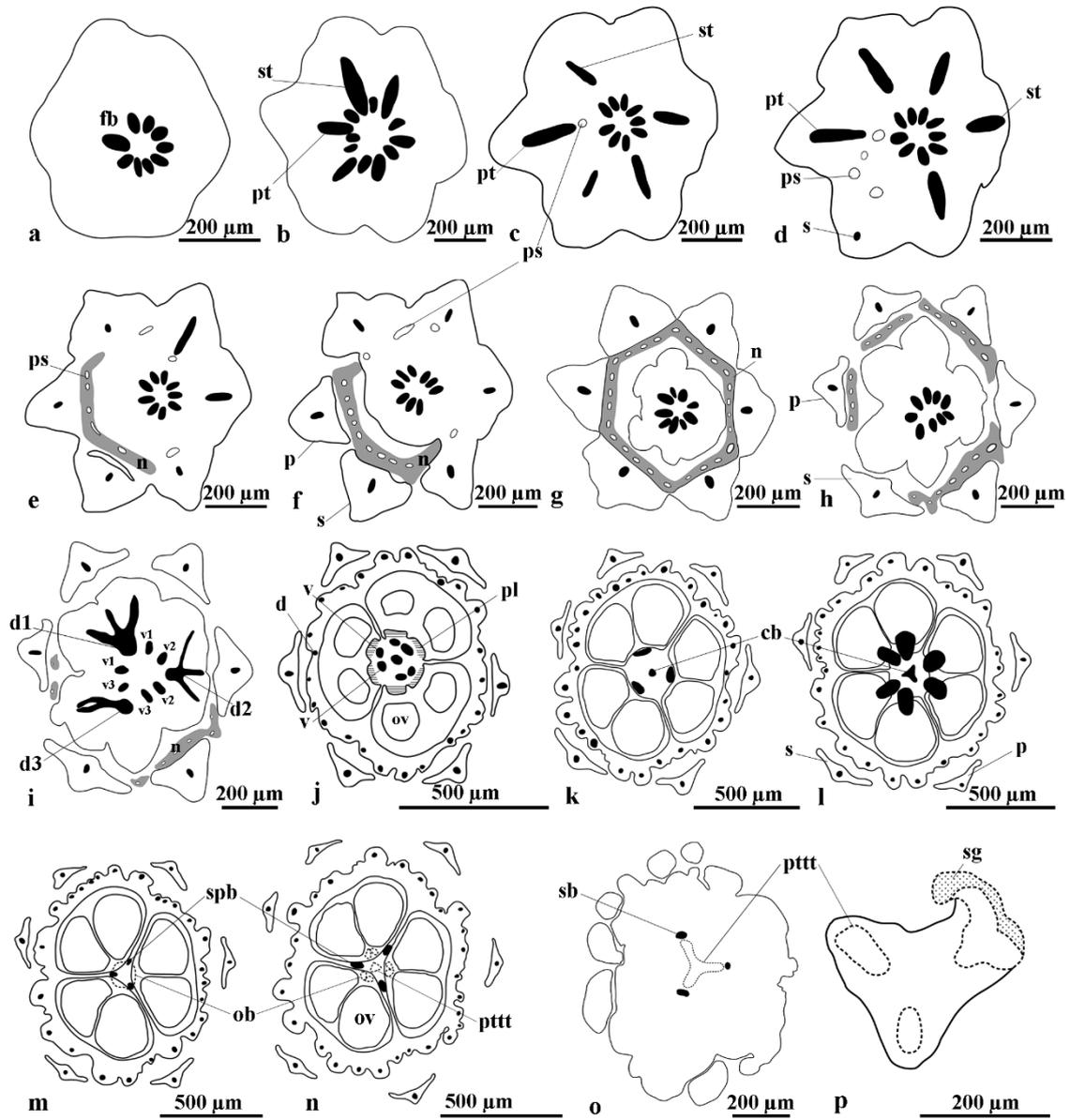
In the corolla, three fundamental bundles of the stele diverge to vascularize the three petals, and the gaps left from the traces are filled, leaving nine bundles in the stele (fig. 6c–6e). Each corolla trace divides in two before reaching the petal base (fig. 6d, 6e). One of the traces is formed only by phloem and extends to the adaxial region of the petal reaching the base of the nectary (fig. 6d–6f); unlike the staminate flowers, the phloem strands enter the nectary (fig. 6e–6h). The bundle in the petal remains unbranched along its whole extent (fig. 6h–6n).

The nectary, at first, is initiated by one phloem strand that divides, generating more phloem cells in front of the petals (fig. 6d–6f). These cells then divide, reaching the adaxial surface of the sepals. Each nectary merges into one by postgenital connation (figs. 5h, 5i, 6g). This event occurs in a very short time, and soon after, the nectary has six lobes between the sepals and the petals (figs. 5h, 5i, 6h, 6i).

The vasculature of the carpel starts from the nine bundles that are in the stele. Three traces expand to reach each carpel; one trace is median, and two are lateral, originating the dorsal and two ventral bundles, respectively (fig. 6i, 6j). Although each carpel receives three traces, it generates only one gap at the stele from the dorsal trace. After the divergence of the ventral bundles, some remnant cells of the vascular tissue extend to the stellar axis, become fused and continue to extend above the uppermost carpels, and are eventually consumed to supply the septa (fig. 6j–6l).

The dorsal bundle divides into many bundles that reach the entire wall of the ovary (fig. 6j–6n). In a later stage, the two ventral bundles fuse into one (fig. 6k) and then divide into two bundles, each extending to a different ovule of the carpel (fig. 6l).

Figure 6: Vasculature of pistillate flowers of *Phyllanthus urinaria*. A, eustele with nine vascular bundles in the pedicel. B, sepals and petals traces divergence in the receptacle. C, sepals are vascularized by one trace and the petals by two traces, being ventral one composed only of phloem that extends in the adaxial region of the petal. D, ramification of the phloem strand. E, nectary base with several phloem branches. f, the sepal and the petal bundle remain unbranched along the organs. G, continuous nectariferous ring at the adnate base of the perianth. H, upper lobes of the patelliform nectary in front of the petals. I, divergence of the carpels traces. Note that the dorsal trace ramifies in three bundles at the ovary base and the two ventral bundles remain unbranched. J-K, the two ventral bundles of each carpel ramifies forming the central bundle at the median level of the ovary and then become fused into one ventral bundle per locule. L, division of the ventral bundles in two and their extension into the ovules. M, branching of the central bundle for the vascularization of the septa at the apical portion of the ovary. N, pollen tubes transmitting tissue strands in the center of the ovary. O, unique pollen tubes transmitting tissue strand in the style. P, portions of the pollen tubes transmitting tissue related to each stigma. d, dorsal bundle. cb, central bundle. fb, fundamental bundles. n, nectary. ob, obturator. ov, ovule. p, petal. pl, placentae. ps, phloem strand. pt, petal trace. s, sepal. sb, style bundle. sg, stigma. spb, septal bundle. st, sepal trace. pttt, pollen tubes transmitting tissue. v, ventral bundle.



The stellar bundle takes the form of a triangle that will be consumed to vascularize the three septa (fig. 6l). At this point it is possible to identify the placental obturator of each ovule (fig. 6m) and some remnant of the pollen tube transmitting tract (PTTT) in the center of the ovary (fig. 6n). This PTTT becomes triangular (fig. 6o), and on the end of the style it divides into three to keep pace with the styles (fig. 6p).

DISCUSSION

***Phyllanthus* flowers and their evolutionary aspects in Phyllanthaceae**

In *Phyllanthus urinaria*, unlike previous descriptions for the genus (e.g., Martins and Lima 2011; Zhang et al. 2012), we found two whorls, which might be distinguished as calyx and corolla, based on ontogenetic and structural studies, especially when regarding the vasculature, which showed a different number of traces among sepals and petals. Flowers of *P. urinaria* are tiny, which reduces the size and vascularization structure in both sterile and fertile whorls. The nectaries are different in staminate and pistillate flowers, with the former being segmented and the latter patelliform, but both originate from the petals.

The staminate inflorescences have more flowers than the pistillate ones in *P. urinaria*. This pattern was commonly found in the genus and has been described for other *Phyllanthus* species by Webster (1957), Radcliffe-Smith (2001), Sasidharan (2004), and Zhang et al. (2012). Inflorescence with one single flower represents a level of reduction of ramification, leading to uniflorous terminal or axillary inflorescences (Weberling and Hoppe 1996), as in the pistillate inflorescences of *P. urinaria*, and are also common in Picrodendraceae, the sister group of Phyllanthaceae, in which the pistillate inflorescences often have fewer flowers than the staminate ones (Esser 2010).

Perianth

Pentamerous flowers are the most common merism in the genus (Michaelis 1924); however, there are flowers with four, five, or six organs arranged in two whorls, such as in *P. chekiagensis* Croizat & F.P. Metcalf (Zhang et al. 2012). Petals, if present, in the flowers of Phyllanthaceae and Picrodendraceae are not conspicuous (Pax and Hoffmann 1931; Merino Sutter et al. 2006). Webster (1994b) observed that in the tribe Phyllantheae, a flower that has imbricate sepals generally does not have petals, and if there are petals, often these are longer than the sepals (Michaelis 1924). When the analysis is based only on a morphological observation, occasionally it is not possible to distinguish an organ as a petal or a sepal (Endress 1994) or to confirm the existence of two whorls. In many species of *Phyllanthus* the elements of the perianth have the same color and are poorly differentiated (Bancilhon 1971), despite the presence of two whorls; as a result, the outer and inner organs of this genus have regularly been called bisseriated calyx (Webster 1957). However, trimery was observed before in Phyllanthaceae; Huft (1989) interpreted the flowers of *Tacarcuna amanoifolia* Huft and *Tacarcuna tachirensis* Huft as having three sepals and three petals. Species of *Phyllanthus* have undifferentiated parts that are conspicuous, and if investigated developmentally, they could be interpreted with greater precision.

In the phylogeny of the tribe Phyllantheae (Katriarachchi et al. 2006), the clade *Phyllanthus* sect. *Urinaria* + *Phyllanthus* sect. *Emblica* includes species with six perianth parts as in *P. urinaria*. This indicates that perhaps all these species have a similar type of development with sepals and petals. Furthermore, perhaps this prediction can be extended to the clade composed of *Breynia*, *Glochidion*, and *Sauropus*, now included within *Phyllanthus*, since they also present six parts in the perianth (Webster 1957; Radcliff-

Smith 2001) and represent the sister group to *Phyllanthus* sect. *Urinaria* + *Phyllanthus* sect. *Emblica*, demonstrating that this is a widespread syndrome within *Phyllanthus*.

Many basal angiosperms, basal eudicots, and monocots have trimerous or dimerous flowers, while the core eudicots have pentamerous and tetramerous flowers (Ronse De Craene 2010, 2016; Endress 2011). When the perianth has six parts, some authors assert that the flower is apetalous (Frye and Kron 2003; Ronse De Craene 2010; Zhang et al. 2012). The number of elements seems to be directly related to the aestivation pattern, which is imbricate in the hexamerous and trimerous flowers and quincuncial in the pentamerous ones, due to the loss of a tepal in the inner whorl (Frye and Kron 2003; Ronse De Craene 2010, 2016; Endress 2011). The flowers of *Phyllanthus* can have four, five, or six perianth parts (Radcliffe-Smith 2001), but in some genera of *Phyllanthaceae* and *Picrodendraceae*, they are trimerous (Pax and Hoffmann 1931; Merino Sutter et al. 2006). According to our study, the hexamerous flowers, in particular, should be ontogenetically reevaluated because the number of trimerous flowers may be much higher than is currently believed, since the related groups of the section *Urinaria* also have six parts in the perianth.

In *P. urinaria* several organs originate almost simultaneously according to the alternation rule (Leins and Erbar 1997), resulting in an arrangement of the organs in isomerous alternating whorls. The traces that alternate with sepals are interpreted as petal traces (Puri 1951), indicating that the second whorl in *P. urinaria* is formed by petals.

Initially, the floral meristem of *P. urinaria* has three protuberances on the flower dome, which arise in a counterclockwise direction more or less simultaneously in the outer whorl, followed by the development of the inner whorl, which arises in a clockwise direction. This difference in the direction of development between the inner whorl and

the outer whorl of the perianth has also been observed in dichlamydeous and trimerous flowers of other families (Ronse De Craene 2010).

Anatomically, sepals commonly have one median and two lateral vascular traces, and petals usually have a single vascular trace in most rosids (Bachelier and Endress 2009). However, in the staminate flowers of *P. urinaria* the opposite occurs: there is one vascular trace per sepal and three traces per petal, one median and two laterals, which are composed only of phloem that extends to supply the nectaries.

Schmid (1972) commented that the fusion of vascular bundles indicates a high degree of evolutionary advancement since bundles, which are conservative, may remain separate or partly separate, even when the structures they supply have become connate or adnate. The bundles of the sepals and petals are not fused despite the adnation between the petals and sepals during the development of the flower, generating a perianth of mixed origin. The whorls are slightly fused at the base for a short length, only visible in light and electron microscopy, which is unusual among the eudicots.

Nectary

According to Weberling (1989), nectary disks develop from the floral receptacle; however, studies have shown that in basal eudicots the nectaries can have several forms and can be found in different locations, generally on petals or carpels or resulting from a floral organ that has become nectariferous, such as a staminode, e.g. (Erbar et al. 1999; Endress 2010, 2011). However, the results from ontogenetic and vascular studies reveal that the floral nectaries in *P. urinaria* are corolline and the phloem strands emerge from the petal trace, to vascularize the gland.

In the staminate and pistillate flowers of *P. urinaria*, the nectaries originate from the proliferation of petals. For other species of *Phyllanthus* a segmented nectary is

present in the staminate and a disk in the pistillate (Webster 1957), or a disk in the flowers of both sexes (Silva 2009), but there is no information about how the nectary develops in the genus and family. *Leptopus chinensis* (Bunge) Pojark., a species of Phyllanthaceae, has nectaries attached to the petals (Ronse De Craene 2010), as seen in *P. urinaria*. Reinforcing the identity of the petals by the presence of the nectaries. Despite the late initiation of the nectaries, their position in *P. urinaria* supports the conclusion that the inner whorl (corolla) where they are located is different from the outer whorl (calyx).

Webster (1956) acknowledged that although the nectary segments of the staminate flowers of *Phyllanthus* do not possess vasculature, there was vascular tissue near their base. This observation agrees with our results in *P. urinaria*, in which we found phloem strands only until the base of the nectaries, which have stomata in their epidermis. On the other hand, the nectary of the pistillate flower in *P. urinaria* is vascularized. It may occur because this nectary is bigger than that in staminate flowers (Webster 1956).

Fertile whorls

In *P. urinaria*, the filaments are congenitally connate, and the anther structure is similar to that found in the vast majority of angiosperms (see Endress 2011). The carpels are also connate, as previously described in other Phyllanthaceae (e.g., Martins and Lima 2011; Zhang et al. 2012), which might be considered a characteristic of this family.

The flower in anthesis of *P. urinaria* is very similar to *Phyllanthus stipulatus* (Raf.) G.L. Webster, as both have the same number of stamens and are completely fused, generating a column, although these species are differentiated by the number of sepals, three in *P. urinaria* and five in *P. stipulatus* (Martins and Lima 2011). The pistillate flowers of *P. urinaria* have three bifid styles, fused at the base, with six papillose stigmas, and a biovulate trilocular ovary, completely connate.

The COM clade (Celastrales, Oxalidales, and Malpighiales) presents ovules pendant and antitropous with obturator and axile placentation (Endress and Matthews 2006; Endress et al. 2013). These features can be found in *P. urinaria*, which has bitegmic, hemitropous ovules with axile placentation and a long nucellar beak. Among Malpighiales this set of characteristics is remarkable in the phyllanthoids, which have ovules often with nucellar caps and sometimes nucellar beaks (Endress et al. 2013). The nucellar beak in *P. urinaria* is long, exceeding the micropyle. In addition, *P. urinaria* has a distinguishing endothelium, which has been acknowledged mainly in asterid families with thin nucelli (Kapil and Tiwari 1978).

CONCLUSIONS

From ontogenetic and structural analyses of *Phyllanthus urinaria*, we can assert the presence of calyx and corolla in the perianth of staminate and pistillate flowers distinguished by their origin, nectary position, and vasculature. The origin of the nectary is a novelty for the genus, modified by the interpretation of the corolla. According to our study, the hexamerous flowers, in particular, should be ontogenetically reevaluated to better understand the trajectory of the developmental differences in the perianth and provide broader conclusions across *Phyllanthus*, since *Phyllanthus* sect. *Urinaria*, *Phyllanthus* sect. *Emblica*, and a related group (*Breynia*, *Glochidion*, and *Sauropus* = *Phyllanthus*) appear to have similar flower development and present six organs in the perianth, indicating that the presence of corolla might not be exclusive to *P. urinaria*.

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Chapter II

Floral structure, staminodes, pistillodes and nectaries in *Piranhea trifoliata*
(Picrodendraceae)

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Running Title: Flowers of *Piranhea*

ABSTRACT

Picrodendraceae is a small family that was segregated from Euphorbiaceae *s.l.*, formerly consisting of the subfamily Oldfieldioideae. The flowers are considered trimerous with one or two whorls, may have nectaries and the ovules are anatropous with a long appendage. The aim of this work was to investigate the floral structure of *Piranhea trifoliata* based on structure, vasculature and ultrastructure analyses. Flowers and buds of *Piranhea trifoliata* were embedded in Paraplast and historesin, and sectioned in a rotary microtome for analysis under light microscopy. SEM and TEM were performed for additional structural data. *Piranhea trifoliata* has sepals and petals, distinguished by the vascular traces that reach each organ; nectariferous disc in the staminate and pistillate flowers, in the staminate it is in the androecium base and in the pistillate it is in the base of the staminodes. The ovary has three carpels congenitally connate with two anatropous ovules per locule and a long aril *s.s.*, that is, an outgrowth from the funiculus. The nectary is similar in staminate and pistillate flowers, and it produces a heterogeneous secretion composed of carbohydrates and lipids. These data may help to understand the evolution of the flowers in the group, since the results show for the first time the presence of petals in *Piranhea* due to the presence of two distinct whorls, the presence of staminodial nectaries in the pistillate flowers and a long aril *s.s.*, which was thought to be a strophiole.

Keywords: anatomy – aril – nectary – Picrodendraceae - androecium – petals – vasculature.

INTRODUCTION

Picrodendraceae has about 28 genera (Merino Sutter *et al.*, 2006) and 100 monoecious or dioecious species as trees, shrubs or subshrubs (Radcliff-Smith, 2001). The family was for long time part of Euphorbiaceae *s.l.* as the subfamily Oldfieldoideae (Webster, 1994a; 1994b; Radcliff-Smith, 2001). However molecular studies showed differences between this group and the Euphorbiaceae *s.l.* (Savolainen *et al.*, 2000; Soltis *et al.*, 2000; Chase *et al.*, 2002; APG II, 2003) denominating it, at first, as Pseudanthaceae (Soltis *et al.*, 2000; Savolainen *et al.*, 2002). Chase *et al.* (2002) analyzed molecular data based on plastid *rbcL* sequences and recognized the group as Picrodendraceae, and this circumscription is accepted to date.

In the APG II (2003), the authors state a possibility of Phyllanthaceae and Picrodendraceae form a clade, since the families have similarities in the flowers, merism and embryology; this relationship is supported by Tokuoka & Tobe (2006), Wurdack & Davis (2009) and Xi *et al.*, (2012) that denominated it as Phyllanthoids (Phyllanthaceae + Picrodendraceae), since they are strongly supported as sister groups.

Studies on flowers of Picrodendraceae are scarce and there are about eight papers covering aspects of floral development and/or floral structure, including fruits and seeds (Endress *et al.*, 2013). Among these studies, the work of Merino Sutter *et al.* (2006) stands out by exploring the gynoecium of nine species from Picrodendraceae, focusing on the nucellar beak and obturator.

The flowers of the family are described as pedicellate, actinomorphic with imbricate sepals; diclinous without petals in both morphotypes. The staminate flower has (2)3-30 stamens, the pistillate one has 2-4(-5) carpels., and both flower types may have or not nectaries. When present, the nectary is a disc with interstaminal lobes in the staminate flower and a disc annular to lobed or dissected in the pistillate one. (Webster,

1994b; Radcliff-Smith, 2001). Species from the family are found worldwide, mainly near the equator region (Webster, 1994a), although five genera are endemic to the Neotropics with restricted distribution, occurring in several lowland vegetation types (Esser, 2010). One of this five is *Piranhea* Baill, genus that has the common characters for the family, but is also unique for having combined features, such as staminodes, hexalobate disc in the pistillate flowers and a long strophiole covering the ovules (Radcliff-Smith, 2001).

Piranhea trifoliata Baill. is the type species and it is well known by the indigenous and river communities in the Amazon. Its common name is “piranheira” or “piranha tree” because of the piranha fish which is attracted to the plant to eat the caterpillars that fall from the tree (Goulding, 1980). The few studies about the species are related to the chemical properties in the leaves, which have triterpenes (Jeffreys & Nunes, 2016), and morphology of the pollen grains (Moura *et al.*, 2004).

Our aim was to investigate the structure of staminate and pistillate flowers of *Piranhea trifoliata* based on anatomy and vascular analyses, focusing on the perianth, staminodes, aspects of the nectary and the ovule.

MATERIAL AND METHODS

Inflorescences and flowers in different stages of development were collected in Manaus, at Catalão River, in Brazil. Voucher of the specimens were provided and exsiccates were deposited in the Herbarium of the University of Pará State: *Piranhea trifoliata* = T.S.S. Gama – 9, (MFS 006024).

Light microscopy

Flowers in several developmental stages were isolated, fixed in FAA (formalin, acetic acid, 50% ethyl alcohol) for 24 h (Johansen, 1940) or BNF (neutral buffered formalin) for 48h (Lillie, 1965), and subsequently stored in 70% ethyl alcohol.

The flowers were dehydrated in butyl series (Johansen, 1940), embedded in Paraplast (Leica Microsystems Inc., Heidelberg, Germany), and transversely and longitudinally sectioned in a Leica RM2145 rotary microtome (Leica Microsystems, Wetzlar, Germany). The sections were stained with safranin and astra blue (Gerlach, 1984) and the slides were mounted in resin Permout (Fisher Scientific, Pittsburgh, Pennsylvania, USA). The photomicrographs were obtained in a Leica DMLB light microscope (Leica Microsystems, Wetzlar, Germany) using the Scan System Images (IM50).

Some flowers were also embedded in methacrylate resin to obtain thinner, better quality sections for the flower vasculature analysis. These flowers were dehydrated in ethyl alcohol series, embedded in Historesin (Leica Microsystems, Wetzlar, Germany), following the technique used by Gerrits (1991), and transversely sectioned at a thickness of 6-10 μm . The sections were stained with 0.05% toluidine blue (O'Brien *et al.*, 1964) in a 0.1 M, pH 4.7 sodium acetate buffer and the analysis was performed in a Leica DMLB light microscope. The additional observations of flower morphology were made using a Leica M125 stereoscopic microscope (Wetzlar, Germany).

Electron microscopy

The micromorphological analyses were done using material fixed in FAA. After flower dissection, the material was dehydrated in ethyl alcohol series up to 100%, critical point dried in a Balzers CPD 030 (Balzers, Liechtenstein, Germany), mounted on

aluminum stubs, sputter coated with gold using a Balzers SCD 050 (Balzers, Liechtenstein, Germany), and examined in a Zeiss DSM 940 scanning electron microscope (Carl Zeiss, Oberkochen, Germany; Robards 1978).

The material designated for transmission microscopy (TEM) was fixed in Karnovsky solution for 24 h at 4°C, postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 2 h (pH 7.2), dehydrated in a graded acetone series and embedded in Spurr resin (Roland, 1978). Ultrathin sections were obtained and counterstained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) for further analysis in a Zeiss EM 900 electron microscope.

All figure plates and schemes were made using Adobe Photoshop and Illustrator software (Redwood City, CA). Vasculature drawings were prepared from digital photographs using Adobe Illustrator software (Redwood City, CA).

RESULTS

Inflorescences

Piranhea trifoliata is a dioecious that presents cymose and glomerular inflorescences located on the branch of the leaves on the proximal or distal region of the branch. The staminate inflorescence is a glomerulate cyme with about 7 flowers (Fig. 1A), while pistillate inflorescences are uniflorous or a cymule with two or three flowers (Fig. 1B), all different types of inflorescences are subtended by one bract and each flower by two bracteoles.

Flower morphology

The staminate and pistillate flowers are small, measuring approximately 2-5 mm. The flowers are trimerous, with two whorls arranged in an imbricate aestivation (Fig. 1C-

D). The bracteoles have the same color as the perianth whorls but they are a bit fleshy and slightly small. The organs of the two perianth whorls are similar (homoclamydeous) in the staminate flowers and fused each other in the mature flowers, forming one single perianthial envelope. However, the pistillate flowers are heteroclamydeous, with the organs of the inner whorl being thinner than those of the outer whorl and all are entirely free. The perianth is recognized here as composed of sepals in the outer whorl and petals in the inner whorl.

The staminate flower has a long pedicel with an articulation below the receptacle (Fig. 2A). The perianth is adnate, seen in anatomy analysis as well in the external morphology (Fig. 2B). The bracts and the sepals are deciduous when mature flowers. The androecium is composed of six to eight stamens (Fig. 3B), with the latter more predominant.

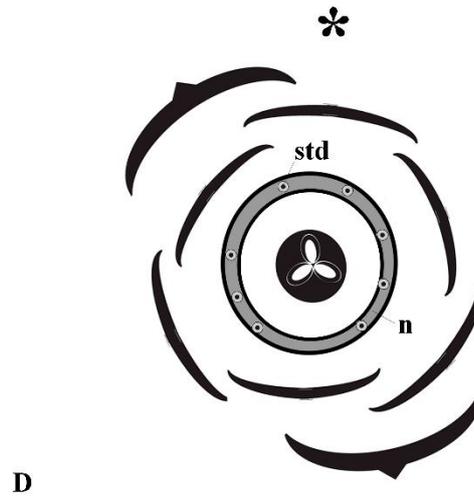
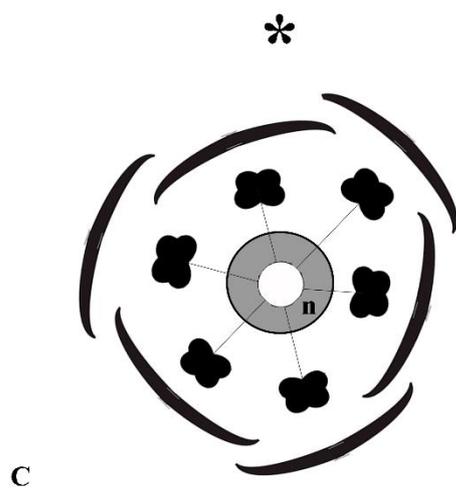
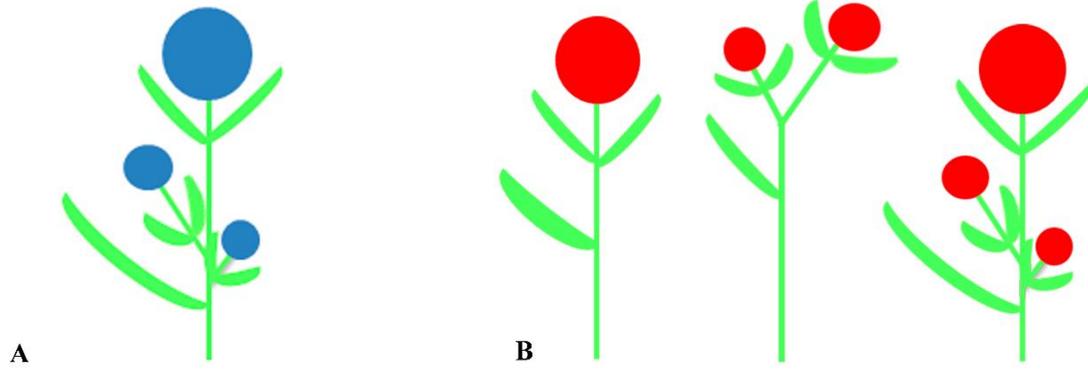
The pistillate flowers are composed of a long pedicel, free perianth, staminodes with a disc annular and a tricarpeal, trilocular syncarpous gynoecium with two ovules per locule. Staminate and pistillate flowers have nectaries. In the staminate one it is an intrastaminal disc with prominent interstaminal lobes, and in the pistillate it is a disc, located in the base of the staminodes.

Staminate flower

Structure

The sepals and petals have uniseriate epidermis, composed of small cells with thin walls (Fig. 2B-C). The mesophyll is homogeneous with three layers in the sepals and three in the petals. Each perianth part is composed of parenchymatous cells with some idioblasts containing phenolic compounds and one collateral bundle.

Figure 1. Schematic view of *Piranhea trifoliata* inflorescences and the floral diagrams of flowers. A, staminate cymule with three flowers. B, uniflorous pistillate cymule, Cyme with two and three flowers. C, staminate flower diagram. D, pistillate flower diagram. bt, bracteole. n, nectary. std, staminodes.



The stamens are organized in two or three whorls, they are connate in a short extent (Fig. 2B). The filament and connective has one concentric bundle and the anther is bithecal and tetrasporangiate (Fig. 2C). The mature anther wall is formed by papillose epidermal cells and an endothecium composed of palisade cells. The anthers dehisce by a longitudinal slit (Fig. 2E-F).

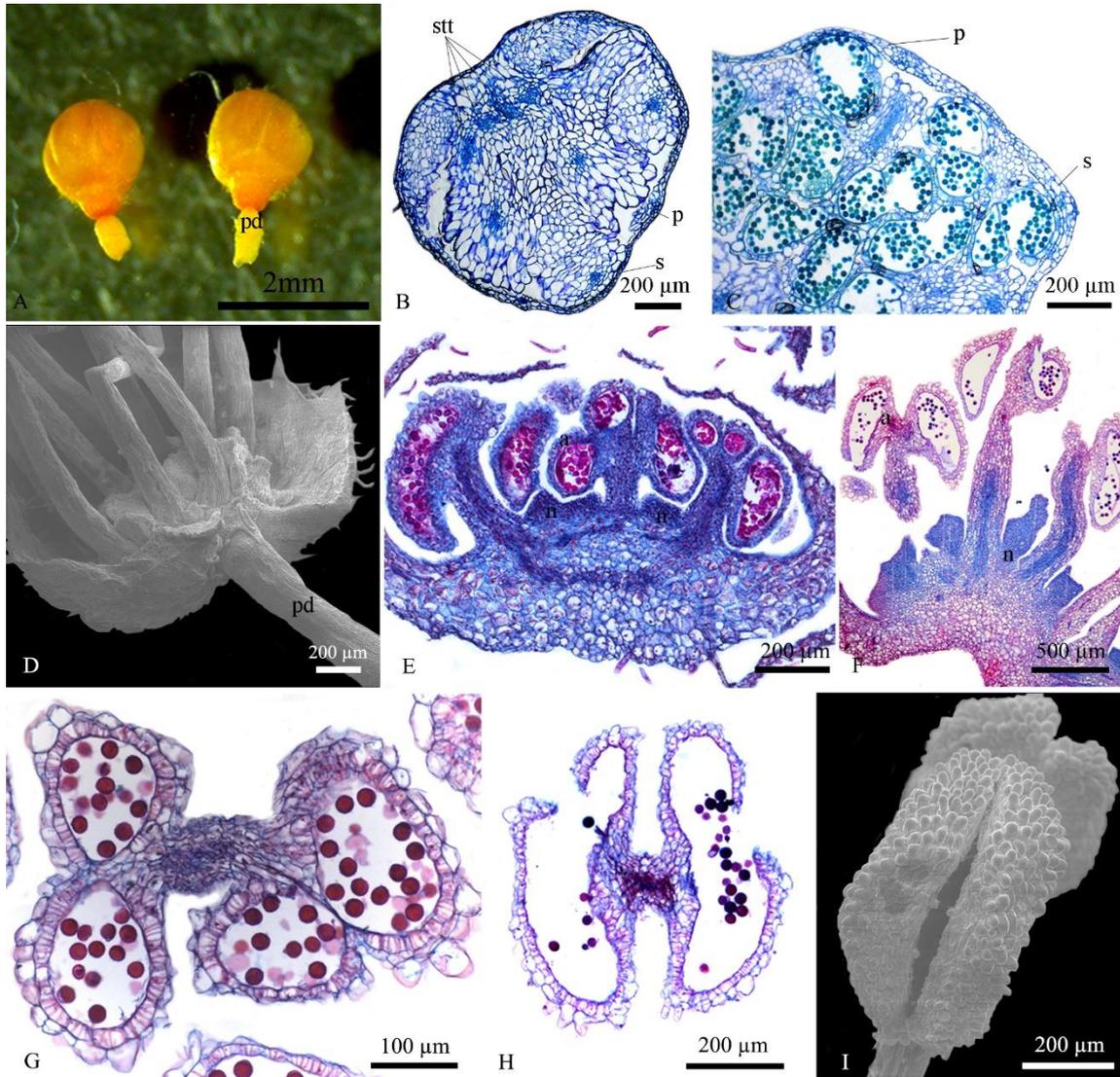
The nectary protrudes from the base of the filaments and covers the receptacle (Fig. 2 G-I). It is non-vascularized and have both the epidermis and about seven layers of parenchyma secretory (Fig. 2H-I).

Vasculature

The floral stele is a siphonostele composed of six vascular bundles observed at the pedicel level (Fig. 3A). In the receptacle at the level of first whorl (calyx), three traces diverge from the stele and vascularize the sepals (Fig. 3B). Each trace reaches the sepal base without ramification and does not leave a gap in the floral stele. This sole bundle of the sepal remains unbranched along the whole sepal.

The traces of the next whorl (corolla) start to diverge almost simultaneously to the calyx ones. Three traces diverge from the stele of the floral axis to vascularize the three petals (Fig. 3B-C) also without leave gaps. The six remaining traces will vascularize the stamens (Fig. 3C-D). Each stamen receives one trace and, depending on the number of stamen in the flowers the fundamental bundles multiply. The first traces to leave are the antesepalous ones, and then the antepetalous. In cases when the flower has more than six stamens, the third whorl appears, although in this third whorl only two stamens develop alternate with the second whorl.

Figure 2. Structure of staminate flowers of *Phyllanthus urinaria*. A, Stereomicroscope image showing a pedicel with an articulation. B, adnate perianth, eight stamens in the flower. C, sepal and petals with uniseriate epidermis and thin mesophyll. D, SEM of the interstaminal nectary. E, longitudinal view of the stamens, nectary and vascularization. F, parenchyma secretor stained in blue. G, bithecal and tetrasporangiate anther. H - I, extrorse anthers with a longitudinal slit. a, anther. n, nectary. P, petal. pd, pedicel. s, sepal. stt, stamen trace.



The filament column is vascularized by one concentric bundle (Fig. 3E), which is undivided until the base of the anthers. The nectary is not vascularized, the transport of the substances occurs cell to cell until reach the epidermis.

Pistillate flower

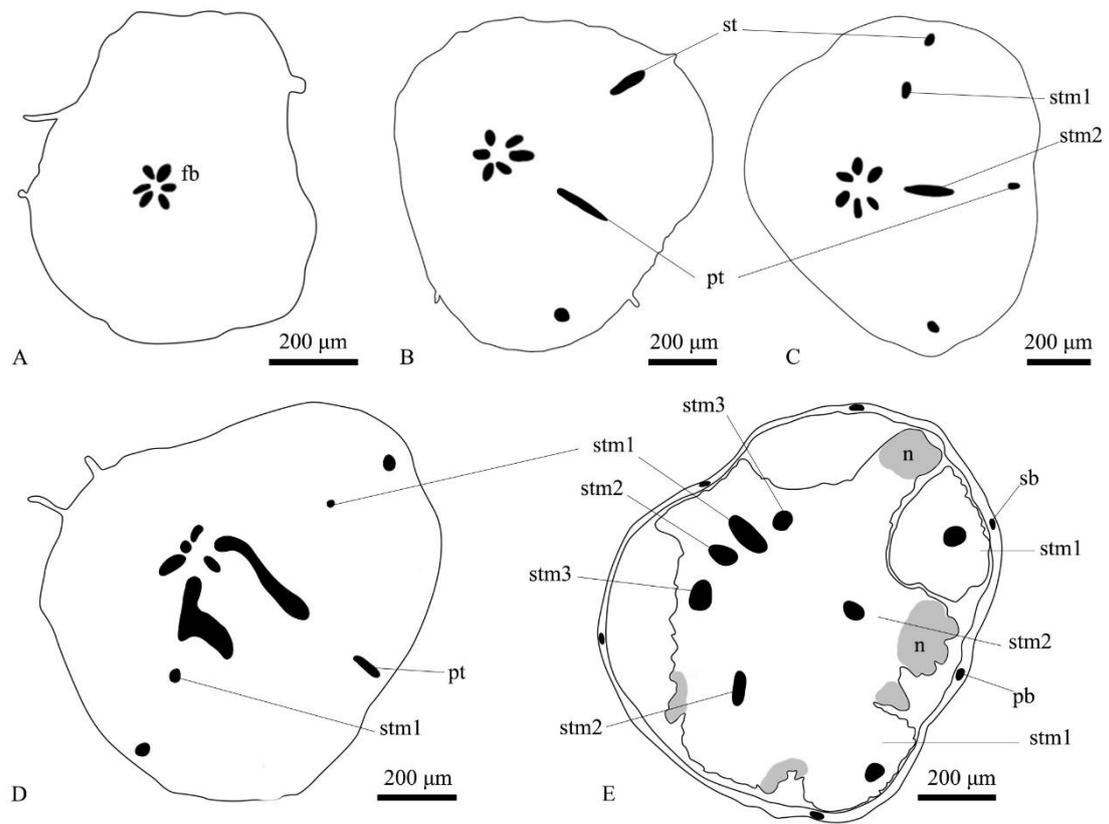
Structure

The perianth of the pistillate flowers is thicker and larger than the ones from the staminate. The petals are more membranaceous when compared to the sepals. Both structures have a uniseriate epidermis, with many tector trichomes and a homogeneous mesophyll (Fig. 4A). Sepals present about 16 layers in the mesophyll and petals have 13 being slightly small (Fig. 4B-C). The sepals are larger and have more vascular bundles than the petals.

The staminodes vary from seven to eleven, but predominantly eight. They are long, filiform and flattened, comprising about 3 layers in the mesophyll (Fig. 4B), they not present a antheroid structure. The nectaries originate from the staminodes that has the base morphologically modified (Fig. 4C-F). The nectary in the base of the staminodes is thick, having about 22 layers at the mesophyll (Fig. 4C); the colors are also different between these two regions, the base is light brown and looks fleshy and the lobes are darker and full of phenolic content. The epidermis is nectariferous like the parenchyma and vascular bundles are only found in the subnectariferous parenchyma.

The gynoecium has three long bilobed sessile stigmata, strongly curved (Fig. 4G), humid and secretor covered by trichomes on its whole surface (Fig. 4 H-I).

Figure 3. Vasculature of staminate flowers of *Piranhea trifoliata*. A, six fundamental bundles. B, divergence of the sepal and petal traces. C, vascularization of the first and second whorl of stamens. D, first whorl of stamens in front of the sepals. E, all the traces of stamens diverging, note the presence of seven traces plus one stamen separated. pb, petal bundle. pt, petal trace. sb, sepal bundle. st, sepal trace. stm1, first whorl of stamen. stm2, Second whorl of stamen. stm3, third whorl of stamen.



The ovary is rounded covered by many lignified trichomes (Fig. 5A). The outer epidermis of the ovary is uniseriate with juxtaposed cells slightly elongated towards the radial direction. The mesophyll is homogeneous and consists of several layers of parenchyma cells, up to 24, where the vascular bundles occur. The inner epidermis of the ovary is uniseriate with minute cells and lignified trichomes (Fig. 5B) which are also present at the base of the locule (Fig. 5C, E-F).

In *Piranhea trifoliata* the obturator is small, possesses few, lesser than ten, elongated cells at the epidermis and it originates from the placenta; the secretory obturator is located at the axil between the aril and the micropyle (Fig. 5G-H). The pollen tubes transmitting tissue (PTTT) cross the placenta (Fig. G) and the cytoplasm of its cells is dense, stained in red. The ovules are bitegmic (Fig. 5B-D), anatropous, antitropous (Fig. 5C-D) with axile placentation. The outer integument is formed by about six layers of cells, while the inner integument has approximately six to nine layers of cells (Fig. 5D). The outer epidermis of the inner integument has phenolic compounds, stained in purple (Fig. 5 C-D). The nucellar beak is short and does not reach the endostome (Fig. 5D).

This most remarkable characteristic of the ovule is the presence of a very long aril (Fig. 5A-C) this structure is not reaching the micropyle, instead of that the structure cover the micropyle and almost the entire ovule (Fig. 5 A, C-D). In the chalaza region, the vascular bundle branches, forming two post-chalazal bundles that enter and reach until the middle layers of the into the inner integument (Fig. 5C).

Vasculature

The floral stele is a siphonostele formed by 18 or more vascular bundles in the receptacle (Fig. 6A). At the level of calyx, three traces diverge from the stele to the sepals (Fig. 6B), and the gaps left by them are replaced by other bundles. Each sepal trace branches into three and the marginal bundles branches again (Fig. 6B), generating at least eleven bundles that enter in the sepal (Fig. 6C-D).

Figure 4. Floral structure of pistillate flowers of *Piranhea trifoliata*. A, tricarpellary gynoecium with two ovules per locule. B, sepals and petals. C, nectariferous disc. D-F, staminodes fused in the nectary. D, small lobes, detail of the short portion of the nectary. E-F, long and thin staminodes. G, three curved stigmas. H, surface of the stigma covered by trichomes. I, glandular trichomes on the stigma. gtc, glandular trichome. n, nectary. ovr, ovary. p, petal. s, sepal. sg, stigma. std, staminodes.

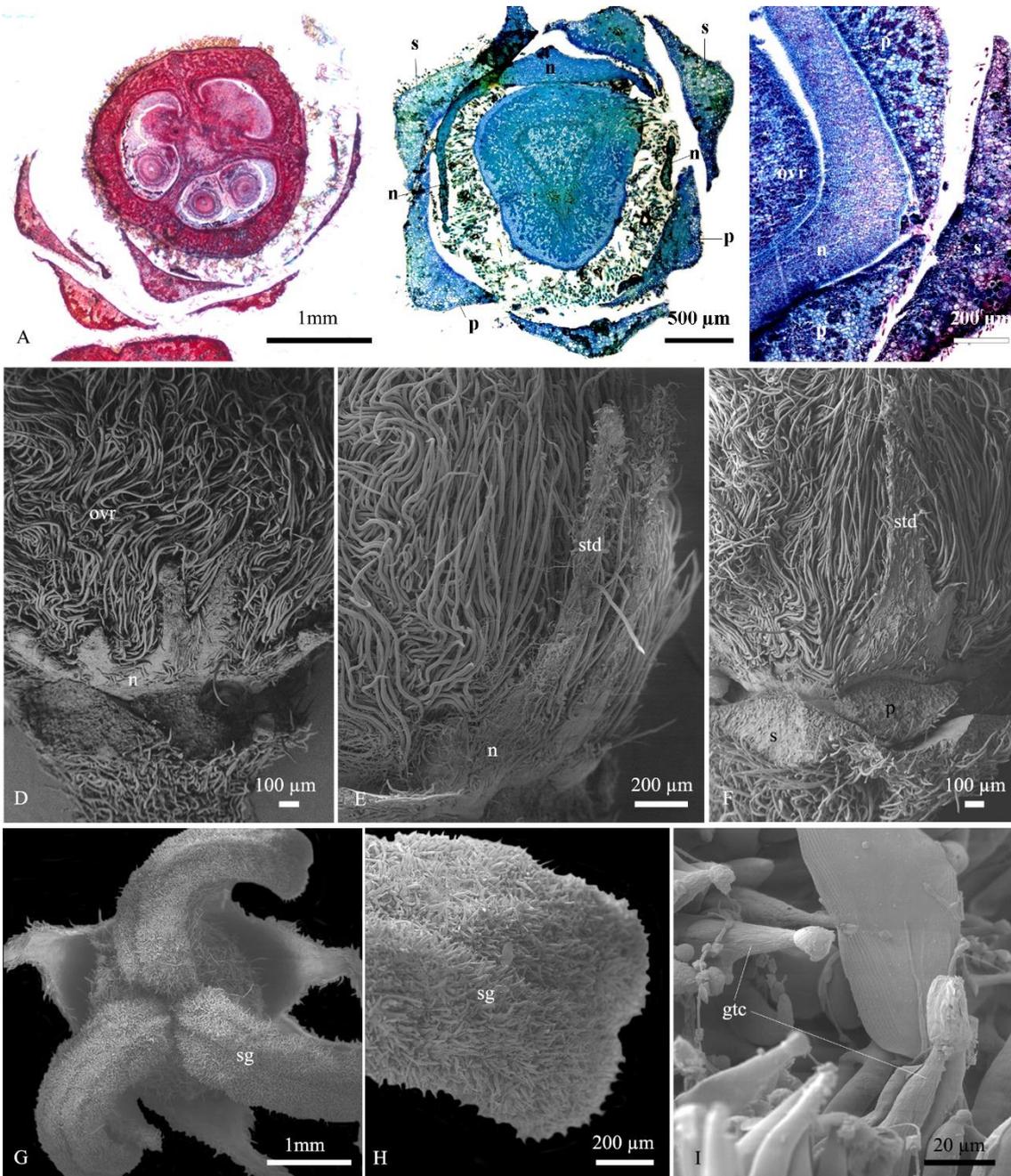
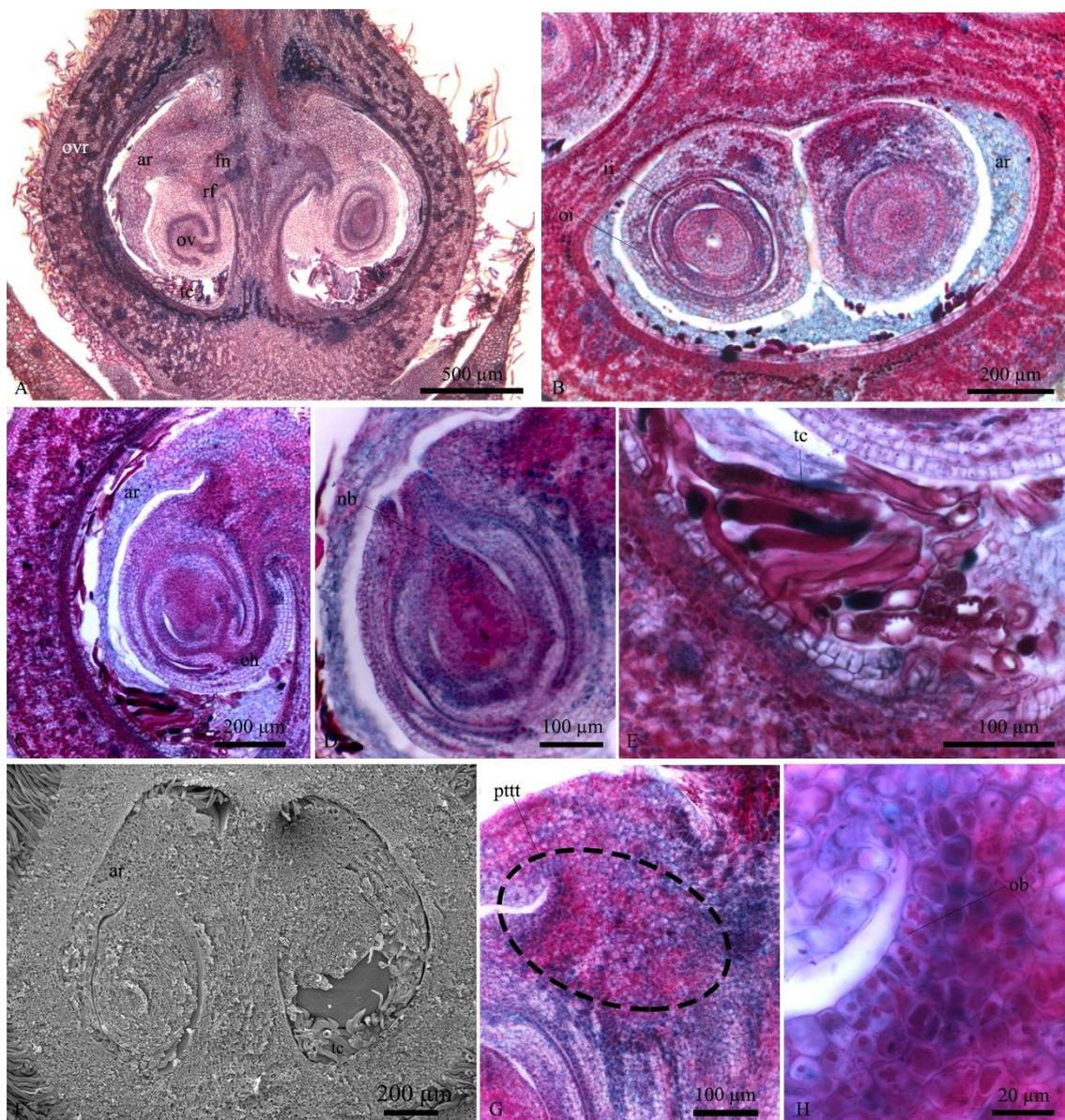


Figure 5. Ovule structure of *Piranhea trifoliata*. A, gynoecium, note the ovary with many trichomes. B, transversal view of the ovules and the trichomes in the inner epidermis of the ovary. C, Ovules epitropous, long aril and the post-chalazal bundle. D, long nucellar beak. E, detail of the lignified trichomes. F, SEM of the ovary showing the trichomes and the aril. G, note the PTTT region in the dashed circle. H, Elongated cells of the obturator.

ar, aril; ch, chalaza; fn, funiculus; ii, inner integument; nb, nucellar beak; ob, obturator; oi, outer integument; ov, ovule; ovr, ovary; pttt, pollen tubes transmitting tissue; tc, trichomes



In the corolla, three fundamental bundles of the stele diverge to vascularize the three petals (Fig. 6C). Each trace may divide in three before reaching the petal base (Fig. 6D), just like the sepals, although in the petal the number of traces is always smaller than that of the sepal (Fig. 6D-E). Each petal receives seven vascular traces (Fig. 6E).

The staminode is not vascularized, although traces of the staminodes were observed. They give out phloematic strands that reach until the base of the nectaries (Fig. 6D) without entering it.

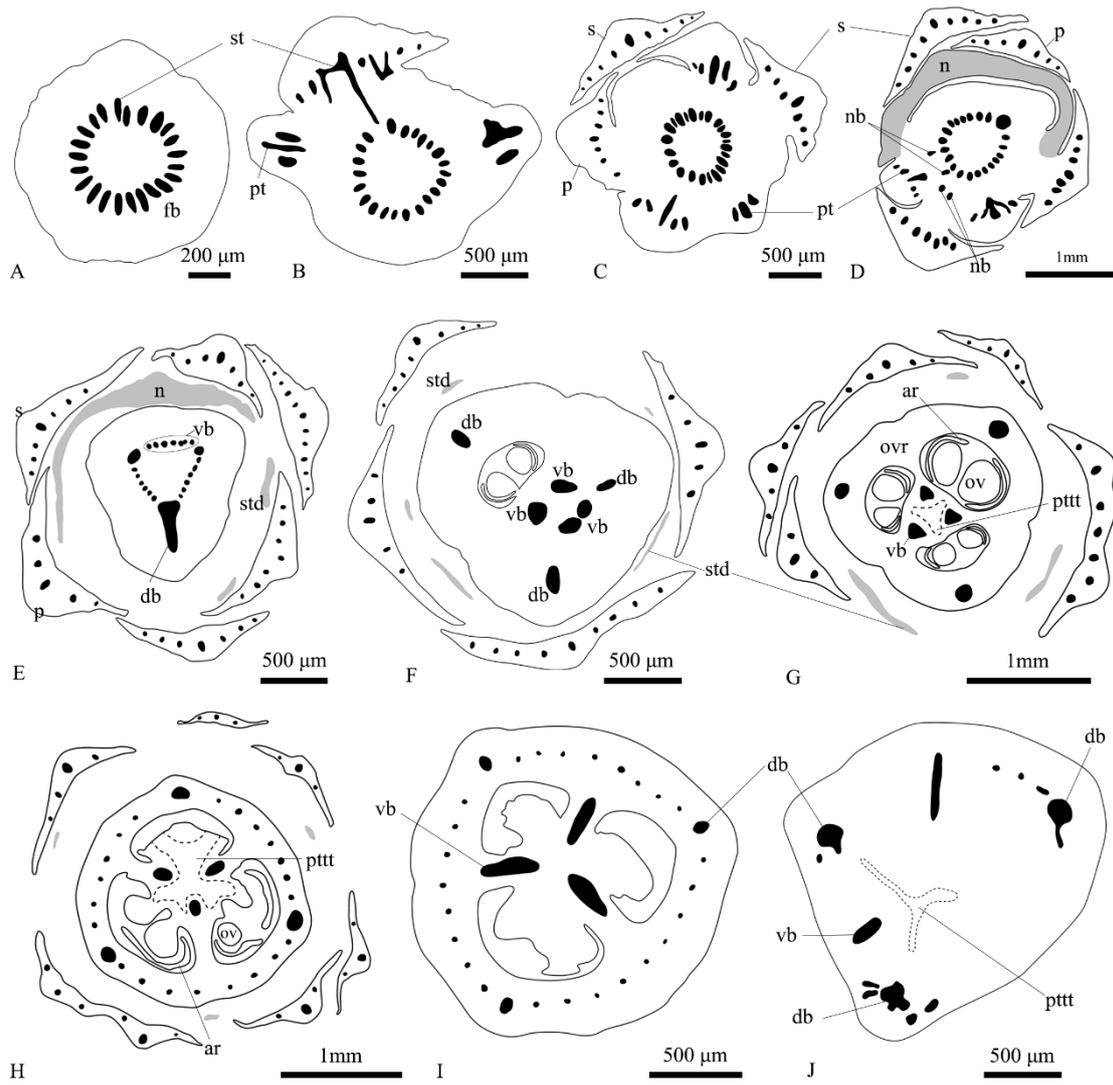
The vasculature of the carpel starts from many remaining bundles in the stele. Three of all these bundles expand to reach each carpel (Fig. 6E), occupying the position of the dorsal bundles Fig. 6F-I). These dorsal bundles will branch into many ones to vascularize the ovary wall (Fig. 6H). The bundles that are still in the stele fuse (Fig. 6E), generating six bundles, that will be the ventral ones (Fig. 6F). The ones from different carpels fuse laterally (Fig. 6E) generating one big ventral bundle positioned in front of the septa (Fig. 6G-H). At this point, the pollen tubes transmitting tissue is seen in the center of the organ (Fig. 6G-H). At the top of ovary, all bundles of the ovary fuse with the dorsal one to vascularize the stigma (Fig. 6 I-J).

Ultrastructural aspects of the nectary in staminate and pistillate flowers

Although the morphological difference between the nectaries in the distinct flower morphotypes, they are similar in the production and release of the substances.

The ultrastructure of the nectariferous cells indicates the production of a heterogeneous secretion (Fig. 7A-B), composed of carbohydrates (Fig. 7B-C) and lipids (Fig 7D-E). This production occurs in the cells of the parenchyma and in the epidermis. The cytoplasm of these cells has electron-lucent droplets, possesses leucoplasts (Fig. 7B), mitochondria (Fig. 7F, Fig. 8A) and endoplasmic reticulum (Fig. 7G). The dictyosomes are rare. The vacuole is central and large.

Figure 6. Vasculature of pistillate flowers of *Piranhea trifoliata*. A, Fundamental bundles at the receptacle. B, divergence of sepals and petals. C, Sepals with more bundles than the petals, note the flower stele filled with bundles. D, Traces diverging from the stele to vascularize the nectariferous disc, note the bundle do not enter the nectary. E, bundles of the stele differentiate in dorsal and ventral ones, note the staminodes. F, ventral bundles fuse into two (vb3), which fuse into one (vb1, vb2). G, the ventral bundles are positioned in front of the septa and in the center, is the PTTT, note the aril next to the ovules. H, the dorsal bundles divided into many smaller ones to vascularize the ovary wall. I, the remnant cells of the ventral bundles extend to the septa. J, the bundles of the ovary wall fuse back with the dorsal bundle to vascularize the stigma. ar, aril; db, dorsal bundle; n, nectary; nb, nectary bundles; ov, ovule; ovr, ovary; p, petal; pt, petal trace; pttt, pollen tube transmitting tissue; s, sepal; st, sepal trace; std, staminodes; vb1, ventral bundles carpel 1; vb2, ventral bundles carpel 2; vb3, ventral bundles carpel 3.



After the production, the secretion is stored at the vacuole and during this stage, the cells exhibit many vesicles located near the plasma membrane (Fig. 8A-B) which transport the compounds from the inside of the cell to its periphery and merge with the plasma membrane, extruding the nectar or, at least, the lipophilic fraction of the nectar compounds (Fig. 8C-D). Then the secretion is observed within a periplasmic space (Fig. 8E) before crossing the cell wall to reach the nectary surface. The cuticle is very thin and during the nectar transport through the cell wall some regions of the cuticle detach and the nectar is accumulated in the subcuticular space before its release to the surface.

At the end of the secretory phase, after the end of the nectar production, the cells produce electron-dense substances and deteriorate their content in a process of senescence or cellular degeneration (Fig. 8F).

DISCUSSION

Inflorescence

In the flowers of *Piranhea trifoliata*, opposing previous descriptions for the genus (Webster, 1994b; Radcliffe-Smith, 2001), we found two perianth whorls, which can be distinguished as calyx and corolla, based on structural studies, especially when regarding the vasculature, which showed a different number of traces among sepals and petals. The nectaries are different in staminate and pistillate flowers, with the first being interstaminal disc and originated from stamens and the second an annular disc originated from the androecium.

Figure 7. Production of the secretion of the nectaries of *Piranhea trifoliata*. A, cell showing a heterogeneous secretion. B, Vacuole full of hydrophilic compounds, note the leucoplasts with osmiophilic compounds. C, Vacuole with carbohydrates. D, lipids. E, Osmiophilic drops. F, mitochondrias next to the cell wall. G, endoplasmic reticulum in the cytosol. ca, carbohydrates; er, endoplasmic reticulum; le, leucoplasts; lp, lipids; mi, mitochondrias; om, osmiophilic drops.

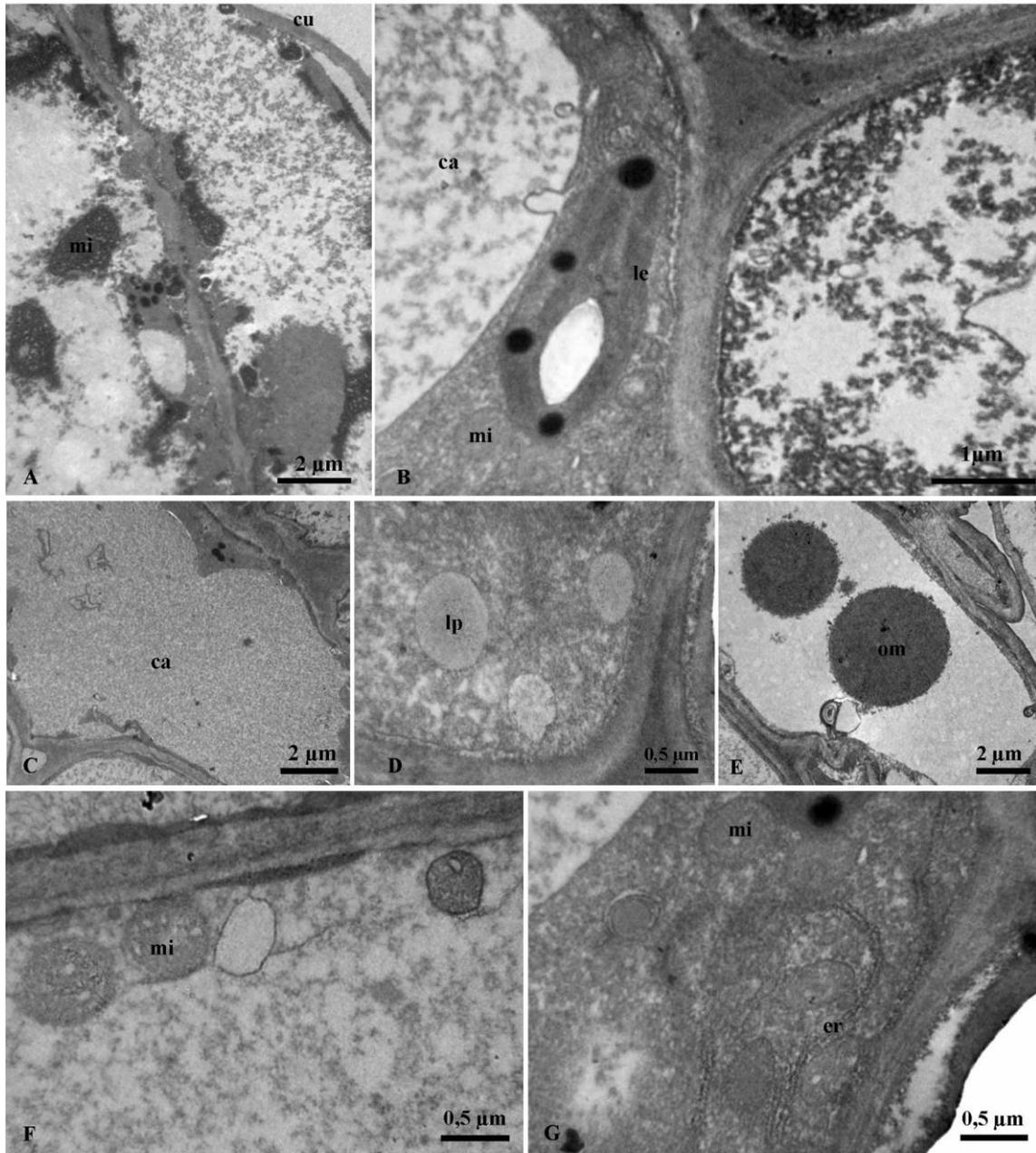
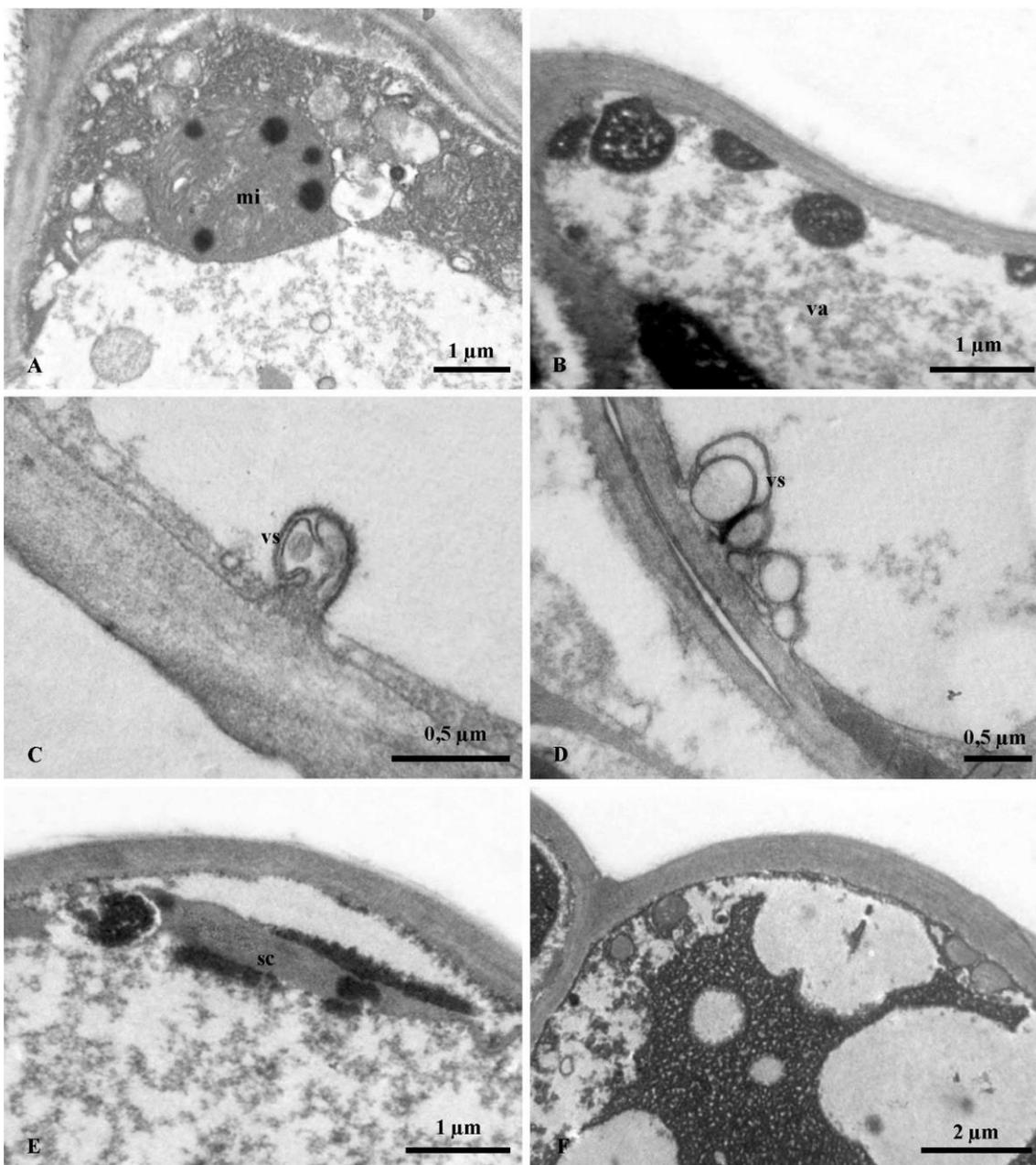


Figure 8. Release of the secretion in the nectaries of *Piranhea trifoliata*. A, vesicles near the plasma membrane. B, vesicles with content near the plasma membrane. C-D, vesicles fusing with the cell walls. E, substance in the periplasmic space. F, Electron dense substances in the cell. mi, mitochondria; sc, secretion; va, vacuole; vs, vesicle.



Pistillate flowers of *P. trifoliata* may have inflorescence with one single flower representing a level of reduction of ramification, leading to uniflorous terminal or axillary inflorescences (Weberling & Hoppe, 1996), this is also common in Phyllanthaceae, the sister group of Phyllanthaceae (Xi *et al.*, 2012), where the pistillate inflorescences often have fewer flowers than the staminate ones (Esser, 2010; Gama *et al.*, 2016).

Perianth: presence of petals and merism

Piranhea trifoliata flowers are trimerous in all whorls, although the third whorl of stamens may present only two organs. The perianth has peculiar differences between the different morphotypes, one of them is the fusion present in the staminate flowers and absent in the pistillate ones.

Merino-Sutter *et al.* (2006) analyzing species from Picrodendraceae said the petals are absent. However, based in the vascularization of the pistillate flowers of *P. trifoliata* that shows different number of traces diverging to the sepals and petals and slightly variation in the morphology in the inner whorl from the outer we affirm the presence of petals and sepals in the perianth, although the perianth of staminate flowers are very similar. In the sister group, Phyllanthaceae, the presence of sepals and petals is known (Michaelis 1924; Ronse de Craene 2010; Gama *et al.* 2016), supporting the idea that *Piranhea* have petals and this character fluctuates in this clade (Phyllanthaceae+Picrodendraceae). Pax & Hoffmann (1931) and Merino Sutter *et al.* (2006) agree that if there are petals in the flowers of this clade, they are not conspicuous.

Petals and sepals in *P. trifoliata* are very similar, mainly in the staminate flowers. Some analysis based only on a morphological observation may not distinguish an organ as a petal or sepal (Endress, 1994) being necessary the results from floral ontogenesis and/or vascularization. This case of *P. trifoliata* when the perianth of staminate flowers

is fused and the pistillate is free is uncommon, although similar event was seen in Cannabaceae, but in this family the staminate are free and the pistillate fused in a short, unlobbed tube or in a ring (McClintock, 1993).

Picrodendraceae can have variation between dimerous to pentamerous in eight genera (Radcliff-Smith, 2011), though trimerous is predominant (Endress *et al.*, 2013) In *P. trifoliata*, the flowers are trimerous and the variation occurs in the third whorl of stamen, when it is present, this whorl has only one or two stamens.

Androecium

The frequency of stamens in the flowers of *P. trifoliata* varies between six and eight but the prevailing number is eight. Webster (1994b) reported that the genus has eight to fifteen stamens, while Radcliffe-Smith (2001) observed eight to sixteen. The stamen reduction observed in the studied species may happen because the six stamens would be sufficient to effect satisfactory outcrossing, just like in the *Stellaria media*, the chickweed, which has a considerably variation in the number of stamens and the research done resulted that neither warmth, duration, and intensity of light, nor times of flowering affect average stamen number and this variation is non-heritable between flowers on a plan (Haskell, 1949).

Gynoecium

Piranhea trifoliata has a huge aril, structure that was considered a strophiole by Radcliffe-Smith (2001). Strophiole, in a general term is an outgrowth on the raphe (van der Pijl, 1972; Fahn, 1990) or a protuberance that will reinforce the normal tissues of the seed situated between the hilum and the chalaza on the trajectory of the principal bundles

(Pitot, 1935). After analyzing many flowers, we assure the difficult of determining the origin of this structure, since the funiculus is extremely short.

The morphological terms of seed appendages are often described from their mature stage, thus paying little or no attention to their origin and histogeny, which reflects in an inconsistent use of the terms (Kapil & Bouman, 1980). Berg (1975) said Picrodendraceae had an aril differentiated as a caruncle, and this description was based in an aril *sensu lato* that may originate from funicle, hilum, raphe, chalaza or exostome (Corner, 1976). However, van der Pijl (1969) classified the various concepts of aril in a view of location, the aril *sensu stricto* is originated from the funiculus while the strophiole from the hilum, raphe or chalaza. In *P. trifoliata* we saw it comes from the funiculus and the obturator have a placental origin.

The concept of obturator is functional, it is a necessary structure for pollen tubes to bridge the gap between the pollen tube transmitting tract and the micropyle (Endress, 2011). Obturators were observed in other Picrodendraceae (see Merino Sutter *et al.*, 2006), and it has a very similar structure with the aril seen in *P. trifoliata*, though the obturators in the others species are short and wide in width. Nevertheless, in *P. trifoliata* the aril structure is very long, covers almost the entire ovule, does not possess the function of an obturator and originates from the funiculus. In *P. trifoliata* the obturator is a group of few secretory cells placed between the aril and the micropyle.

The ovules vascularization in *P. trifoliata* called attention by having post-chalazal bundles that branches and end at the middle of the inner integument length. It was previously reported in Picrodendraceae in *Dissiliaria baloghioides* F. Muell. ex Baill. and *D. muelleri* Baill. by Merino Sutter *et al.* (2006), although in the first species the ovular bundle ends at the base of the nucellus and in the second it branches in the chalazal region and end at the base of the inner integument. Ovules of *Phyllanthus* also have an extended

vascular bundle but it occurs only in the outer integument (Tokuoka & Tobe, 2001). In Euphorbiaceae vascular bundles in the ovules integuments are present (Tokuoka & Tobe, 1995, 2002, 2003), these bundles are in the outer or inner integument and never in both (Endress, 2013).

Staminodes and Nectaries

From the anatomical point of view nectaries diverge broadly in ontogeny, morphology, and structure (Fahn, 1979; 1988; Durkee, 1983; Smets *et al.*, 2000), both between species and within species, depending on flower sexual expression or flower morphology (Nepi *et al.*, 1996; K uchmeister *et al.*, 1997; Pacini *et al.*, 2003; Nepi, 2007). In the staminate flowers of *P. trifoliata*, the nectary is an intrastaminal disc, agreeing with the description of Radcliffe-Smith (2001) to the genus, and in the pistillate flowers it is a disc in the basal region of the staminodes being a novel for the genera.

Floral evolution often exhibited changes in the androecium, generating in either stamen loss or transformation of stamen function from pollen production to alternate purposes (Weberling, 1989; Ronse Decraene & Smets, 1993, 1995). With the loss of their main function as producers of viable male gametophytes, stamens become staminodes (Walker-Larsen & Harder, 2000). In this study, we identified the staminodes by the number, since it has the same merism of stamens in the staminate flowers, position and vascularization, where we verify that the nectary has origin from the united base of the staminodes, like in the staminate flowers.

Staminodes are broadly scattered in the angiosperms (e.g. Walker-Larsen & Harder, 2000; Ronse De Craene & Smets, 2001) and the occurrence of staminodial nectaries or stamens with a common nectariferous base are present in some groups, such as Burseraceae and Anacardiaceae (e.g. *Canarium caudatum* King, *Buchanania*

arborecens (Blume) Blume, *Santiria* cf. *apiculata*, *Pleiogynium solandri* (Benth.) Engl., *Anacardium occidentale* L. and more; Bachelier & Endress, 2009) and although the authors do not affirm it clearly it is possible to see in the images and in the description, where they assert the presence of nectary lobes between sterile stamens appressed to gynoecium. The same structures happen in other Malpighiales, for example Euphorbiaceae (De-Paula *et al.*, 2011), Bonnetiaceae (Dickinson & Weitzman, 1998), Caryocaraceae (Prance & Freitas da Silva, 1973; Dickinson, 1990), Clusiaceae (Robson, 1961; Ronse De Craene & Smets, 1991), Chrysobalanaceae (Matthews and Endress 2008), Linaceae (Brown, 1938; Cronquist, 1981), Passifloraceae (Cronquist, 1981; Bernhard, 1999) and Violaceae (Vogel, 1998; Freitas & Sazima, 2003).

In Euphorbiaceae, group phylogenetically near to Picrodendraceae, *Astraea* Klotzsch and *Croton* L. presents nectaries that are homologous with the stamen whorl, results based in the topology of the nectaries of both species and the vascularization (in *Croton*), these secretory staminodes are a synapomorphy for Crotonoideae (De-Paula *et al.*, 2011).

Staminodes are considered an evolutionary mark and according to Walker-Larsen & Harder (2000) and Ronse De Craene & Smets (2001) these structures are an indication of a changing process, either by the loss or modification of a whole whorl of stamens. In *P. trifoliata*, there is a loss of their primary function of pollen production and a reduction to a filamentous structure. The phylogenetic distribution of staminodes suggests that they typically arise during evolutionary reduction of a partial stamen whorl than from reduction of an entire whorl (Walker-Larsen & Harder, 2000).

In cases where an entire stamen whorl becomes obsolete, stamen remnants may still be identified in flowers, but they will eventually disappear and this process is irreversible (Ronse de Craene, 2010). In the staminodes of *P. trifoliata* there is a

filamentous structure, which is homologous to the filament and the antheroid did not develop indicating a type of reduction and transition series from fertile stamens to staminodes to eventual stamen loss Walker-Larsen & Harder (2000). This process was previously observed in many groups, such as the genus *Bauhinia* L. (Fabaceae: Endress, 1994), Lauraceae (Hutchinson, 1964), and rosid families (e.g., Sterculiaceae: Robyns, 1964; Crassulaceae: Cronquist, 1981; Zygophyllaceae: Ronse De Craene and Smets, 1995).

The nature of the nectary was confirmed in the anatomy and ultrastructure analysis, which showed presence of secretion in the vacuoles. The secretory structure has a mixed nectar, beyond the carbohydrates it also secretes lipids. The nectar in *P. trifoliata* is packaged in vesicles and released to the outside by granulocrine mode of transport.

In the secretory cells, the vacuole was wide and some organelles were present, such as mitochondria, which is involved in energy (Nepi, 2007) and osmiophilic production; leucoplasts and endoplasmic reticulum are involved lipids production of *P. trifoliata*, while the hydrophilic ones are produced by the cytosol. The secretion process is merocrine and the extrusion of the lipophilic and osmiophilic substances are carried out through vesicles reaching the cuticle to goes to the external side, revealing the release process by exocytosis in a granulocrine way. According to Nepi (2007) the vacuole size in nectariferous parenchyma cells varies according to the stage of nectary development, he observed that small vacuoles are present in the pre-secretory phase, and may increase in volume at the time of secretion.

Among Picrodendraceae we found morphological novelties based on structural analyses of *P. trifoliata*, such as the presence of calyx and corolla in the perianth of staminate and pistillate flowers that was distinguished by the number of traces that vascularize each organ; the presence of staminodial nectaries in the pistillate flowers,

which was never acknowledge before; and the resolution of morphological aspects regarding the gynoecium and the presence of a long aril in the ovules.

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Chapter III

Floral structure and development and the wind pollination in *Alchornea sidifolia*
(Euphorbiaceae)

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Abstract

The Euphorbiaceae are pollinated mostly by insects, even though Acalyphoideae has a considerable number of species that are wind pollinated. The aim of this study was to investigate the ontogeny, anatomy and vasculature of flowers of *Alchornea sidifolia* Müll. Arg. in order to find structural aspects related to wind pollination. Flowers and buds of *A. sidifolia* were embedded in Paraplast and sectioned in a rotatory microtome for analysis under light microscopy. SEM was performed for additional structural and ontogenetic data. *Alchornea sidifolia* is a dioecious tree with monochlamydeous, tetramerous flowers. In the staminate flower, calyx begins as a ring on the floral meristem in a congenital connation. The stamens are connate at the base in a short portion and free towards the apex. In the pistillate flowers, the sepals may be postgenitally connate or not; the ovary is postgenitally syncarpous and has a short style and two long stigmata. Our results highlight the development of features of the inflorescence and flowers of *A. sidifolia* which allow wind pollination, such as number of staminate flowers and the expansion of the stigmatic surface. We also discuss the presence of two carpels in *Alchornea*.

Key words: Acalyphoideae, carpels, development, inflorescence, stigma expansion, wind pollination.

Introduction

Euphorbiaceae features a wide morphological diversity in its inflorescences, bracts and flowers. Since the early 2000s the family is restricted to the uniovulate subfamilies Acalyphoideae, Crotonoideae and Euphorbioideae (APG II 2003), and phylogenetic studies have also suggested the segregation of some members of Acalyphoideae, raising the subfamily Cheilosoideae (Wurdack et al. 2005) and the family Peraceae (APG IV 2016).

The flowers in the family are organized in several types of inflorescences, axillary or terminal, in cymes or fascicles, both of which are often arranged along an axis, branched or unbranched, forming a thyrse, panicle or a pseudanthium with very reduced flower (Webster 1994*a,b*), as in *Dalechampia* and *Euphorbia*.

The diversity of pollinators and pollination syndromes is extremely large in this family. It is possible to find species pollinated by wind (Daumann 1972; Korbecka et al. 2011), by generalist insects (bees, flies, wasps, and beetles) (Fiala et al. 2011), and also by bats (Steiner 1982) and hummingbirds (Webster 1994*a*). Most Euphorbiaceae are entomophilous (Webster 1994*a*), although the subfamily Acalyphoideae stands out for having a considerable number of wind-pollinated species. Some morphological features are characteristic of anemophilous Euphorbiaceae, like spiciform inflorescences, inconspicuous monoclamydeous flowers, multifid or lacinate stigmata and a higher number of staminate flowers when compared with the pistillate ones (Faegri and Van der Pijl 1979; Webster 1994*a*). Acalyphoideae is also characterized by the absence of milky latex, staminate flower with valvate sepals, free styles which are long and slender, unlobed stigma or bifid at apex only, ovary 2- or 3-locular (Van Welzen and Bulalacao 2007).

Studies of pollination in Euphorbiaceae are still limited (Webster 1994a), although some references to anemophily are found in the subfamily Acalyphoideae in the genera *Acalypha*, *Alchornea* Sw., *Dalechampia* L., *Mallotus* Lour. and *Ricinus* L. (Webster 1994a; Lewis 1986; Bianchini and Pacini 1996; Araújo et al. 2012; Calaça and Vieira 2012; Rizzardo et al. 2012). Despite the fact that some morphological features allowing anemophily have been identified, little is known about the development of flowers pollinated by wind.

Our aim was to investigate the ontogeny, anatomy and vasculature of flowers of *Alchornea sidifolia* Müll. Arg. in order to find structural features that allow wind pollination in the species.

Material and methods

Inflorescences and flowers in different stages of development were collected at University of São Paulo, Brazil. Voucher specimens were provided and exsiccates were deposited in the SPF Herbarium, *Alchornea sidifolia* = T.S.S.Gama – 5.

Flowers in several stages of development, i.e., meristems, flower buds, and pre-anthetic, anthetic and post-anthetic flowers, were isolated, fixed in FAA (formalin, acetic acid, 50% ethyl alcohol) for 24 h (Johansen 1940) and FNT (neutral buffered formalin) for 48 h (Lillie 1965), and stored in 70% ethyl alcohol.

For anatomical analysis, the material was dehydrated in a butyl alcohol series (Johansen 1940), embedded in Paraplast (Leica Microsystems Inc., Heidelberg, Germany), and sectioned in a Leica RM2145 rotary microtome (Leica Microsystems, Wetzlar, Germany). The sections were stained with safranin and astra blue (Gerlach 1984), and the slides with the sections were mounted in Permount resin (Fisher Scientific, Pittsburgh, Pennsylvania, USA). Photomicrographs were acquired using a Leica DMLB

light microscope (Leica Microsystems, Wetzlar, Germany) using the image archiving software Scan System Images (IM50).

For the ontogenesis were done micromorphological analyses using material fixed in FAA. After the isolation of floral parts, the material was dehydrated in an ethanol series up to 100%, dried in a Balzers CPD 030 critical point dryer (Balzers, Liechtenstein, Germany), mounted on aluminum stubs, sputter coated with gold using a Balzers SCD 050 sputter coater (Balzers, Liechtenstein, Germany) and examined using a Zeiss DSM 940 scanning electron microscope (Carl Zeiss, Oberkochen, Germany).

Photographs were processed using Adobe Photoshop software (Redwood City, CA, USA). Vasculature drawings were prepared from digital photographs using Adobe Illustrator software (Redwood City, CA, USA).

Results

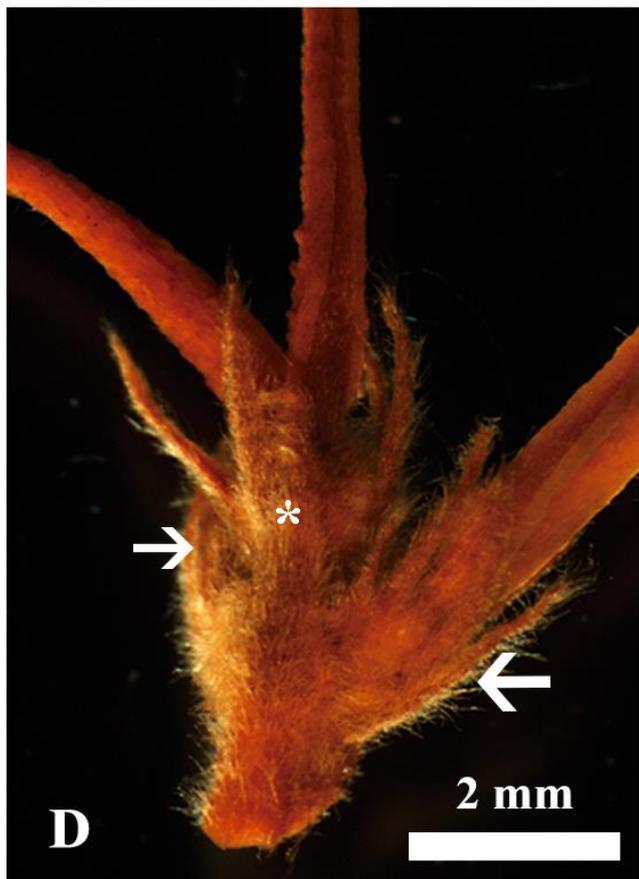
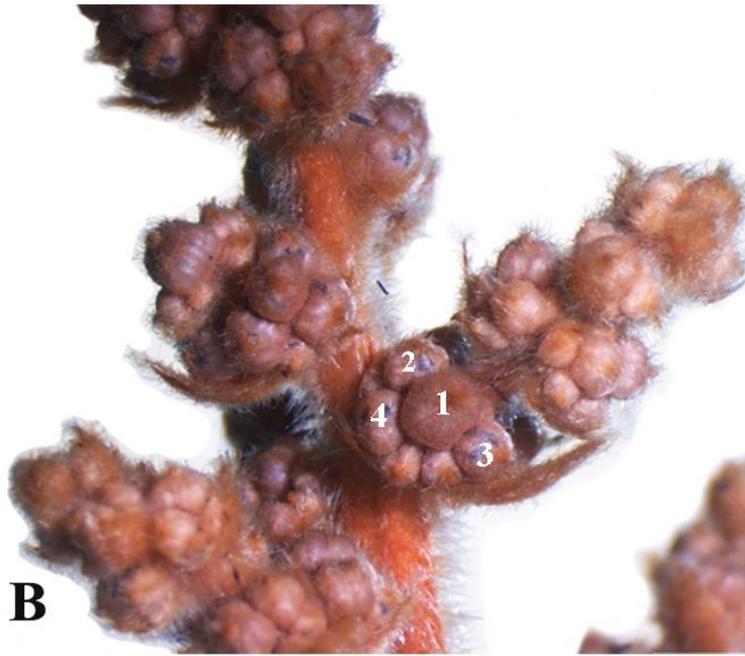
Inflorescences

Alchornea sidifolia is a dioecious tree (Fig. 1, 2 A-B); the staminate inflorescence is a paniculate thyrses with several-flowered, glomerulate cymes (Fig.1 A-B, 2 A). The pistillate inflorescence is a spiciform thyrses with 1 (-3) flowered cymes (Fig.1 C-D, 2 B). Both of the inflorescences present small, rounded extrafloral nectaries in some of their axes (Fig. 1C, inset) in which observed some ants were observed, although these insects were not observed on the flowers.

Flowers

The staminate calyx is 2(3-4)-lobate, valvate, connate at the base, 2- 2.5 mm (Fig. 2C); the androecium is composed generally of eight stamens, rarely more than that, up to twelve. The staminate flower is short pedicellate with a calyx presenting two to four lobes in the anthesis and 8 (-12) monadelphous stamens. The pistillate calyx is 4-lobate,

Fig. 1. Inflorescences of *Alchornea sidifolia*. (a-b) Staminate inflorescences. (a) In the field collection. (b) Stereomicroscope analysis of the inflorescence pattern. (c-d) Pistillated inflorescences. (c) In the field collection. Asterisk, location of the nectary. Inset, detail of the nectary. (d) Detail of the bracts and bracteoles in the inflorescence. Asterisk, bract. Arrow, bracteoles. Image (a) from O.L.M. Silva.



imbricate (Fig. 2D), connate or eventually free at the base, 4-5mm; the ovary is bicarpelar with one ovule per locule.

The flowers are subtended by one bract and two bracteoles (Fig. 2 C-D), which in the beginning of the development have a colleter at the lateral region of these both structures, mainly in the bract (Fig 5B); these colleters are deciduous and fall down before the pre-anthetic phase.

Staminate flowers

Floral structure

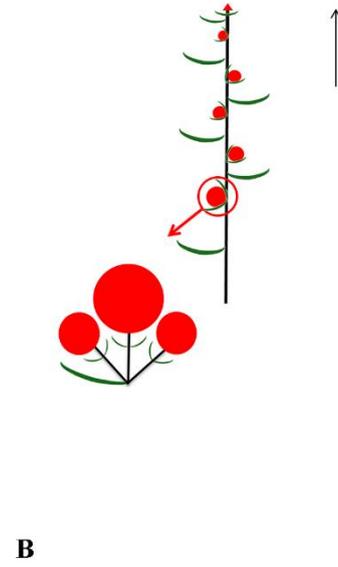
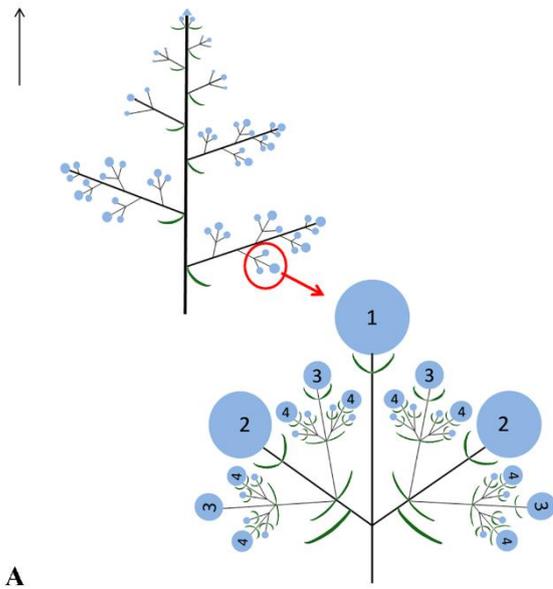
The pedicel has an articulation just below the receptacle (Fig. 3A-B). The four sepals are fused (Fig. 3C), and generally two are larger than the others with tector trichomes on their epidermis, which is uniseriate, composed of small cells with thin walls. The mesophyll is homogeneous with 2-4 layers (Fig. 3C-D).

The connate portion of the filaments is basal (Fig. 3D), short, and free towards the apex; each one is vascularized by a single bundle. The anther is bithecal and tetrasporangiate (Fig. 3E); the epidermal cells are tangentially elongated with a dense cytoplasm. After the development of the pollen grains, the tapetum is consumed, and the middle layers of the anthers wall degenerate (Fig. 3E-F).

Ontogenesis

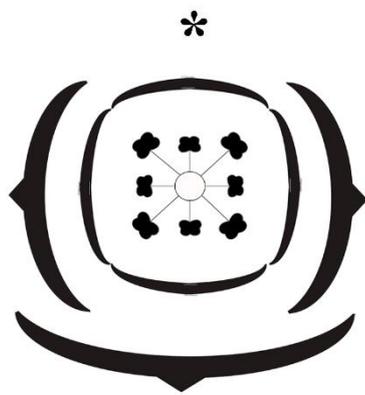
The calyx is the first structure to be formed, beginning as a ring on the floral meristem in a congenital connation (Fig. 3G-H). Next, the sepals elongate generating three or four lobes (Fig. 3I), which grow, protecting the meristematic dome. At this stage, the stamens begin their development, elongating the filament after the initial expansion of the anthers (Fig. 3J). They are free from each other at the beginning and posteriorly become connate at the base (Fig. 3K). The anthers are dorsifixed (Fig. 3K) and release

Fig. 2. Schematic view of *Alchornea sidifolia* inflorescences and the floral diagrams of flowers. (a) Thyse with lateral glomerulous cymes. (b) Spiciform thyse with three-flowered lateral cymes. (c) Staminate flower diagram. (d) Pistillate flower diagram.

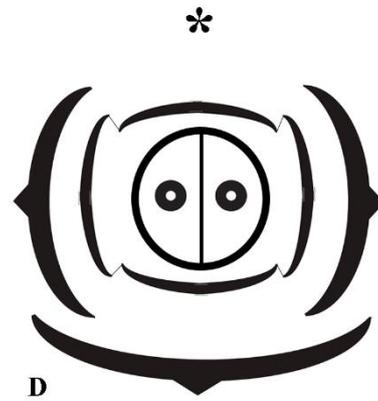


A

B

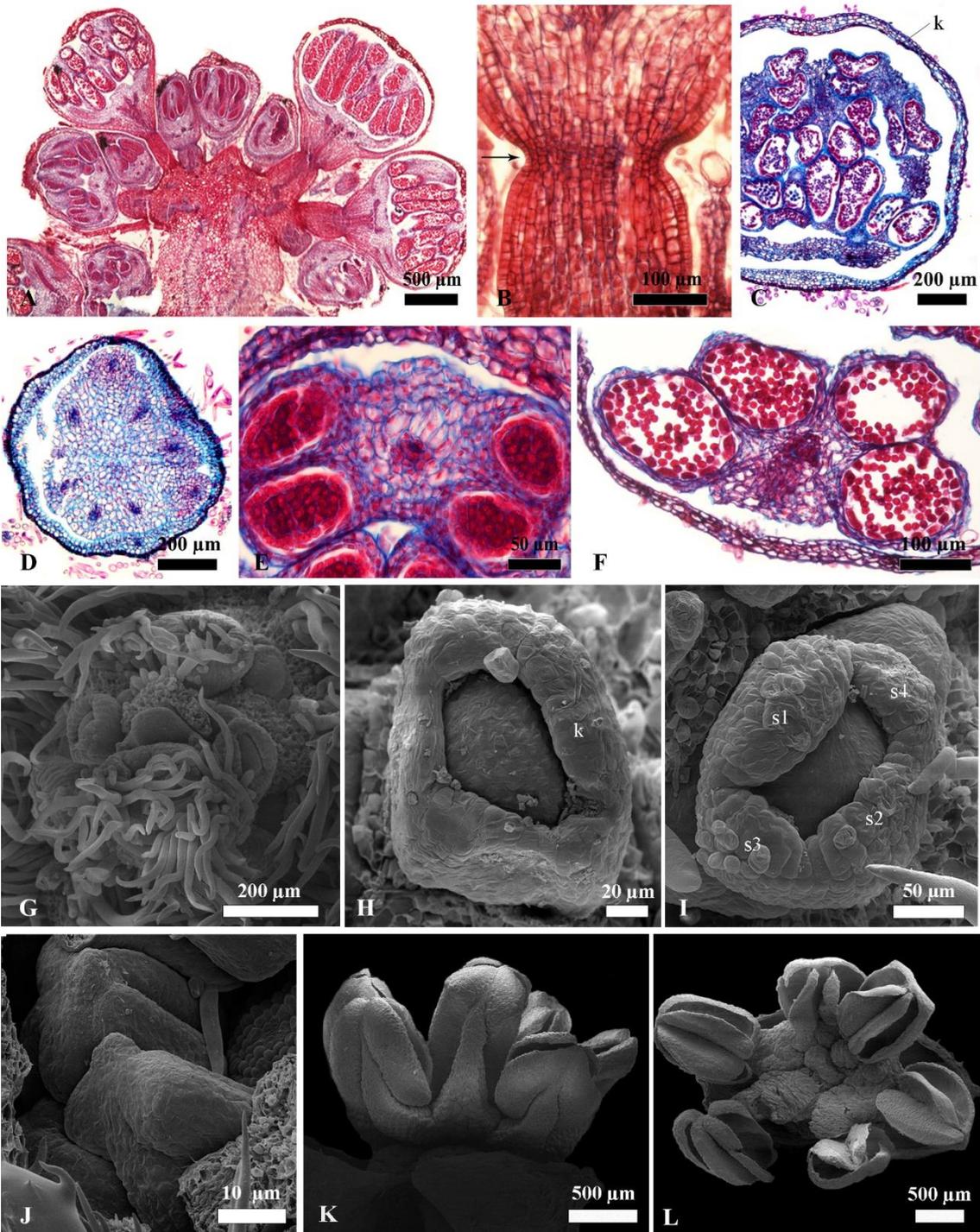


C



D

Fig. 3. Structure and ontogenesis of staminate flowers of *Alchornea sidifolia*. (a) Flowers with a short pedicel. (b) Detail of the articulation in the pedicel (arrow). (c) Fused calyx. (d) Connation of the filaments at the base. (e) Tetrasporangiate anther. (f) Anther without tapetum and middle layer. (g) Inflorescence apex with developing flowers. (h) Congenital connation of the calyx. (i) Sepal elongation showing four lobes. (j) Young anther. (k) Stamens connate at the base. (l) Longitudinal slit of the anther. K, calyx. S, sepal.



the pollen grains through a longitudinal slit (Fig. 3L). When the flowers are senescent, the abscission occurs in the pedicel articulation.

Vasculature

The floral stele is composed of one amphicribal bundle (Fig. 4A). In the receptacle at the level of the calyx, four traces diverge from the stele and vascularize the four sepals (Fig. 4B-C); during this divergence, a pith appears in the center of the floral axis. Each trace reaches the sepal base without ramification and does not leave a gap in the floral stele. This sole bundle of the sepal remains unbranched along the whole organ (Fig. 4D-E).

The androecium is formed by two, or rarely three, whorls, each one with four stamens. The first whorl is alternate with the sepals, and the second is opposite to them. When there is a third whorl, it is alternate to the second one. One trace reaches to each stamen, which will be vascularized by one unbranched collateral bundle in the filament and connective (Fig. 4F).

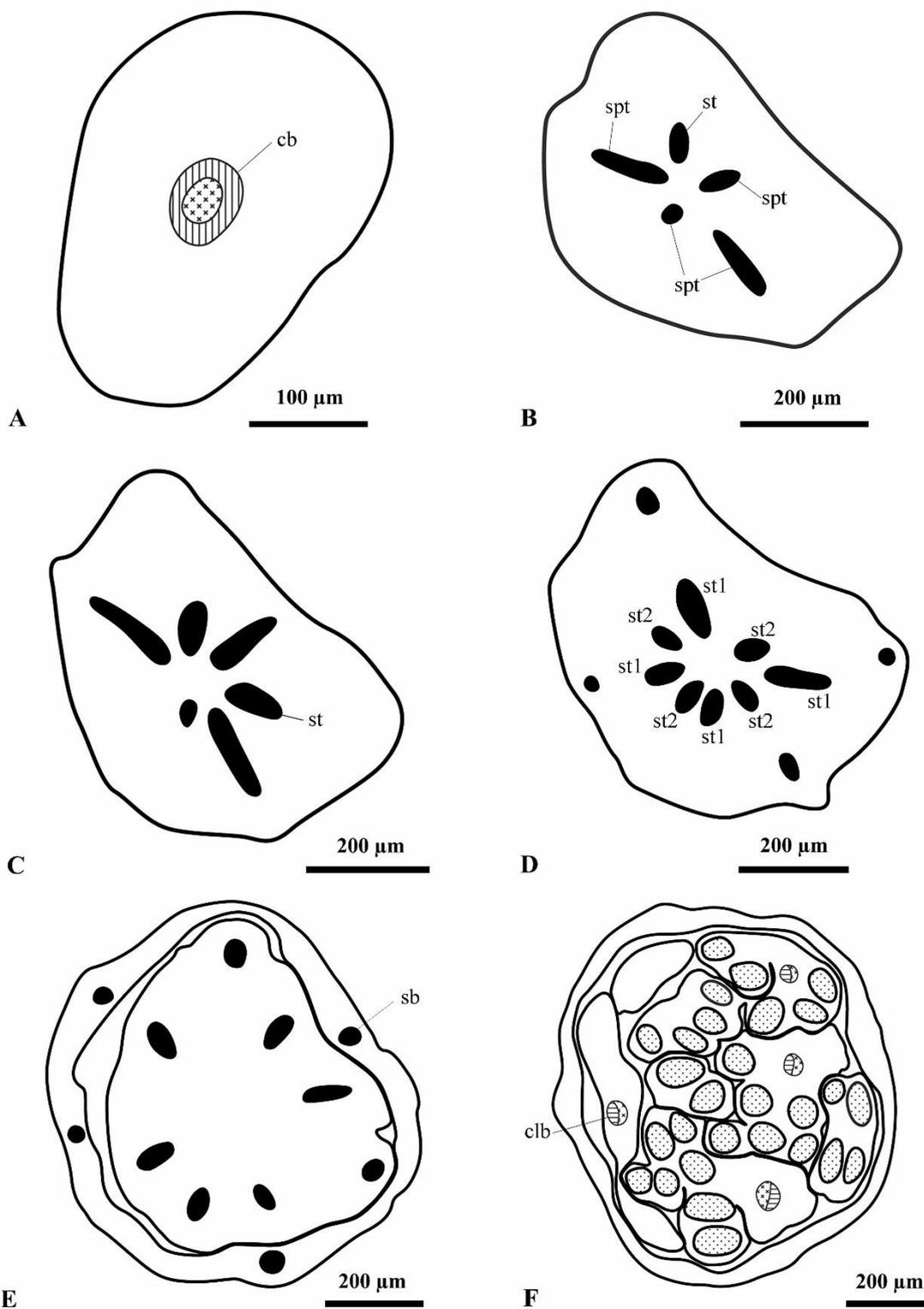
Pistillate flowers

Floral structure

The pistillate flowers have also a short pedicel with an articulation just below the receptacle (Fig. 5A). All four sepals are the same size, connate only in the base or eventually free with a uniseriate epidermis containing many tector trichomes. The mesophyll is homogeneous with 6-8 layers of parenchyma cells and one vascular bundle (Fig. 5B).

The ovary has two flat, reddish stigmata, revolute at the apex (Fig. 5C-E), stigmatic all over the adaxial face (Fig. 5C), slightly unequal in size. The stigmatic epidermis is formed by cubical to slightly elongated secretory cells with dense cytoplasm,

Fig. 4. Vasculature of staminate flowers of *Alchornea sidifolia*. (a) Amphicribal bundle at the pedicel level. (b-c) Divergence of sepals traces from the stele. (d) Unbranched bundle of the sepal and traces of the stamens. (e) Bundles of the sepals and stamens. (f) A collateral bundle in the connective. Cb, central bundle. Clb, colateral bundles. Sb, Sepal bundle. Spt, sepal trace. St, stamen trace.



while the non-receptive surface has cubical cells containing phenolic compounds with stellate trichomes (Fig. 5D-E). The style is very short, solid with two colateral bundles.

The ovary has a uniseriate outer epidermis composed of small, isodiametrical cells with phenolic content and stellate trichomes (Fig 5F). The ovarian mesophyll has three distinct regions: the outer one with small vacuolated cells; the middle composed of several layers of parenchyma cells of different sizes and shapes, idioblasts containing druses, and vascular bundles; and the inner region formed by small parenchyma cells. The inner epidermis is uniseriate with slightly elongated cells (Fig. 5G).

The ovules are antitropous, crassinucellate and bitegmic with four to six layers of cells in the outer and inner integuments (Fig. 5G). The micropyle is zig-zag, and the nucellar beak is short, protruding through the endostome. The placentary obturator is long and makes contact with the nucellar beak. The inner epidermis of the inner integument is differentiated in an endothelium (Fig. 5 G-H) with elongated cells and phenolic content. Some tector trichomes are seen inside the locules (Fig. 5I)

Ontogenesis

The perianth of the pistillate flower has a different origin from that of the staminate one. The sepals are postgenitally connate in a late stage (Fig. 6A-E). When fused, the extent of this union is variable. After the development of the sepals, the gynoecium originates from the remnant cells of the meristematic dome and is postgenitally syncarpous (Fig. 6F). Two protuberances that will originate the stigmata appear on the top of the ovary (Fig 6F-H). These protuberances and the ovary are covered by many tector trichomes. The protuberances will elongate and become the stigma (Fig. 6I). When mature, the adaxial surface of the stigma becomes papillose (Fig 6J).

Fig. 5. Floral structure of pistillate flowers of *Alchornea sidifolia*. (a) Flower with an articulation in the pedicel. (b) Bicarpelar ovary. (c) Sessile style and long stigma. (d) Convex apex of the stigma. (e) In middle region the stigma is flat. (f) Ovary with trichomes in the epidermis. (g) Endothelium stained red at the inner integument. (h) Young ovule showing the protruding nucellar beak. (i) Tector trichomes inside ovarian locules. Bt, bract. Btl, bracteole. Ed, endothelium. Ii, inner integument. nb, nucellar beak. ob, obturator. oi, outer integument. s, sepal. Sg, stigma. tc, tector trichome. Tt, transmitting tissue.

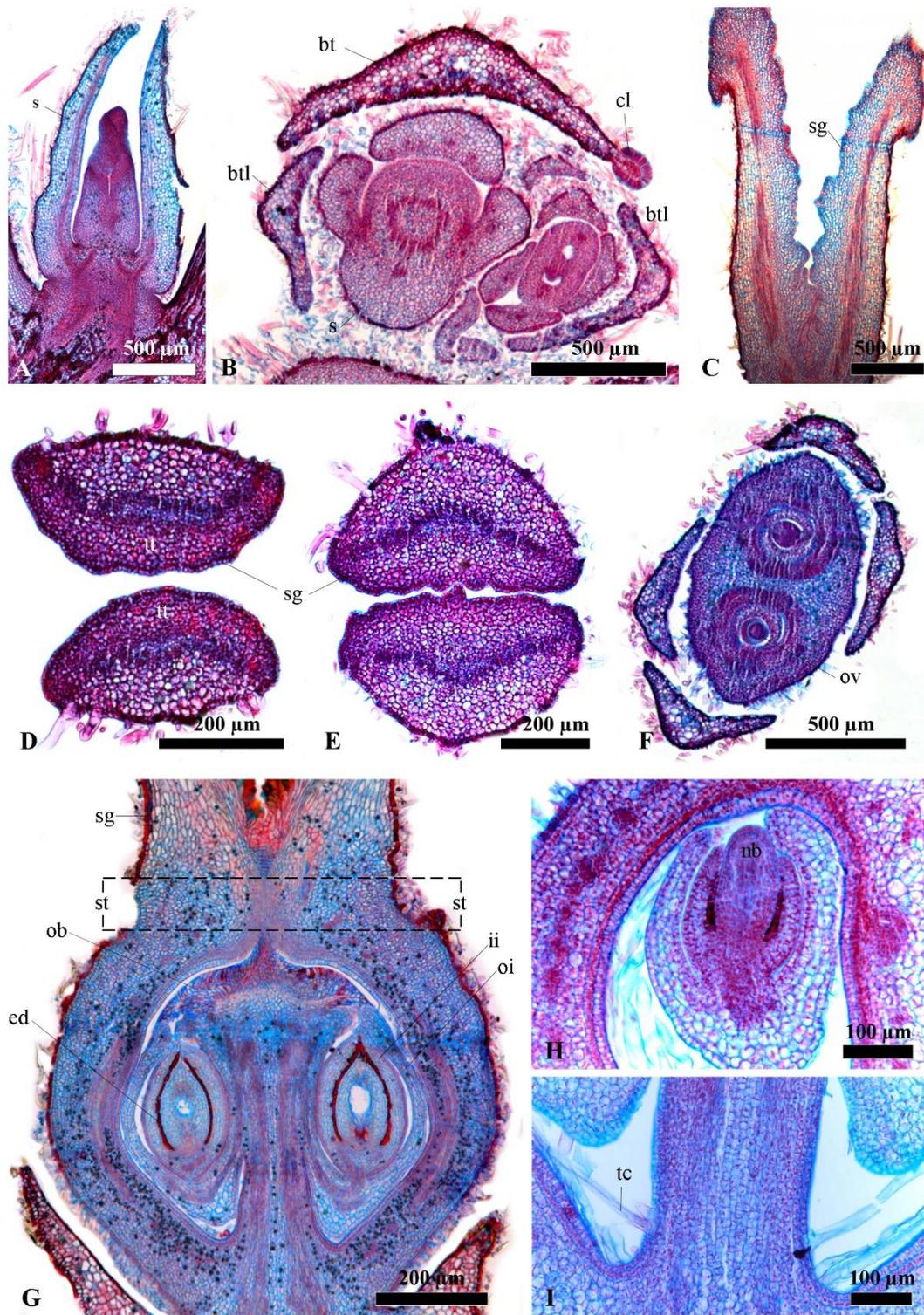
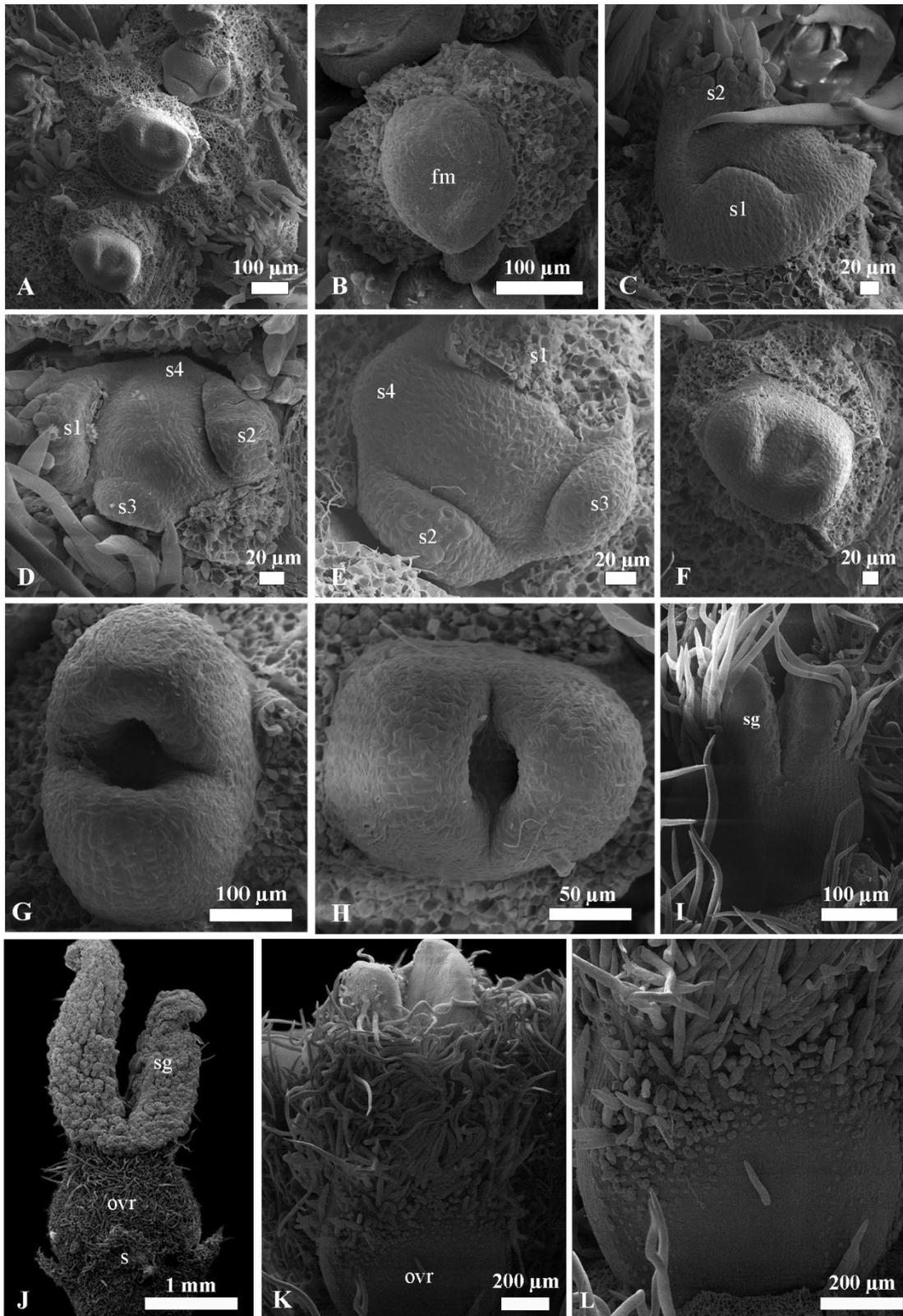


Fig. 6. Ontogenesis of pistillate flowers of *Alchornea sidifolia*. (a) Inflorescence apex with flowers in different stages of development. (b-e) Flower meristem showing sepal primordia. (f) Formation of the gynoecium. (g-h) Carpel closure. (i) Elongation of the stigma. (j) Papillose and irregular surface of the stigma. (k-l) Trichomes on the ovarian epidermis.



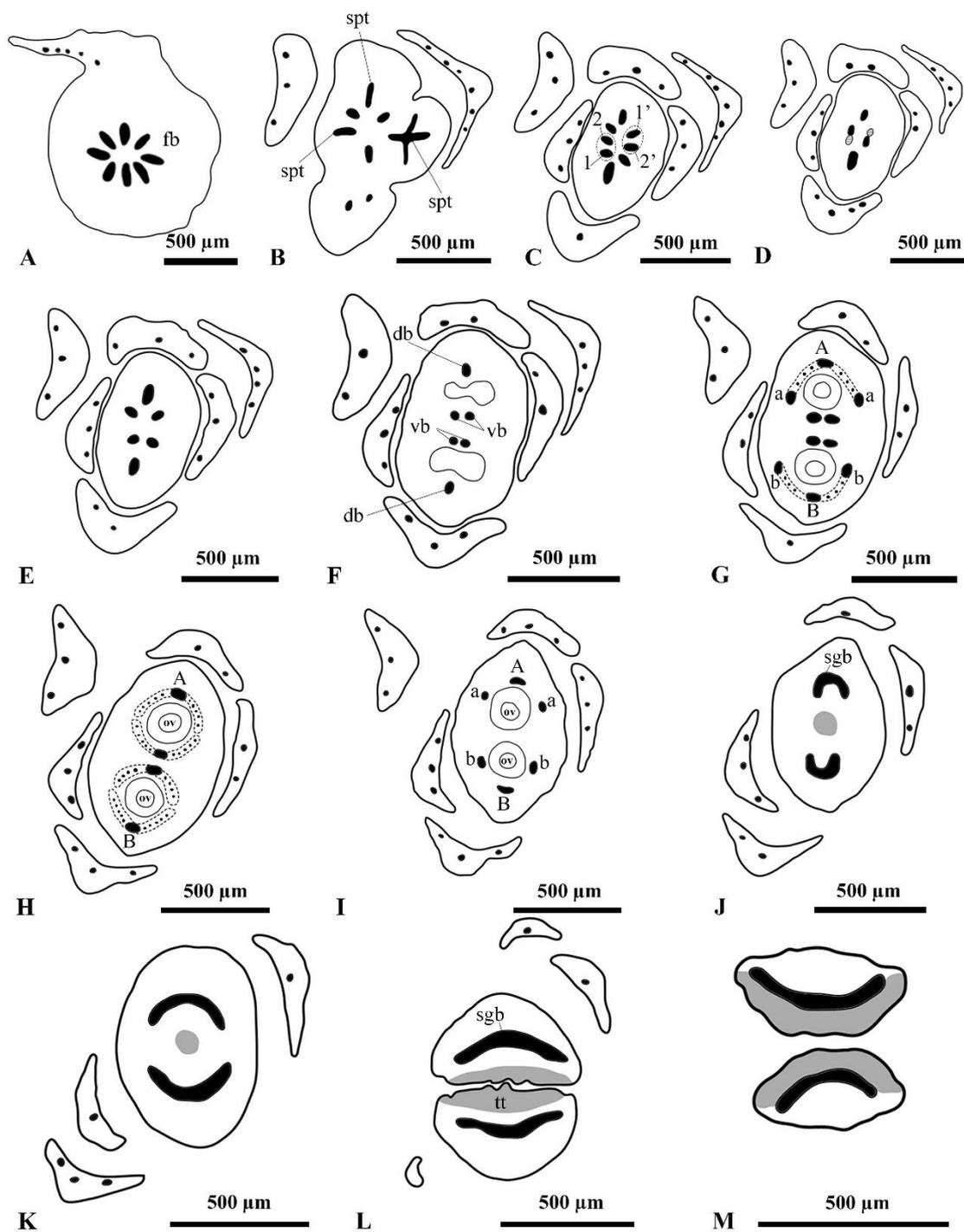
In the initial stages, the vestiture of the ovary has small, sparse trichomes, then becomes densely tomentose in a later stage (Fig. 6K-L). At this point the placental obturator is evident.

Vasculature

The floral stele is an eustele composed of eight vascular bundles at the pedicel level (Fig. 7A-B). In the receptacle, four traces diverge from the stele to vascularize the four sepals without leaving gaps in the floral stele. Each of these ramifies, originating three vascular traces to each sepal (Fig. 7B-D). These three bundles remain unbranched along the whole sepal.

The vasculature of the carpel derives from the eight bundles that remain in the floral stele. Bundle 1 fuses with bundle 2, and bundle 1' fuses with 2' (Fig. 7C-D), generating six vascular bundles in the ovary, three to each carpel (Fig. 7E-F). Then, in each carpel one dorsal bundle and two ventral ones remain (Fig. 7F). The dorsal bundle divides into many small bundles around the locule, three of which are larger than the other (Fig. 7G). The two ventral bundles fuse into one (Fig. 7H), which also divides into many other small bundles in the inner region near the septum (Fig. 7H). The largest ventral bundle vascularizes the ovule. In a higher level of the flower, the inner bundles gradually merge with the ventral bundle, which extends through the funiculus, where it will vascularize the ovule and then end. At the same time, the outer bundles merge with the dorsal one (Fig. 7I), entering into the style from its base (Fig. 7J), then reaching the level of the stigma in the form of an arc (Fig. 7J), present until the apex of the stigma (Fig. 7K-M). At the base of the stigma the vascular bundle is concave (Fig. 7L), becoming convex at the apex (Fig. 7M).

Fig.7. Vasculature of pistillate flowers of *Alchornea sidifolia*. (a) Eustele with eight vascular bundles in the pedicel. (b) Divergence of four sepal traces in the receptacle. (c) Fusion of the bundles of the carpel; bundle 1 fuses with bundle 2, and bundle 1' fuses with 2'. (d-e) Result of the bundles fusion, six vascular bundles for the gynoecium. (f) External bundles differentiate into dorsal bundles and the inner into ventral bundles. (g) Division of the dorsal bundle into small ones located around the locule. Note that three are bigger than the others. (h) Fusion of the ventral bundles and its subsequent division into many small bundles, located in the inner region near the septum. (i) Ventral bundle extends through the funiculus and ends, and the outer bundles merge with the dorsal bundle through the stigma. (j-k) Dorsal bundle forming an arc in the stigma. (l) Concave bundle at the base of the stigma. (m) Convex bundle at the apex of the stigma. Db, dorsal bundle. Fb, fundamental bundle. Sgb, stigma bundle. Spt, sepal trace. Tt, transmitting tissue. Vb, ventral bundle.



The transmitting tissue is central and it is present in the connate base of styles as a central strand. In the stigma, it is located just below the epidermis, as a wide tissue near the adaxial surface of each style.

Discussion

The great number of staminate flowers produced on large panicles versus the few pistillate ones produced on spiciform inflorescences (in addition to the inconspicuous perianth of both), the absence of nectaries in the flowers and the large stigmata of *Alchornea sidifolia* are evident anemophilous characteristics and fit with the characterization of anemophilous pollination syndrome (Stebbins 1970). Although, in general, most Euphorbiaceae flowers are inconspicuous and all are diclinous, most of the family is pollinated by insects (Webster 1994a), while the anemophily is mostly restricted to the subfamily Acalyphoideae with a particular emphasis on the genera *Acalypha*, *Macaranga* and *Mallotus* (Webster 1994a). Secco (2004) used the term anemophily when describing the neotropical species of *Alchornea*. However, its anemophilous traits are not evident as in *Acalypha*, *Macaranga* and *Mallotus* the former genera; only through the present structural analysis was it possible to realize the similarity between their flowers and how these features were originated during floral development.

Considering the phylogenetic hypothesis for the family proposed by Wurdack et al. (2005), anemophily seems to be the plesiomorphic state in Euphorbiaceae since most of the original Acalyphoideae emerged as sister to the rest of Euphorbiaceae s.s.

High production of pollen is one of the main features of anemophily and is observed in *A. sidifolia* due to its inflorescence type. According to Bullock (1994), the flowers must produce a large amount of pollen grains for this stochastic pollination to succeed. The high pollen amount may be produced due to 1) a high number of pollen

grains per anther and/or stamens per flower or 2) a higher percentage of male flowers per plant (in relation to the female plants) and/or percentage of male plants (Cruden 2000). In *A. sidifolia*, the staminate plant has a higher number of staminate flowers per plant in relation to the pistillate plant due to the much more ramified inflorescences, resulting in a high pollen-ovule ratio.

Nectaries similar to those on the leaves of *Alchornea* already described by Van Welzen and Bulacao (2007) and Secco (2004) were observed in the inflorescences of *A. sidifolia* for the first time in the species. These nectaries can be a food source for ants, which were observed climbing through the flowers, like in *Alchornea cordifolia* Müll. Arg. (Schnell et al. 1963). Bentley (1977) suggested that the ants protect the plants against herbivores, while Faegri and Van der Pijl (1979) doubt that insects associated with the extrafloral nectaries participate in the pollination process. On the other hand, two species of Acalyphoideae, *Mallotus japonicus* and *M. wrayi* have been cited by Yamasaki and Sakai (2013) as ambiphilic since they are pollinated by wind and insects.

Particular features of the fertile whorls of the flowers

Androecium

Radcliffe-Smith (2001) described the base of the united filaments of *Alchornea* as having a “small disc” and Van Welzen and Bulacao (2007) mentioned that the stamens of *A. rugosa* (Lour.) Müll. Arg. and *A. tiliifolia* (Benth.) Müll. Arg. are inserted on a ring-like collar of the receptacle, although no secretions were observed in both species, similar to *A. sidifolia*.

Gynoecium (stigma and style)

The large receptive surface of the pistillate flowers of *A. sidifolia* results from the differentiation of the protodermis of the adaxial face of the styles in a receptive area. Major studies in the genus describe the species as having long styles with a rough adaxial

face and the stigma on the top (e.g., Secco and Giulietti 2004; Santos and Caruso 2015). However, our data demonstrate the adaxial face of what is considered the style has a secretory epidermis corresponding to the receptive surface, indicating that this entire structure is a stigma. In addition, the transmitting tissue subtends the entire length of this secretory tissue. The developmental stages of gynoecium formation show that the transformation of the protodermis into a receptive surface is related to the unfolding of the presumed style region. Webster (1994*b*) noticed this different nature of the styles in the subtribe Alchorneinae and affirms they are often stigmatiform or dilated. The absence of previous records about the correct location and length of the stigma in the genus likely occurs due to the lack of a profuse secretion which could be seen with the naked eye, which led Heslop-Harrison and Shivanna (1977) to classify it as dry stigma.

Gynoecium (ovary)

The 3-carpelar gynoecium is one of the main characteristics of Euphorbiaceae, although a few genera may have two carpels, as in *Alchornea*; one, as in some species of *Croton* and *Jatropha*; or six to more, as in *Hippomane* and *Hura* (Webster 1994*a*). It is likely that the ancestor of *Alchornea* had three carpels since there are a few species in the genera with this characteristic (Secco 2004). In addition, only two carpel primordia originate in the floral meristem of *A. sidifolia*, but there are eight fundamental bundles, which are reduced to six vascular traces prior to vascularize the gynoecium (three for each carpel); the extra original trace from each carpel might belong to a suppressed third carpel, which does not develop.

Considering the phylogeny of Van Ee et al. (2011), the reduction of carpels in *Croton* must be important in the evolution of the genus because three groups with this characteristic have been recognized: the monospecific sections *C. sect. Crotonopsis* with *Croton michauxii* G.L. Webster having one carpel but three styles; *C. sect. Eremocarpus*

with *C. setiger* also having one carpel; and *C. sect. Quadrilobi* with the Brazilian species *C. sapiifolius* generally having two carpels. In the Alchorneoid clade, which emerged in the phylogeny of Wurdack et al. (2005), only *Alchornea* and the monospecific *Gavarretia* have two carpels.

As is common in the family, *A. sidifolia* has ovules with thick inner integument, nucellar beak and placentary obturator formed by trichomes which grow into the micropile and make contact with the nucellar beak in a similar way to those observed in *Euphorbia* (Bhanwra 1987; Tokuoka and Tobe 2003).

Relationships with pollination

Studies of pollination in Euphorbiaceae are still scarce and few genera have been investigated in this regard (e.g., Lewis 1986; Calaça and Vieira 2012; Rizzardo et al. 2012; Bianchini and Pacini 1996). Although it is known that the family is predominantly entomophilous, anemophily is widespread in Acalyphoideae, very rare in Crotonoideae and missing in Euphorbioideae (Webster 1994a).

The development and structure of inflorescences and flowers of *Alchornea sidifolia* are characteristic of anemophilous syndrome; however, the nectaries present in the rachis of the inflorescence might be related to a possible ambophily. New studies of the pollination biology for the genus are needed to verify this hypothesis.

Acknowledgements

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Chapter IV

Floral structure of *Microdesmis caseariifolia* (Pandaceae)

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Running title: Flowers of *Microdesmis* (Pandaceae)

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Abstract

Pandaceae is a family with 16 species organized in three genera, *Microdesmis* is a largest and palaeotropical genus of 10 species, eight in Africa from the subg. *Microdesmis* and two in Asia, subg. *Ganitrocarpus*. The flowers are considered functionally unisexual and have many characteristics that distinguish it from Euphorbiaceae, Family from which it was segregated. However, the absence of studies related to the floral structure prevents the analysis of its morphological evolution and generates some divergences in the literature. The aim of this work was to investigate the floral structure of *Microdesmis caseariifolia* based on anatomical and micromorphological analyses. The flowers have calyx and corolla that are morphologically different. The staminate flowers have ten stamens and eventually nine, which changes the vascularization of the central pistillode. The pistillate flowers have a bicarpellary gynoecium with one ovule per locule and placental obturator. This is the first register of floral anatomy to the family that presents structural novelty, such as the presence of an obturator.

Keywords: Vasculature, anatomy, androecium, obturator, pistillode.

Introduction

Pandaceae (Malpighiales) is a short family comprising three genera, the monotypic *Panda* Pierre, *Galearia* Zoll. & Moritzi and *Microdesmis* Hook. f. (Van Welzen 2011). The family was part of the tribe Galearieae (Radcliffe-Smith 1987; Meeuse 1990; Govaerts et al. 2000), subfamily Acalyphoideae in Euphorbiaceae *s.l.* (Webster 1994; Radcliffe-Smith 2001), although morphological differences between the Euphorbiaceae *s.s.* and Pandaceae were already known, like the columella present in fruits of Euphorbiaceae and absent in Pandaceae (Van Welzen 2011), the rays in the wood which is wider in Euphorbiaceae and narrow in Pandaceae (P. Bass in Van Welzen 2011) and the obturator in the seed, which studies shows Pandaceae does not have (Radcliffe-Smith 2001; Merino-Sutter et al. 2006). The molecular analysis added data supporting these differences, it divided Euphorbiaceae *s.l.* into six families, one of these is Pandaceae (APG IV 2016).

The family lacks simple diagnostics characters and it is generally identified by the generic level (Van Welzen 2011). *Microdesmis* has two species occurring in Southeast Asia, *M. caseariifolia* Planch. ex Hook. F and *M. magallanensis* (Elmer) Steenis, both are part of the subgenera *Ganitrocarpus* and although they seem very similar, *M. caseariifolia* present a long apiculate appendage in the connective of the stamens.

Microdesmis has dioecious plants that are shrubs to small trees. The flowers are organized in axillary fascicle, are dichlamydeous and may be tetramerous or pentamerous (Merino-Sutter et al. 2006; Li and Gilbert 2008; Van Welzen 2011). The staminate flowers of the species from Asia generally have 10 stamens distributed in two whorls and the pistillate flowers can have one to five locules, with one ovule per locule (Li and Gilbert 2008; Van Welzen 2011).

The family is not well known in general, although some studies regarding the potential pharmacological and medicinal were done revealing that the leaves of *Microdesmis* species are used against malaria and some other parasites (Zamblé et al. 2007). The effects of alkaloids in *M. keayana* were addressed regarding the erectile dysfunction topic (Zamblé et al. 2008). *M. caseariifolia* also play its role in the medicinal and construction area, its fresh plant juice is used against caries and its wood is utilized for house construction (Van Welzen 2011). A few publications cover the aspects of morphology and anatomy of the family, two about pollen structure (e.g. Nowicke 1984; Nowicke et al. 1998; van der Ham in van Welzen 2011), one of wood anatomy (Forman 1966; P. Bass in van Welzen 2011), one of leaf idioblasts (Rao and Cheluviah 1988) and two covering aspects of floral development and inner floral structure (Mildbraed 1931; Pax and Hoffmann 1931)

According to Endress et al. (2013) the data of floral structure and biology combined with a good phylogenetic resolution provides foundation for conducting a comprehensive comparative analysis between the subclades of Malpighiales. The same author affirms there are big gaps regarding the cited points in 13 families of Malpighiales development of ovules and embryology.

Aiming to know the floral structure of *Microdesmis caseariifolia* we studied the ontogeny, structure and vascularization, focusing on the androecium and gynoecium, in order to clear divergences in the literature and unravel characters regarding to the flowers morphology in the group.

Material and methods

Inflorescences and flowers in several stages of development were collected at Singapore Botanic Gardens, Singapore. Voucher of the specimens were provided from

the Herbarium of Singapore Botanic Gardens: *Microdesmis caseariifolia* Planch. ex Hook. f.= Samsuri, A. & Gwee, A.T., 2003 (32).

Several flower buds, pre-anthetic, anthetic and post-anthetic flowers were isolated, fixed in FAA (formalin, acetic acid, 50% ethyl alcohol) for 24 h (Johansen 1940) and subsequently stored in 70% ethyl alcohol.

For anatomical studies, the material was dehydrated in butyl series (Johansen, 1940) and embedded in Paraplast (Leica Microsystems Inc., Heidelberg, Germany), transversely and longitudinally sectioned in a Leica RM2145 rotary microtome (Leica Microsystems, Wetzlar, Germany). The sections were stained with safranin and astra blue (Gerlach 1984) and the slides were mounted in resin Permount (Fisher Scientific, Pittsburgh, Pennsylvania, USA). The photomicrographs were obtained in a Leica DMLB light microscope (Leica Microsystems, Wetzlar, Germany) using the Scan System Images (IM50).

For the scanning electron microscopy, the material was dehydrated in ethanol series up to 100%, critical point dried in a Balzers CPD 030 (Balzers, Liechtenstein, Germany), mounted on aluminum stubs, sputter coated with gold using a Balzers SCD 050 (Balzers, Liechtenstein, Germany), and observed in a Zeiss DSM 940 scanning electron microscope (Carl Zeiss, Oberkochen, Germany; Robards 1978).

Photographs were processed using Adobe Photoshop and vasculature drawings were prepared from digital photographs using Adobe Illustrator software (Redwood City, CA).

Results

Flowers

Flowers of *Microdesmis caseariifolia* are dioecious arranged in axillary fascicles on a short peduncle (Fig. 1A, Table 1), with a single to many flowers, pistillate fascicles usually with fewer flowers than staminate ones.

Table 1 Characters between all the genera in Pandaceae

Genera/character	<i>Panda</i>	<i>Galeaeria</i>	<i>Microdesmis</i>	Literature
Inflorescences	Cauliflorous racemes	Terminal or cauliflorous racemes	Axillary fascicles	Webster (1994b) Van Welzen (2011)
Aestivation	± Imbricate	Valvate	Imbricate	Webster (1994b)
Androecium	5+5	(8) 10 (15) single or in two whorls	5 – Africa 10 – Asia	Forman (1966) Van Welzen (2011) Forman (1966)
Ovary	(2-)3-4 locular	2-6 locular	(1-) 2-5 locular	Van Welzen (2011) Webster (1995b)
Ovule	Orthotropous	Anatropous/ Ortotropous	Anatropous	Van Welzen (2011)
Pollen grain	Larger reticulate	Small perforate to microrreticulate	Small perforate to microrreticulate	R.W.J.M. van der Ham (2011)

Flowers are shortly pedicelled, pentamerous and dichlamydeous. The calyx is gamosepalous for a short portion in the pistillate flowers (Fig. 1B) and free at the

staminate, hairy on both sides (Fig. 1C). The petals are twice as long as sepals, also hairy with papilla-like hairs in especially upper half on both sides and the aestivation is imbricate (Fig. 1D).

Staminate flowers

Structure

The staminate flowers have a free perianth, 9-10 stamens organized in two whorls (Fig. 2A), the outer whorl with longer filaments, antesealous, and the inner whorl with shorter filaments, antepetalous (Fig. 2B), the anthers are basifixed, opening by longitudinal slits, connective slender, and a columnar pistillode.

All five sepals have the same size and are formed by a uniseriate epidermis, composed of small cells with thin walls (Fig. 2C); the mesophyll is homogeneous with five to seven layers of parenchymatous cells and some idioblasts with a phenolic content and three collateral bundles

The petals are formed by a uniseriate epidermis of small cells and the mesophyll is homogeneous with five to six layers of parenchymatous cells (Fig. 2D). The petals also have idioblasts with phenolic content, although it appears in a major quantity than the sepals. The mature petal presents three colateral bundles.

Fig. 1 Flowers of *Microdesmis caseariifolia*. **(a)** View of the staminate inflorescence. **(b)** Pistillate flower showing the gamosepalous calyx. **(c)** Upper region of the sepal with trichomes. **(d)** Flowers with imbricate aestivation.

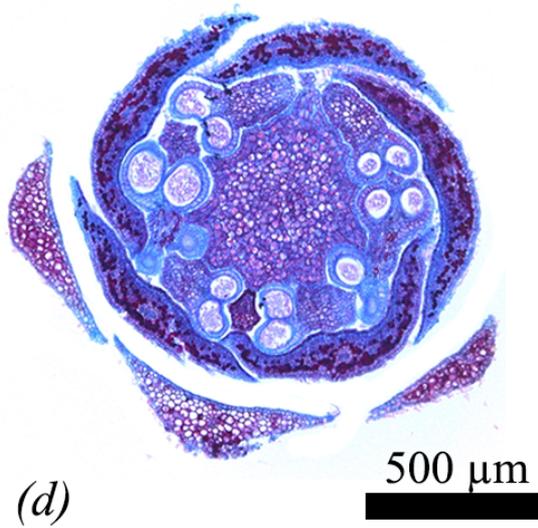
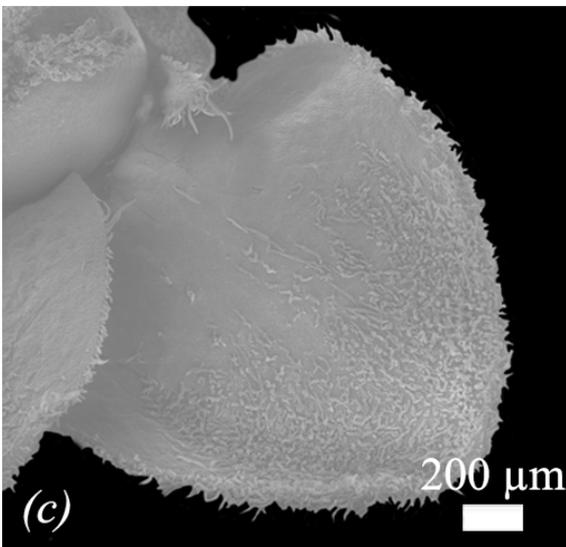
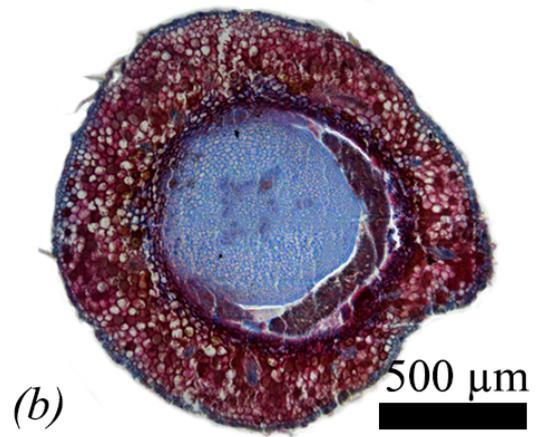
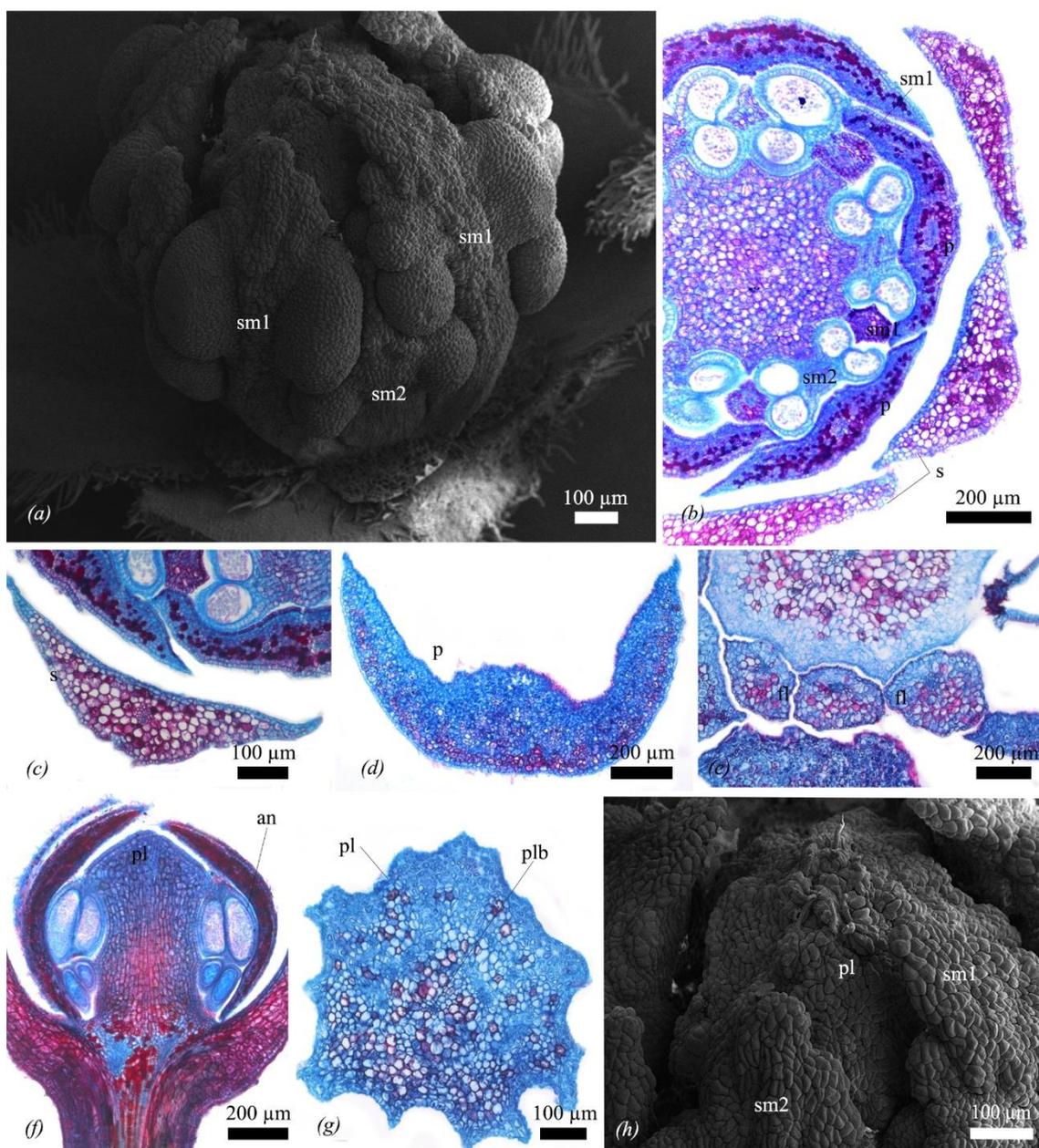


Fig. 2 Structure of staminate flowers of *Microdesmis caseariifolia*. **(a)** Scanning electron microscopy showing two whorls of didynamous stamens. **(b)** First whorl of stamens opositisepalous and second whorl opositipetalous. **(c)** Detail of sepal. **(d)** Detail of petal. **(e)** Filaments with one collateral bundle. **(f)** longitudinal view of the flower showing anthers and the large pistillode. **(g)** Pistillode with sinuose and ondulate epidermis. **(h)** Detail of the pistillode sinuosity in SEM. Abbreviations: an, anther; fl, filament; p, petal; s, sepal; sm1, stamen first whorl; sm2, stamen second whorl.



The stamens are didynamous, with the outer whorl antesealous having five stamens and the inner whorl antepetalous with four to five. They are monadelphous on the base and the filaments column shares only one concentric bundle (Fig. 2E). The anthers are bithecal and tetrasporangiate. The mature anther wall is formed by epidermal cells tangentially elongated and an endothecium composed of palisade cells.

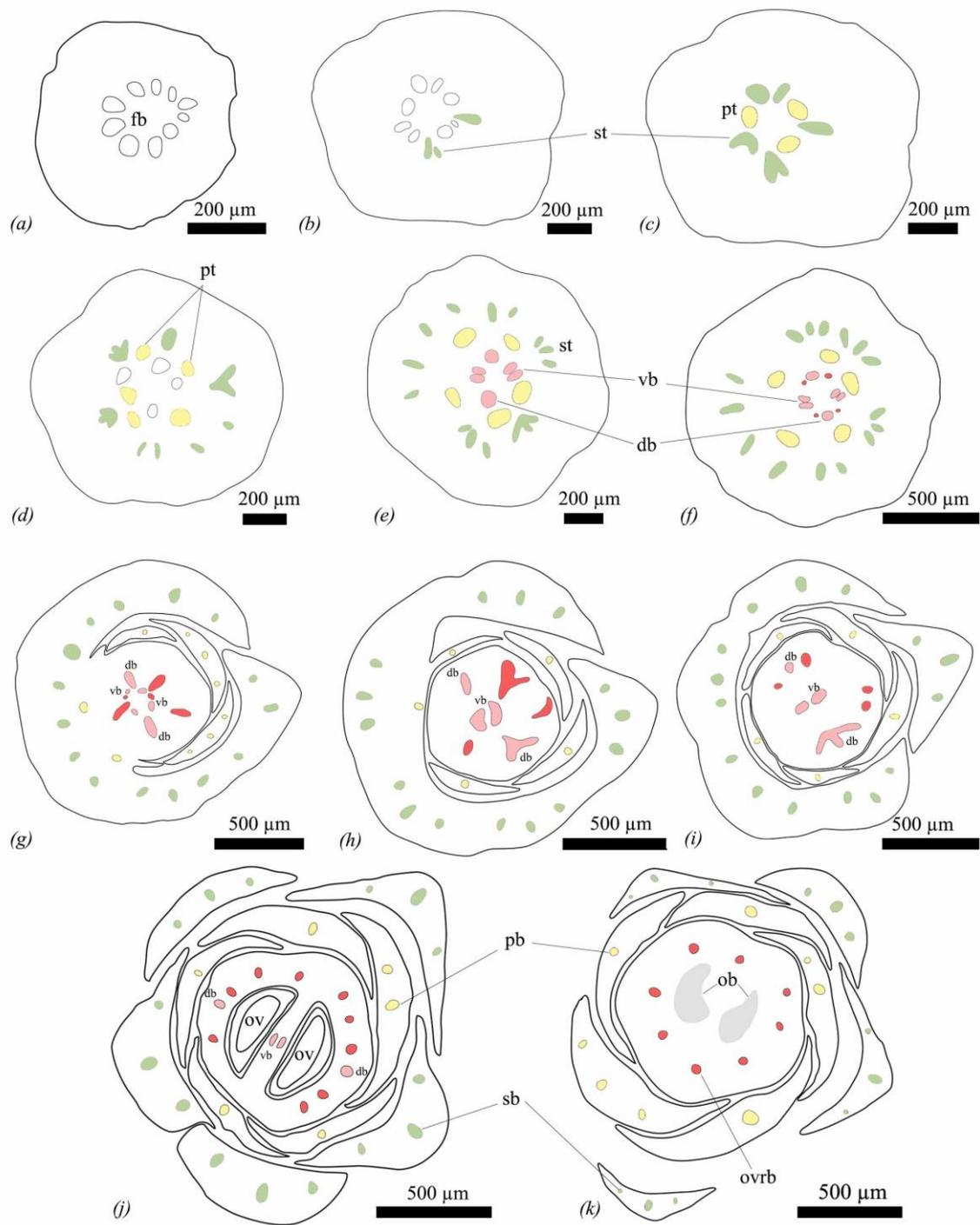
The pistillode is wide (Fig. 2F), round and central, with a uniseriate epidermis, and one eccentric colateral bundle, which becomes central towards the apex. When the flower is in anthesis the pistillode surface becomes sinuose and undulate (Fig. 2G-H).

Vasculature

The floral stele is an eustele composed of ten vascular bundles observed at the pedicel level (Fig. 3A). In the receptacle at the level of first whorl (calyx), 10 traces diverge from the stele and vascularize the sepals. Two trace reaches the sepal base (Fig. 3B-E) and one of them branches (Fig. 3C-E). The traces that branches leave a gap in the floral stele and the one that does not branch does not leave a gap (Fig. 3D-E), which is replaced by another vascular bundle that will supply the petals (Fig. 3E-F), before the divergence of the petal traces it will divide into many, which is enough to vascularize the petals, stamens and pistillode (Fig. 3F-H).

In the next whorl (corolla), five fundamental bundles of the stele diverge to vascularize the five petals (Fig. 3E). After the divergence, each petal bundle divides into three, one central and two laterals, these three bundles of the petals remain unbranched along the whole organ (Fig. 3G-H).

Fig. 3 Vasculature of staminate flowers of *Microdesmis casariifolia*. **(a)** fundamental bundles at the pedicel level. **(b)** beginning of sepal traces (in black) divergence in the receptacle. **(c-d)** two traces leaves the receptacle to the sepals, one of these traces branches. The trace that branches leaves a gap at the receptacle. The trace that does not branch does not leave a gap. In a higher Sepal trace branching and some petal traces (in gray). **(e)** beginning of petal traces divergence. **(f)** petal divide into three bundles. The receptacle traces organize to vascularize the stamens and pistillode. Ten stamen traces (in yellow). **(g)** After the petals divergence start the stamen traces one, note the nine stamen traces and that the pistillode trace is not central. **(h)** at the final stage, the filament column is vascularized by one collateral bundle and the pistillode trace becomes central. Abbreviations: fb, fundamental bundles; Pb, petal bundle; Plb, pistillode bundle; Plt, pistillode trace; Pt, petal trace; Sb, sepal bundle; Smb, stamen bundle; Smt, stamen trace; St, sepal trace.



The filament column is vascularized by one concentric bundle, which corresponds to the remnant of the floral stele (Fig. 3F-H). At this stage, there are 10 traces at the stele (Fig. 3F), although in a higher position of the stele only nine remain (Fig. 3G). One of the traces disappears.

The pistillode is vascularized by one bundle that came from one trace (Fig. 3F). At the beginning this sole trace is not central (Fig. 3F-G) and it is opposite the stamen trace that disappears. Once the stamen trace completely disappears the trace of the pistillode takes a central position (Fig. 3H).

Pistillate flowers

Structure

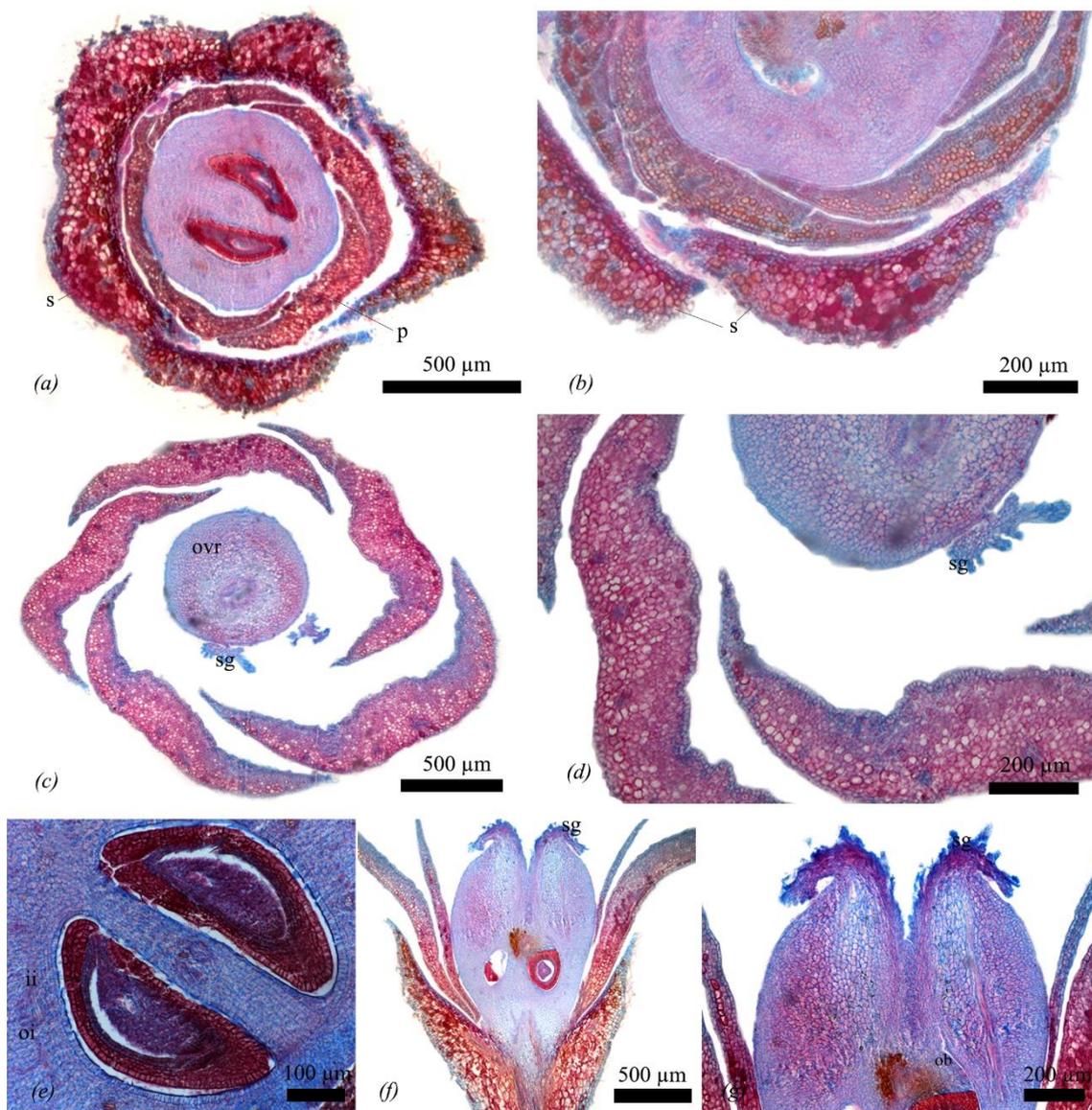
The pistillate flowers are composed of a gamosepalous perianth in a short extent, bicarpellary, bilocular syncarpous gynoecium, with one ovule per ovule (Fig.4A).

The structure of the calyx is similar to that of the staminate flower. Petals are slightly larger than those from staminate flowers. The sepals have uniseriate epidermis, the largest region of the mesophyll contain six to nine layers of parenchymatous cells (Fig. 4B) and three vascular bundles.

The petals also have a uniseriate epidermis and the mesophyll is homogeneous with to four to seven layers of parenchymatous cells (Fig. 4C-D). The petals, like the staminate flowers have idioblasts with phenolic content. The mature petal presents three collateral bundles (Fig. 4D).

The ovary is cylindrical, there is a lack of style and the stigmas are completely split and papillae. The outer epidermis of the ovary is uniseriate with small cells (Fig. 4A). The mesophyll is homogeneous and consists of several layers of parenchyma cells, where the vascular bundles occur. The inner epidermis of the ovary is uniseriate.

Fig. 4 Structure of pistillate flowers of *Microdesmis caseariifolia*. **(a)** bicarpellar and bilocular gynoecium, with one ovule per ovule. **(b)** Sepal with uniseriate epidermis. **(c)** Petals in an imbricate aestivation. **(d)** Detail of the petal, showing the three vascular bundles. **(e)** Detail of the gynoecium, showing the outer and inner integuments. **(f)** longitudinal view of the flower, note the absence of the style. **(g)** Detail of the stigma and the placentary obturator. Abbreviations: li, inner integument; ob, obturator; oi, outer integument; ovr, ovary; p, petal; s, sepal; sg, stigma.



The ovules are bitegmic, anatropous with axile placentation. The outer integument is formed by four layers of cells, while the inner integument has two layers of cells, at anthesis (Fig. 4E). The inner and outer integument have cells with phenolic content. The micropyle is formed for both integuments (Fig. 4E). The flower does not present a prominent nucellar beak (Fig. 4E) and the placentary obturator is evident (Fig. 4F), composed of a group of cells in palisade, located just above the micropyle (Fig. 4G).

The flower has no style (Fig. 4F) and the stigma is bifid.

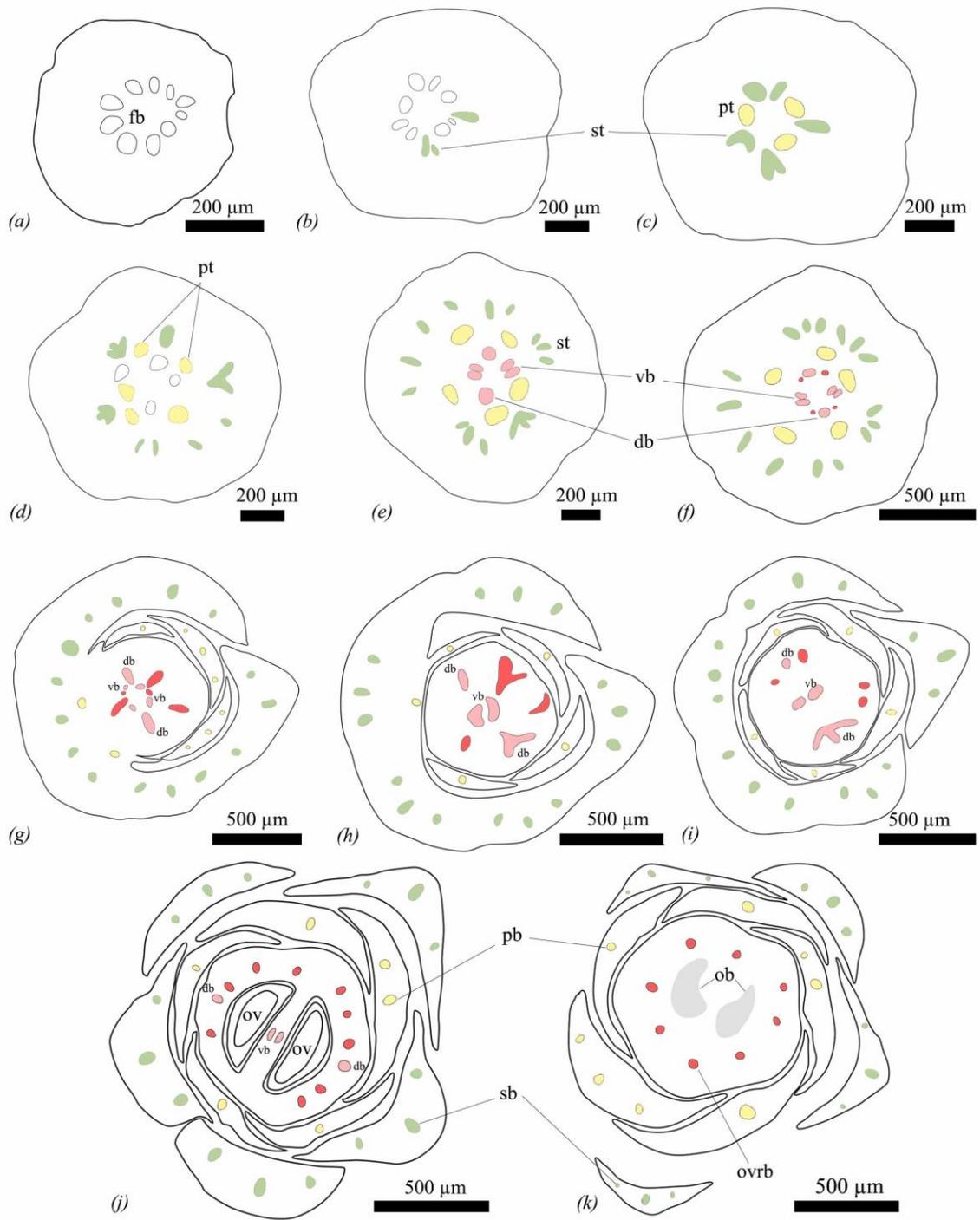
Vasculature

The floral stele is an eustele formed by ten vascular bundles in the receptacle Fig. 5A). At the level of calyx, five traces diverge from the stele to the sepals, these traces branches into two (Fig. 5B-D) and then into three (Fig. 5E-F) before reach the sepals, the remnant vasculature in the stele will vascularize the petals (Fig. 5C-F).

In the corolla, five fundamental bundles of the stele vascularize the petals (Fig. 5D-E). After this organ leaves the receptacle the sole bundle of the petals divide in three (Fig. 5G), one central and two laterals. This division may occur in the middle portion of the petal and these three bundles of the petals remains unbranched along the whole organ.

Just before the petals traces leave the receptacle, three traces expand to reach each carpel, one trace is median and two are lateral (Fig. 5E), originating the dorsal and two ventral bundles, respectively. Still at this moment, the dorsal bundles start to divided into many small bundles (Fig. 5F-G) that reach the parietal position to vascularize the entire wall of the ovary. The ventral bundles of the different carpels fuse, forming only two big ones (Fig. 5H) that soon after move to a central region (Fig 5I). At the ovule height, the vascularization appearance is the same, although it is more organized now (Fig. 5J).

Fig. 5 Vasculature of pistillate flowers of *Microdesmis casariifolia*. **(a)** Eustele with ten vascular bundles in the pedicel. **(b)** sepal traces (black) divergence in the receptacle. **(c)** Sepal and petal traces divergence in the receptacle. Sepal traces branch into two. **(d)** Sepal trace branches into three. Sepals are vascularized by three traces and the petals by one trace. **(e)** divergence of the carpels traces. **(f)** The dorsal trace ramifies at first in three bundles at the ovary base and the two ventral bundles remain unbranched. **(g)** The dorsal trace ramifies in many bundles and the two ventral bundles prepare to fuse into one. **(h)** organization of the receptacle traces and the fusion of the ventral bundles. **(i)** The dorsal bundles start to divide into many small bundles. **(j)** Ovary wall vascularized by the bundles from the dorsal trace. **(k)** in the upper region of the ovules appears the obturator. Note the petal bundles divide into three. Abbreviation: db, dorsal bundle; fb, fundamental bundle; ob, obturator; ov, ovule; ovr, ovary bundles; pb, petal bundle; pt, petal trace; sb, sepal bundle; st, sepal trace; vb, ventral bundle.



At this point it is possible to identify the placental obturator of each ovule in the center of the ovary and some bundles at the ovary wall (Fig. 5K), which will extend to the stigma, since the flowers has no style.

Discussion

***Microdesmis* flowers and morphological aspects in Pandaceae**

Although the several molecular phylogenetic studies with Malpighiales (e.g. Wurdack et al. 2005; Tokuoka and Tobe 2006; Korotkova et al. 2009; Wurdack and Davis 2009; Soltis et al. 2011), Pandaceae still remain without certain phylogenetic affinity (Tokuoka and Tobe 2006). According to Stevens (2001), it is sister group of Irvingiaceae, though the bootstrap is not well supported 50-75%. This clade (Pandaceae+Irvingiaceae) is related to a bigger one that comprise six families, named Ochnaceae, Bonnetiaceae, Clusiaceae, Calophyllaceae, Hypericaceae and Podostemaceae (Stevens 2001). The flower morphology on this big group has several variations in the merism, number of ovules, presence of staminodes or pistillodes and more (Matthew and Endress 2011; Stevens 2001; Bove and Philbrick 2010; Fiaschi et al. 2010).

Flowers of Pandaceae are not well known, though the morphological studies from Li and Gilbert (2008) and Van Welzen (2011) clarify some of the doubts regarding Pandaceae. Based on the study of *Microdesmis caseariifolia* we found some different results from the previous ones, mainly related to the fertile organs. The presence of nine stamens is one of these differences, as the genera is known for having 10 (Li and Gilbert 2008, Van Welzen 2011) in species from Asia, and the placental obturator, which for long time is considered inexistent in Pandaceae (Merino-Sutter 2006), this last character was important in the former morphological studies, as it makes Pandaceae diverge from the other segregated families from Euphorbiaceae *s.l.*

Although the inexistence of a phylogeny for this family, some authors suggested relations between the genera based on morphological characteristics, when it was considered part of the tribe *Galearia*. Punt (1962) stated the close relationship between *Galearia* and *Microdesmis*, when he studied the pollen morphology. After the molecular analysis was found that *Panda* and *Galearia* form a sister group, and this group is sister of *Microdesmis* [*Galearia* + *Panda*] *Microdesmis* (Wurdack et al. 2005), which comprises two-thirds of the family. However, the morphology of the flowers allows us to say the *Galearia* shares intermediated characters, such as anatropous or orthotropous ovules (Van Welzen 2011), while *Panda* presents orthotropous and *Microdesmis* anatropous (Webster 1994, Van Welzen 2011). This data is confirmed to *Microdesmis caseariifolia* that has anatropous ovules and of *Galearia fulva* that also presents anatropous ovules (pers. obs.).

Microdesmis has two subgenera, the subg. *Microdesmis* which comprises eight species from Africa and the subg. *Ganitrocarpus* with two species from Asia. The subg. *Microdesmis* has staminate flowers with five stamens and the subg. *Ganitrocarpus* has 10 stamens in two whorls (Van Welzen 2011). Although some flowers of *Microdesmis caseariifolia* present only nine stamens, showing a level of reduction.

Reduction in Flowers of *Microdesmis caseariifolia*

Floral evolution often changes the androecium, resulting in either stamen loss or alteration of stamen function from pollen production to alternate functions (Weberling 1989; Ronse Decraene and Smets 1993, 1995). *Microdesmis caseariifolia* presented flowers with nine stamens, although in the vasculature is possible to see the vascular trace of the 10th stamen. In the origin, it would be the last stamen to appear in the second whorl.

For Puri (1951) the reduction in the number of vascular bundles in the flower is caused by definite loss either of organs or of bundles in an organ, or still through fusion.

The loss of the structure in *M. caseariifolia* is seen in other families as well, Puri (1951) says that generally individual members of existing whorls disappear more or less completely and this happens along with the vascular bundles. Although in some cases, the vascular bundles may persist in the tissue of the receptacle in positions corresponding to those of the lost organ (Puri 1951; Hirao et al. 2018 *in press*), similar to *M. caseariifolia*.

One of the causes for this suppression in *M. caseariifolia* may be related to the transition series in some groups, whereas *Galearia* has species with eight stamens. The decreases in stamen number typically involve progressive suppression of stamen development during morphogenesis (Tucker 1988; Ronse Decraene and Smets, 1995) and for Larsen and Harder this variation is reflected by evolution changes in some groups, the same authors give the example of species of *Bauhinia* (Fabaceae) where the stamen number varies from ten (which is the ancestral state) to one, and ‘‘missing’’ stamens are absent or represented by stamen remnants (Endress 1994).

According to Larsen and Harder (2000) the reduction of a partial stamen whorl occurs only in more derived clades, 34.4% of rosid families have species with either an incompletely or completely reduced partial whorl.

Gynoecium

Another feature in these flowers is the presence of a pistillode, character synapomorphic to the family. This pistillode in the species has one vascular trace, which is just opposite to the stamen that does not develop in *Microdesmis caseariifolia*. This

vascular trace of the pistillode is not central initially, but becomes central after the disappearance of the stamen trace. This event may occur because of the spatial organization of the whorls in the flower. The presence of a pistillode is uncommon between the clade which comprises Irvingiaceae, Pandaceae, Ochnaceae, Bonnetiaceae, Clusiaceae, Calophyllaceae, Hypericaceae and Podostemaceae (Stevens 2001), only Pandaceae has pistillodes. However, staminodes are generally presents (Ochnaceae, Clusiaceae, Hypericaceae), but absent in Pandaceae (Zappi and Lucas 2002; Farrar and DeCraene 2013; Radcliffe-Smith 2001; Stevens 2001).

Regarding the pistillate flowers, in *M. caseariifolia* it is bicarpellary and uniovulate, with anatropous ovules. To Merino-Sutter et al. (2006) the ovules of *Microdesmis* are hemitropous or anatropous as seen here. The ovule characteristics has for long time been an important character in the circumscription of Euphorbiaceae *s.l.* and the currently segregated families. Previous non-molecular studies questioned the monophyly of the Euphorbiaceae *s.l.* based on structural features (Meeuse 1990, Huber 1991, Stuppy 1996). Huber (1991) recognized two different families, one with one ovule per carpel, where should stay Euphorbiaceae *s.s.*) and another with two ovules per carpel, where was the remainder of the Euphorbiaceae *s.l.*. In the currently circumscription the segregate families from Euphorbiaceae *s.l.* do not form a clade, some are still related, such as Phyllanthaceae and Picrodendraceae, although Pandaceae is in a further clade as sister group of Irvingiaceae, these two families share the character of having one ovule per locule (Matthew and Endress 2011).

In the taxonomic descriptions of the family and related studies some authors say that all segregated families from the Euphorbiaceae *s.l.* have obturator, except for Pandaceae (Radcliffe-Smith 2001, Merino-Sutter et al. 2006, Van Welzen 2011). However, in this study we reveal the presence of a placental obturator in *M. caseariifolia*,

as observed in *Irvingia smithii* Hook.f., species of the sister group of Pandaceae (Matthew and Endress 2011). Our results agree with the statement that the COM clade (Celastrales, Oxalidales and Malpighiales) presents ovules pendant and antitropous with obturator and axile placentation (Endress and Matthews 2006; Endress et al. 2013). Still regarding the ovule, Tokuoka and Tobe (1999) did not observe a nucellar beak, although we observed a narrow one in *M. caseariifolia*.

The following features of Pandaceae are of interest because it is unusual in Malpighiales. Pandaceae, although it is a small family with 16 species, has morphological novelties for the group, such as the reduction of the stamen number, the presence of a placental obturator and a pistillode. In an evolutionary view, based on the floral morphology, Pandaceae and Irvingiaceae are supported as forming a clade. However, *Microdesmis caseariifolia* have a pistillode, character not seem in the other families in the clade, suggesting this character may have evolved independently in Malpighiales.

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Final Considerations

In this work, we intended to explore several aspects of the floral morphology of different species from the segregated families of Euphorbiaceae *s.l.* and try to link it to the currently systematic position of the species. The different species have a diverse morphology according to the family they belong, then, as said previously, it is not possible to have a broader conclusion on the flower aspects of Euphorbiaceae *s.l.*, although many consistent considerations were done for the genera or family studied.

For *Phyllanthus*, the presence of petals was a novelty. We showed in the first chapter that the presence of sepals and petals may be widely distributed in the genus even though it is still unknown. Some studies had acknowledged the presence of two whorls in the perianth, but they did not consider the ontogenesis and vasculature of these structures. The position of the nectary in this species was also a remarkable information that reinforces this thinking. The data found here may help to understand the evolution of the perianth in the group, since Phyllantaceae have other genera with corolla, although more studies are necessary to comprehend if it is or not an isolated case in *Phyllanthus*.

In relation to the perianth, the same may be applied to *Piranhea*, since we recognized the difference between the two whorls in its flowers. Analysing the characters of flowers of *P. trifoliata*, some structures drew attention, such as the staminodial nectaries that may indicate a changing process during the evolution of the group.

In the third chapter, the main conclusion was about the type of pollination in *Alchornea sidifolia* based in the characters related to the inflorescence and flowers of this species that displays a set of features, like the extensive stigmatic receptive surface that are functional and typically associated in many other unrelated angiosperm lineages that are wind pollinated. In this paper, we also pointed the presence of two carpels in *Alchornea*, when the representative number for Euphorbiaceae and many other Malpighiales is three. This reduction was an important character to the evolution of the

genus, since only *Gavarretia* and *Alchornea* have two carpels from the Alchorneoid clade.

Microdesmis was for the first time studied in such a detailed anatomical view. Then, morphological novelties were found in the group, such as the reduction of the stamen number, that may be related to the transition series between Pandaceae, whereas *Galearia* has species with eight stamens. The presence of a placental obturator, structure in which previous studies for the family affirm to be absent. And the presence of the pistillode, character not seen in the other families in the clade.

By the studies of the different species we tried to improve the knowledge regarding the floral structure of these groups linking to their evolution and pollination types. The results obtained allowed us to discuss over the anatomy, vasculature, and ontogeny of flowers of these groups, as well as investigate the functionality of some features. In this way, we expect that the diversity of form, color, whorls, presence of staminodes or pistillodes, among other characteristics described here, may represent an important advance to the knowledge of the floral diversity and the variables involved in the morphological evolution of Malpighiales, although studies are still required to come to an accurate conclusion of the changes in the development patterns that are responsible for the great floral diversity found in the order.