

**Estrutura floral de *Guarea macrophylla* Vahl e
Trichilia clausenii C.DC. (Meliaceae): aspectos
funcionais e implicações na sistemática e
evolução de Sapindales.**



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Orientador: Prof. Dr. José Rubens Pirani
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Orientador

Dedico a minha família,
que são indispensáveis em minha vida.

*“O que vale na vida não é o ponto de partida e sim a caminhada. Caminhando e
semeando, no fim terás o que colher.”*
(Cora Coralina)

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ABSTRACT

The reproductive systems of angiosperms are quite diverse. In the flowers of Meliaceae (Sapindales) we found a wide diversity of these systems, including reports of monoecious, dioecious and polygamous plants. However, the sexual dimorphism of flowers in the family is very subtle, and structurally each flower always has a gynoecium and a developed androecium, even though functionally one or the other may be sterile (pistillodes or staminodes). Consequently, the determination of floral sexuality in the family is often difficult or even inconclusive in many taxa, and the available data often come from the analysis of herbarium material. This lack of detailed information on floral structure, development and function still prevent an accurate identification of floral sexuality in most members of Meliaceae, and also limits a comparison with the data known to other representatives of Sapindales, especially in relation to the structure and development of the carpels and stamens in the families most closely related, which are Rutaceae and Simaroubaceae. Studies on flower development associated with pollination studies and phylogenetic through comparative analysis, provide a better understanding on how these systems behave, and allow a more accurate classification of the sexual system present in particular species, leading to evolutionary data and assisting in clarifying kinship issues. The present dissertation addresses a detailed study of the structure of flowers of two Brazilian taxa (*Trichilia clausenii* and *Guarea macrophylla* subsp. *tuberculata*) belonging to the subfamily Melioideae, with emphasis on the organization and development of their sexual systems. Our study includes analysis of the external morphology, anatomy, vascularization, ontogeny, and histochemical tests of both species. We also analyzed the functional aspects of flowers of *T. clausenii*, related to floral sexuality, which was evaluated by means of pollination tests, associated to tests of pollen viability, stigmatic receptivity and morphoanatomic and ontogenetic analyzes. Among the main results, floral dimorphism was detected only in *T. clausenii*, which is likely gynodioecius, and provided with a peculiar sterility of the androecium in the female flower morphotype: pollen grains remain concealed by a secretion from the tapetum. In addition, we promote the discussion of pathways of the evolution of sexual systems within the Meliaceae, as well as of its main flower and fruit morphological characters, through hypotheses evaluated upon the current molecular phylogenetic framework of the family.

RESUMO

Os sistemas reprodutivos das angiospermas são bastante diversos. Nas flores de Meliaceae (Sapindales) encontramos uma grande diversidade desses sistemas, sendo relatada a presença de plantas monoicas, dioicas e poligâmicas. No entanto, o dimorfismo sexual das flores nessa família é muito sutil, e estruturalmente cada flor sempre possui um gineceu e um androceu desenvolvidos, mesmo que funcionalmente um ou outro seja estéril (pistilódio ou estaminódio). Conseqüentemente, a determinação da sexualidade floral na família é muitas vezes difícil ou até mesmo inconclusiva em muitos táxons, sendo a maioria dos dados disponíveis provenientes da análise de material herborizado. A carência de informações mais detalhadas sobre estrutura, desenvolvimento e função, ainda limita muito uma acurada identificação da sexualidade floral em Meliaceae, assim como impede uma comparação com os dados conhecidos sobre outros representantes de Sapindales, especialmente em relação à estrutura e desenvolvimento do androceu e gineceu, nas famílias mais proximamente aparentadas a ela, que são Rutaceae e Simaroubaceae. Estudos sobre o desenvolvimento floral, associados a estudos de polinização e filogenia, por meio de análises comparativas, propiciam melhor compreensão de como esses sistemas se comportam, permitindo uma classificação mais acurada do sistema sexual presente numa determinada espécie, além de fornecer dados evolutivos importantes e auxiliar no esclarecimento de questões de parentesco. Neste contexto, a presente dissertação aborda um estudo detalhado da estrutura das flores de dois táxons brasileiros (*Trichilia clausenii* e *Guarea macrophylla* subsp. *tuberculata*) pertencentes à subfamília Melioideae, com ênfase na organização e desenvolvimento do sistema sexual. Tal estudo é composto por análise da morfologia externa, anatomia, vascularização, ontogenia e testes histoquímicos em ambos táxons. Também foram feitas análises dos aspectos funcionais das flores de *Trichilia clausenii*, relativos à sexualidade floral, por meio de testes de polinização, testes de viabilidade polínica, receptividade estigmática e análises morfoanatômicas e ontogenéticas. Entre os principais resultados, destacamos o dimorfismo floral detectado apenas em *T. clausenii*, espécie ginodioica na qual a esterilidade do androceu no morfotipo floral feminino é alcançada pela impossibilidade de liberação dos grãos de pólen, retidos por uma secreção do tapete. Além disso, promovemos a discussão de transições evolutivas do sistema sexual na história da família, bem como de outros caracteres morfológicos florais e de fruto, por meio de avaliação de hipóteses estruturadas sobre a filogenia molecular atual da família.

GENERAL INTRODUCTION

The flowers are quite diverse reproductive structures, mostly bisexual and only about 10% unisexual (Barrett, 2002). Their sexual systems present a great complexity, which makes possible a series of classifications, from the spatial and temporal distributions of the reproductive organs in the plant, to self-incompatibility and variation in the length of these reproductive structures (Bawa & Beach, 1981).

Sexual systems based on spatial distribution of male and female reproductive organs can be classified as: hemaphroditism, where plants have only bisexual flowers; monoecism in which the plants have male and female flowers on the same individual; andromonoecism, where the same individual has male and bisexual flowers; gynomoecism, in which the plant has female and bisexual flowers; dioecism, where the female flowers are in an individual different from the male flowers; androdioecism, in which individuals have male or bisexual flowers; gynodioecism, in which there are individuals who produce female flowers and individuals who produce bisexual flowers in the same population; polygamous in which the plant population produces female, male and bisexual flowers on the same plant or on different plants; subdioecism presents populations of plants with male flowers, plants with female flowers and some hermaphrodite plants (Bawa & Beach, 1981; Sakai & Weller, 1999). The diversity of these sexual systems derives from the strategy of differential allocation of female and male resources controlled by sexual selection, since male success depends on the dispersion of pollen and the female depends on the nutritional resources available for the development of embryos, fruits and seeds. Pollination has an important impact in this process, since it promotes the movement of pollen among flowers of the same species (Bawa & Beach, 1981).

The understanding of this dynamic and its complex diversity relies on the necessity of detailed studies of floral development associated with studies of pollination and reproductive biology, along with phylogenetic and evolutionary approaches. The combination of these different approaches allows us to understand more properly how sexual diversification may have taken place in certain groups and across other related lineages.

When analyzing the flowers of dimorphic taxa, if there are macroscopic differences as the presence of rudimentary organs of the opposite sex, such as staminodes (anthers smaller than functional ones and with non-viable pollen grains) or pistillodes (with aborted ovules), there is a greater facility to classify the functionality as well as the sexual system (Pennington *et al.*, 1981; Vieira 2013). However, in cases where the dimorphism is subtle the difficulty in sexual determination increases and developmental and pollination studies are needed to clarify and classify sexual systems accurately. The presence of the opposite sex linked to functionality can be approached from a macroevolutionary perspective in order to evaluate the evolutionary rates of this vestigialisation in non-functional characters of the opposite sex (e.g. Eckhart, 1999).

In the order Sapindales (belonging in the Eudicots-Rosids-Malvids clade according to APG IV, 2016), it is common the occurrence of morphologically bisexual flowers that are functionally unisexual (e.g., Engler, 1931; Smith *et al.*, 2004). Among the nine families of the order, several recent studies have improved the knowledge about this phenomenon (e.g., Bachelier and Endress, 2008, 2009; Bachelier *et al.*, 2011; El Ottra *et al.* 2013; El Ottra, 2014; Alves *et al.*, 2017; Gouvea *et al.*, 2008a, 2008b), but there are still many gaps to be filled.

Some of these gaps are found in relation to the flowers of the clade formed by Rutaceae, Simaroubaceae and Meliaceae, which according to Muellner-Riehl *et al.* (2016) is strongly supported. However, the relationship between Meliaceae and Simaroubaceae as sister to each other is moderately supported. Detailed studies of the floral structure with emphasis on the reproductive organs are available for several genera of the first two families. In contrast, the lack of such information for most Meliaceae precludes an accurate comparison between the representatives of that clade.

Meliaceae is a mostly pantropical family, with a few taxa represented in the subtropical and temperate regions. In Brazil the two monophyletic subfamilies, Melioideae and Cedreloideae, are represented by eight genera and ca. 84 species, 30 of them endemic (Pennington & Styles, 1975;

Muellner *et al.*, 2009; Mabberley, 2011; Stefano *et al.*, 2015). The sexual systems of this family present a wide diversity, with dioecious, monoecious and polygamous plants being reported. Usually the flowers in the family are morphologically bisexual, but functionally they are predominantly unisexual. The sexual dimorphism of flowers is often very subtle, making it difficult to determine floral sexuality in several genera of the family (e.g. Pennington & Styles, 1975; Styles, 1972). In this way, more detailed studies of floral structure are necessary, since many of the reproductive data on floral sexuality in the family come only from morphological analysis, often from exsiccate, which makes it impossible to detect the functionality of reproductive structures and may even generate conflicting data. This lack of studies stimulated the elaboration of the present dissertation.

Meliaceae are of great economic importance, since the wood of several species is widely marketed worldwide. They have great potential for timber, such as the species of mahogany (*Swietenia* sp.) and cedar (*Cedrela* sp.) in the Neotropical region (Styles, 1972; Pennington *et al.*, 1981). Another commercial use of the family is the use of its active compounds (e.g., limonoids and meliacins) as insecticides or repellents (e.g., neem oil), which may further have fungicidal, antibacterial, antiviral (Singh *et al.*, 1988; Engelmeier *et al.*, 2000; Aboutabl *et al.*, 2000; Simmonds *et al.*, 2001; Greger *et al.*, 2001).

Trichilia P.Browne and *Guarea* Allamand ex L., are two of the five major genera that compose the family. There are few works related to sexuality in these genres (e.g., Moscheta, 1995; Souza *et al.* 2001, 2002; Morelato, 2004; Schmitt, 2013) and no detailed study regarding the structure and development of internal morphological surfaces of carpels and anthers.

In this dissertation we present an analysis of the sexual-functional aspects of the flower of *Trichilia clausenii*, whose sexual system is doubtful. This analysis was performed through optical microscopy and scanning electron microscopy, combined with pollen tests, pollen viability and stigma receptivity. Data from these analyzes along with their discussion and the external morphology boards, anatomical sections, anther electromicrographies and tests performed are found in Chapter 1.

We performed also an analysis on the structure and development of the gynoecium and androecium of *Trichilia clausenii* and *Guarea macrophylla* subsp. *tuberculata*, in a comparative way, trying to understand the sexual systems of these species. For this, analyzes of the external morphology, anatomy, vascularization, ontogeny, and histochemistry of both species were performed with the aid of stereomicroscopy, optical microscopy, scanning electron microscopy and histochemical tests. The data obtained from these analyzes, their discussion and the boards of the internal morphology, the anatomical sections, the electromicrographs, the vascularization diagrams and the histochemical tests are presented in Chapter 2.

Finally, we performed an evolutionary analysis on the main morphological characters of flowers in Meliaceae, based on the current phylogenetic framework. We aimed to find probable synapomorphies for some clades of the family, and to evaluated hypotheses on evolution of the reproductive structures (flowers and fruits) and of the sexual systems within the group. We coded 20 reproductive morphological characters and used 31 terminals of Meliaceae and six terminals of Simaroubaceae as outgroup, and plotted evolutionary transitions upon the molecular phylogeny published by Muellner-Riehl *et al.* (2016). The discussion of the evolution of the sexual systems in the family and other reproductive morphological characters is found in Chapter 3.

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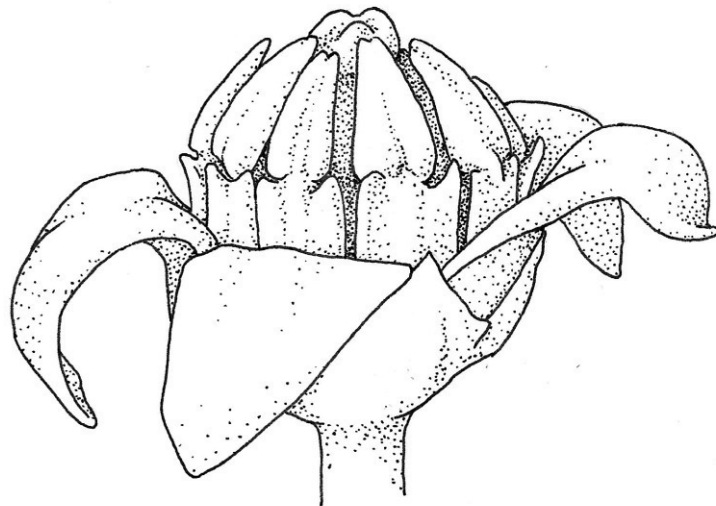
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Chapter I

Gynodioecy in *Trichilia* P.Browne (Meliaceae)
and a peculiar case of male sterility due to
tapetum secretion



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Abstract

Premise of research. Although the flowers of Meliaceae are traditionally described as bisexual, in numerous cases they are in fact functionally unisexual and still little is known about floral sexuality in various genera in the family. For *Trichilia* P.Browne, one of the five major genera of the family, several reproductive systems have been already reported: subdioecy, gynodioecy, polygamy, or hermaphroditism. The aim of this study is to analyse the sexual-functional aspects of the flower of *Trichilia clausenii* C.DC., whose sexual system is little known.

Methodology. Morphological analyses of flowers of *T. clausenii* were made using a stereomicroscope and SEM. In addition, flowers and buds of different individuals were embedded in Paraplast and sectioned using a rotatory microtome for structural and developmental analyses under LM. Tests of pollen viability were performed using specific stains and self-pollination and cross-pollination were manually carried out to verify female sterility. After pollination, live plants were monitored for assessing fruit set and the fruits developed were collected to verify the seed viability.

Pivotal results. Two floral morphotypes were identified in *T. clausenii*: one with a fully developed androecium and a reduced gynoecium and the other with an androecium which does not release pollen and a fully developed gynoecium. They share similar sterile whorls, while their androecium and gynoecium show morphological, anatomical and functional differences. In the supposedly male flower, anthers are well developed and produce viable pollen grains. The ovary is conical with the base almost totally immersed in the nectariferous disc and the ovules are small. The pollination viability test proved that this ovary and ovules are fertile and develop into fruit, which contain viable seeds. These flowers are preferably geitonogamic. In contrast, the supposedly female flower presents a fully developed gynoecium with larger ovules, conical-pyramidal shape, and the transition between ovary and nectary is very subtle. The stamens of the female flowers have pollen grains that are united into an amorphous mass inside the anthers, so they are in fact staminodes. The results found in sporogenesis and gametogenesis are consistent with previous descriptions reported for most angiosperms, but some particularities are noted in *T. clausenii*, specially the fact that anthers of staminodes are functionally sterile due to the presence of a secretion from the tapetum, which prevents the release of the pollen grains. Each fruit bear a single seed. Thirty fruits were analysed in greenhouse, and seeds from 25 of them germinated.

Conclusions. Our results demonstrate that *T. clausenii* exhibits sexual floral dimorphism. The viability tests along with the morphoanatomical and pollination tests data strongly indicate that male sterility occurs in one morphotype, while the other is actually bisexual, therefore the species is gynodioecious.

Keywords: dimorphism sexual, sporogenesis, gametogenesis, viability, pollination tests

Introduction

The angiosperms present a great diversity of reproductive strategies, though the majority of species are hermaphrodite, with bisexual flowers (Karasawa 2009). Among the Sapindales, an order belonging to the Eudicots-Rosids-Malvids clade (APG IV 2016), several authors have reported the common occurrence of flowers with both reproductive whorls but which are functionally unisexual (e.g. Engler 1931; Smith et al. 2004). Among the nine families of the order, several recent studies have improved the knowledge about this phenomenon. The works done on members of Anacardiaceae, Burseraceae and Kirkiaceae (Bachelier and Endress 2008; 2009), Nitrariaceae (Bachelier et al. 2004), Rutaceae (Pirani et al. 2010; El Ottra et al. 2013; El Ottra 2014), Simaroubaceae (Alves et al. 2017) and Meliaceae (Gouvea et al. 2008a) indicate the occurrence of sexual dimorphism and various sexual systems. Those authors also discussed the implications of floral structure and plant sexuality for the systematics and evolution of the order.

Angiosperm species with sexual dimorphism in different individuals show morphotypes that may be either dioecious (strictly unisexual), gynodioecious (one hermaphrodite and the other female) or androdioecious (one hermaphrodite and the other male) (Barret 2002). The two latter conditions are often referred to as polygamous or polygamodioecious (e.g. Sakai and Weller 1999). These three fundamental types of sexual dimorphism have already been reported for members of Sapindales, along with the conditions of monoecy and hermaphroditism (e.g. Bawa and Beach 1981; Pennington and Styles 1975; Alves et al. 2017). The evolution of sexual dimorphism may have occurred via hermaphroditism, where intermediate types that gradually lost the fertility of one of the reproductive organs, provided the presence of hermaphrodite plants, male plants and female plants in the same population (subdioecy or trioecy). The dioecy via monoecy appears to have occurred from mutations that caused female and male sterility in different individuals (Bawa and Beach 1981; Karasawa 2009). A wide revision of the theories on the evolution of sexual dimorphism has been presented by Geber (1999).

Bisexual flowers have great morphological similarities, which hinders the recognition of functional organs. However, some of these flowers present macroscopic differences where flowers have rudimentary organs of the opposite sex, such as staminodes (different color, anthers smaller than functional anthers and with inviable pollen grains) or pistillodes (with aborted ovules), which may facilitate functional classification (Pennington et al. 1981; Vieira 2013). These are floral features, which can be addressed from a macroevolutionary perspective, towards evaluating the evolutionary rates of vestigialization in nonfunctional opposite-sex characters (e.g. Eckhart 1999).

Within the Sapindales, White and Styles (1963) were the first to note that functionally the majority of Meliaceae flowers, which were traditionally described as perfect or bisexual, are in fact unisexual, and they also pointed out that reproductive systems are quite diverse within the family. In that article, and in subsequent ones, examples of monoecy, subdioecy, gynodioecy, polygamy, or hermaphroditism were reported for Meliaceae. The generic monograph of the family by Pennington and Styles (1975) provided the basic descriptions of the floral dimorphism observed in several taxa, as well as did the treatment presented in the *Flora Neotropica* series (Pennington et al. 1981). In most cases, a partial to complete sterilization of the stamens is observed in the female morphotype, while the male morphotype shows a gynoecium that is partially to totally reduced to a pistillode. Styles (1972) provided data on flower dimorphism and sexual systems of several genera of Meliaceae. Gouvêa et al. (2008a) characterized the development of the flower whorls of *Toona* and *Cedrela*, describing the morphoanatomical differences found between functionally female and male flowers. Furthermore, Gouvêa et al. (2008b) in a more detailed study of the structure of the inflorescence and flower of *Switenia macrophylla* King. (mahogany), showed the morphological differences between flowers, and that the female and male flowers occupy different positions in the thyrse inflorescences, a pattern that had been previously reported for many other members of Cedreloideae and also of Melioideae (Styles 1972).

However, there are still many uncertainties about floral sexuality in several genera of the Meliaceae, among them *Trichilia*, a large genus with little available information. Unfortunately, some works developed on species of this genus present results that when compared are conflicting, probably because they are mostly based on the general analysis of floral morphology, often based on herbarium material, or on floral anatomy alone (Styles 1972; Pennington et al. 1981; Pirani 1992; Souza et al. 2001; Morellato 2004; Carmo 2005; Gouvêa et al. 2005; Schmitt 2013). Functional studies of flowers in the field combined with a detailed study of the structure and floral development allow an accurate determination of the floral sexuality for the species. As Pennington et al. (1981) consider *Trichilia* as a genus that exhibits a great variation of reproductive systems, our study aim to better understanding the floral diversity and evolution in Meliaceae, through the analysis the sexual-functional aspects of the flower of *Trichilia clausenii*, whose sexual system is still doubtful.

Materials and Methods

Flowers of 12 individuals of *Trichilia clausenii* were observed and collected from the populations of the Reserva Florestal da Cidade Universitária “Armando Salles Oliveira” (Reserva CUASO - 46° 43' 47,42" W e 23° 33' 56,64" S; one individual) and Horto Oswaldo Cruz in Instituto Butantan (23° 34' 1,22S 46°43'2, 74"O; 11 individuals) in the municipality of São Paulo, Brazil (Rossi 1994; Zanelli 2008). Vegetation of both areas are remnants of the Brazilian Atlantic Forest. Vouchers of both species are deposited at the Herbarium SPF of the Department of Botany at USP.

The flowers at different stages of development were fixed in FAA (formaldehyde, glacial acetic acid, 50% ethanol, 1:1:18 v/v) for 24 hours, dehydrated in ethanol series and storage in 70% ethanol for morphological and anatomical studies (Johansen 1940).

The general aspects of flower morphology were observed using a Leica M125 stereomicroscope. For the anatomical analysis, the material was dehydrated through a terc-butyl series and embedded in Paraplast (Johansen 1940). The material was longitudinal and transversally sectioned

at 8 -14µm using a rotary microtome by Leica RM2145 (Leica Microsystems, Wetzlar, Germany). The sections were stained with astra blue and safranin (Gerlach 1984), and the slides were mounted in Permount resin (Fisher Scientific, Pittsburg, PA). The material was analyzed using a Leica DM 400B microscope (Wetzlar, Germany) coupled with a digital camera.

For the micromorphological analysis, the anthetic flowers were dehydrated through an ethanol series and critical point dried. The sample was mounted on aluminum stubs and coated with gold-palladium and examined with a Zeiss Sigma VP microscope.

To test the pollen viability of both floral morphotypes, the following tests were performed: 0.5% acetic carmine and Alexander's stain. The collected anthers were macerated against a slide with a drop of dye. Results of each test were photographed with a digital camera attached to a microscope. The pollens were observed under UV using a fluorescence microscope to check the presence of exine.

The stigma receptivity test was performed in the supposed male flowers by submitting the stigma to 5% hydrogen peroxide. The presence or absence of effervescence on the stigma was observed with the aid of a stereomicroscope. Hand-pollinations on self - pollination (autogamy) and cross - pollination (geitonogamy and xenogamy) were carried out in three individuals of the bisexual morphotype to observe pollen tube growth.

Inflorescences were isolated with a net-bag while the flowers were in pre – anthesis. When the flowers opened, these inflorescences were taken to the laboratory and the flowers were pollinated by hand out under stereomicroscope with the exposed stigma. Flowers of 3 individuals were used in the tests to verify the viability and reproductive system of this morphotype: 94 flowers were used for the autogamy test, 115 for the geitonogamy test and 160 for the xenogamy test.

After hand-pollination, the flowers were fixed in FAA. After, the gynoecia were isolated and clarified using 5% sodium hydroxide solution for 2 hours, later washed in distilled water and placed in 5% sodium hypochlorite solution at 40°C. Then, the material were washed in distilled water, dehydrated in ethanol series and placed in pure acetone for two days

to remove the stigmatic secretion. Subsequently, the material were hydrated with distilled water and stained with 0.05% aniline blue in phosphate buffer 0.1M, pH 8. The slides were assembled with the same buffer and observed under UV using a fluorescence microscope (Dafni et al. 2005).

Fruits formed in the supposed male individual were collected to verify the seed viability. The aryl of the nine seeds was removed and they were placed in Petri dishes with filter paper moistened with water, to verify their germination. The dishes were kept in an germination chamber. These three individuals were monitored for their fruiting in the natural environment for 16 weeks. This same procedure was performed with two supposed female individuals, where 21 seeds were collected.

In the female morphotype some inflorescences were bagged, to avoid that the pollen of another individual have been brought by pollinators. Autogamic pollination of 28 flowers and geitonogamic pollination of 25 flowers were performed in this individual, to verify if the pollen was viable and observations made during the subsequent days aimed to observe the occurrence of fruit set.

Results

Flowers of *Trichilia clausenii* are heterochlamydeous. In the 12 individuals analyzed two floral morphotypes were identified: even though all the flowers are morphologically bisexual, functionally they can be either unisexual or bisexual. In both morphotypes, the sterile whorls do not present morphological differences, while the androecium and gynoecium show morphological, anatomical and functional differences (figs. 1a – 1h).

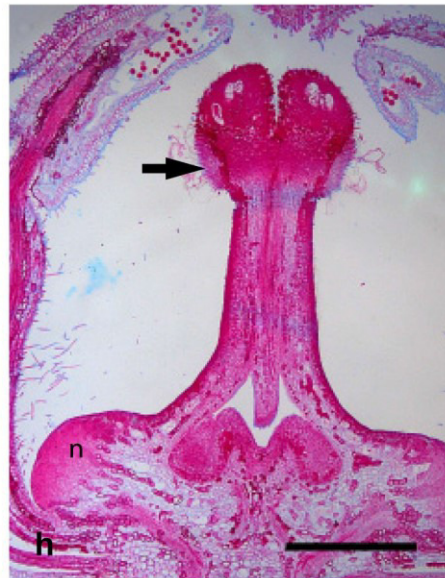
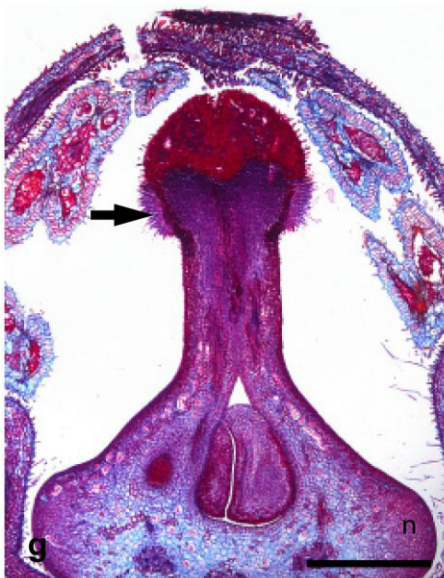
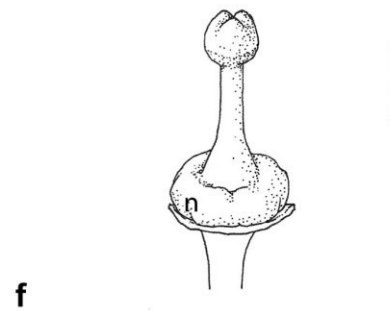
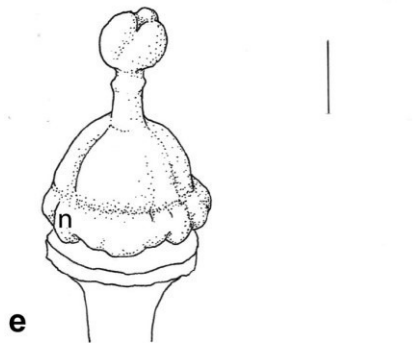
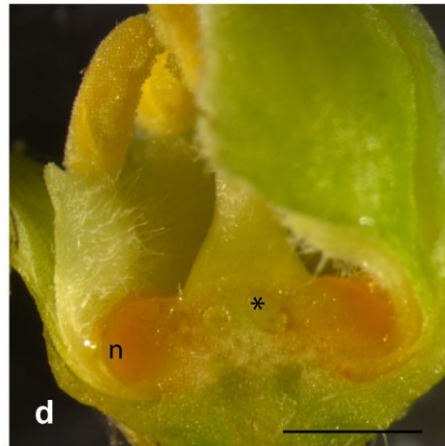
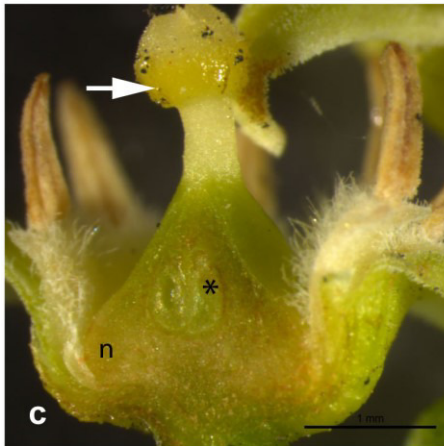
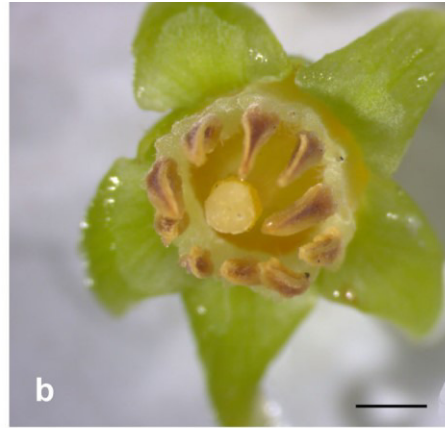
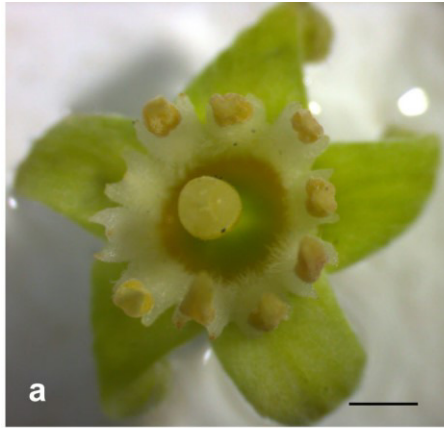
The perianth is pentamerous (figs. 1a, 1b). The calyx is gamosepalous, with sepals greenish-cream, 1–2 mm long, fused up to 1/3 of their length. The petals are free, yellowish-cream and 2–3 mm long. The androecium is composed of ten stamens. The filaments are distally coherent to each other by the intertwining of trichomes and at the base they are connate forming a staminal tube. Anthers are longitudinally dehiscent and tetrasporangiate. The gynoecium is syncarpous with three carpels, antesealous, each locule with two collateral superior ovules. The style is

short and glabrous (figs. 1c, 1d). The stigmatic capitate head presents three conspicuous lobes and a type of collar, in the basal portion, where the pollen receptor is located (figs. 1c, 1e –1h). Also a conspicuous intrastaminal nectariferous disc is present, surrounding the base of the gynoecium (figs. 1c –1h).

In the supposed male morphotype, anthers are well developed and with viable pollen grains that are released in the anthesis (fig. 1d, 1h). The ovary base is almost totally immersed in the nectary which is bright-orange, and the ovules are slightly smaller than in the female morphotype (fig. 1d). These features gives the gynoecium a cylindrical shape at the base, conical in the apical region of the ovary, and at its upper end is attached to a cylindrical style (fig. 1f, 1h).

On the other hand, the supposed female morphotype presents anthers are well developed structurally, but the pollen grains are united as an amorphous mass (fig. 1g), and a fully developed gynoecium bearing larger ovules. The shape of this gynoecium is conical-pyramidal and the transition of the region between the ovary and the nectary is very subtle, almost not being perceptible, and cylindrical in the region of the style (figs. 1c, 1e). The nectary is a very discrete annular disk compared to that found in the bisexual morphotype, and its color is yellow to light orange.

Fig. 1 Structure of dimorphic flowers of *Trichilia clausenii*. *a,c,e,g*, Flowers of the female morphotype. *b,d,f,h* Flowers of the supposedly male morphotype (actually bisexual). *a,b*, Superior view of the pentamerous flower at anthesis, with the filaments coherent to each other by tectonic trichomes forming a pseudotube along the major length of the stamen, a slightly trilobed stigma, and a greenish style in *a* and a yellowish style in *b*. *c*, Longitudinal section of the flower (part of the gynoecium and perianth removed to show the ovules, these marked by an asterisk) showing a conical-pyramidal gynoecium and the subapical pollen receptive region of stigma (arrow). *d*, Longitudinal section of the flower (part of the gynoecium and perianth removed to show the small ovule, marked by an asterisk) showing a conical gynoecium immersed in the bright-orange, annular nectary, and anthers full of pollen grains. *e - f*, Illustrations of the shape of the gynoecium from each floral morphotype. *g*, Longitudinal section showing the anatomical structure of the anthers with the presence of the tapetum secretion involving the pollen, forming an amorphous mass, and the gynoecium with the receptive subapical region of the stigma (arrow). *h*, Longitudinal section showing the anatomical structure of the anthers containing viable pollen grains, and gynoecium with the receptive subapical region of stigma (arrow). n = nectary. Bar a, b = 200µm; g, h = 500µm; c – f = 1mm.



Viability tests

In the stigma receptivity test, the presence of little stigma effervescence was observed in both morphotypes.

Pollen viability tests were positive for the tests with acetic carmine and Alexander's stain, but the female morphotype showed a lighter color than the bisexual morphotype, lighter staining is also observed for this morphotype in relation to the autofluorescence test of pollen grain exina under UV (fig. 2a – 2f).

The bagged inflorescences of the Reserve CUASO individual (female morphotype) did not produce fruits in any of the tests performed (autogamy and geitonogamy). Some flowers began to develop, but aborted in the early stages. On inflorescences that were not bagged, the development of several fruits occurred.

In the test for autogamy pollination, from the 94 flowers used, 22,34% presented pollen tube growth (fig. 2g). In the geitonogamy test, from the 115 flowers tested, 63,5% presented pollen tube growth (fig. 2h, 2i). At last, for the xenogamy test, none of the 160 flowers tested presented pollen tube growth (fig. 2j; Table 1).

No fruits were produced from the inflorescences that were pollinated by xenogamy and monitored for observation of fruit set in the natural environment.

Some fruits have developed on individuals 2 and 3 on non-bagged inflorescences. Of the nine fruits collected from the supposed male individuals, seeds from seven germinated. In the individuals of the female morphotype, 18 out of 21 seeds collected germinated.

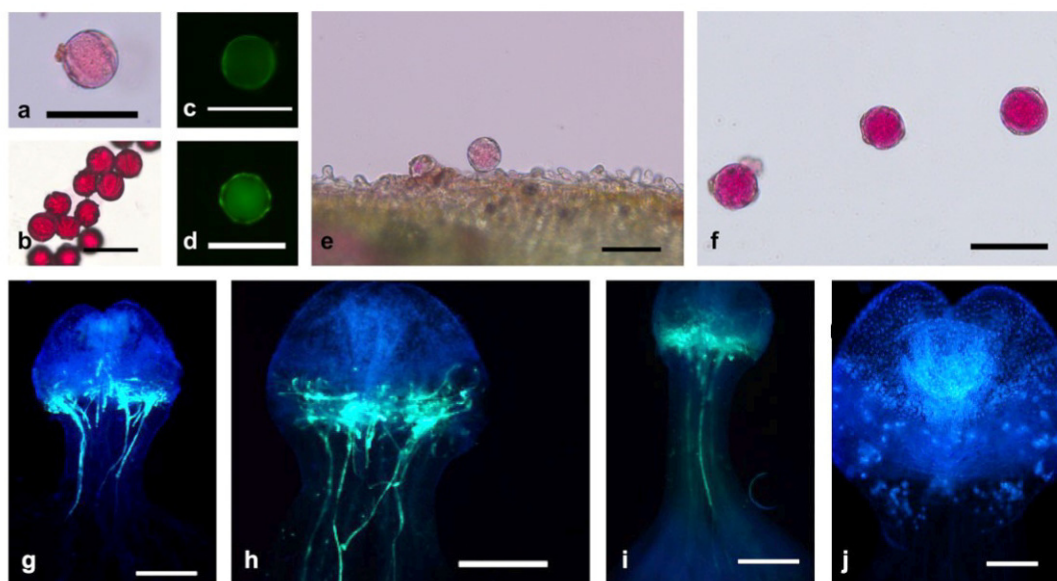


Fig. 2 Pollen viability tests in *Trichilia clausenii*. *a – b*, Acetic carmine coloration. *a*, Pollen of the female floral morphotype with weak staining. *b*, Pollen of the bisexual floral morphotype with positive staining. *c – d*, Autofluorescence of exine under UV light. *c*, Pollen grains of the female morphotype weakly fluorescing. *d*, Pollen grains of the bisexual floral morphotype fluorescing. *e – f*, Pollen grains stained in Alexander's solution. *e*, Poorly stained female floral morphotype. *f*, Bisexual floral morphotype with positive staining. *g*, Gynoecium of the bisexual floral morphotype submitted to the autogamic pollination test. *h – i*, Gynoecium of the bisexual floral morphotype submitted to the geitonogamic pollination test. *j*, Gynoecium of the bisexual floral morphotype submitted to xenogamic pollination test. Bar *a – f* = 50µm; *g – i* = 500µm; *j* = 200µm.

Table 1. Pollination tests and the relation between the total of pollinated flowers and the number of flowers that presented growth of the pollen tube, for individuals of the bisexual morphotype of *Trichilia clausenii* in São Paulo, Brazil.

Tests	Individual 1 flower/growth	Individual 2 flower/growth	Individual 3 flower/growth
Autogamy	17/00	38/00	39/21
Geitonogamy	26/00	41/28	48/45
Xenogamy	40/00	64/00	56/00
Total flower/ Growth of the pollen tube	83/00	143/28	143/66

Development

Microsporogenesis. The first cellular differentiation occurs at the tip of the stamen that is beginning to form. Early in development the outer layer divides periclinally and differs in the protoderm. The meristematic cells just below this layer differentiate into the archesporium (figs. 3a - 3c).

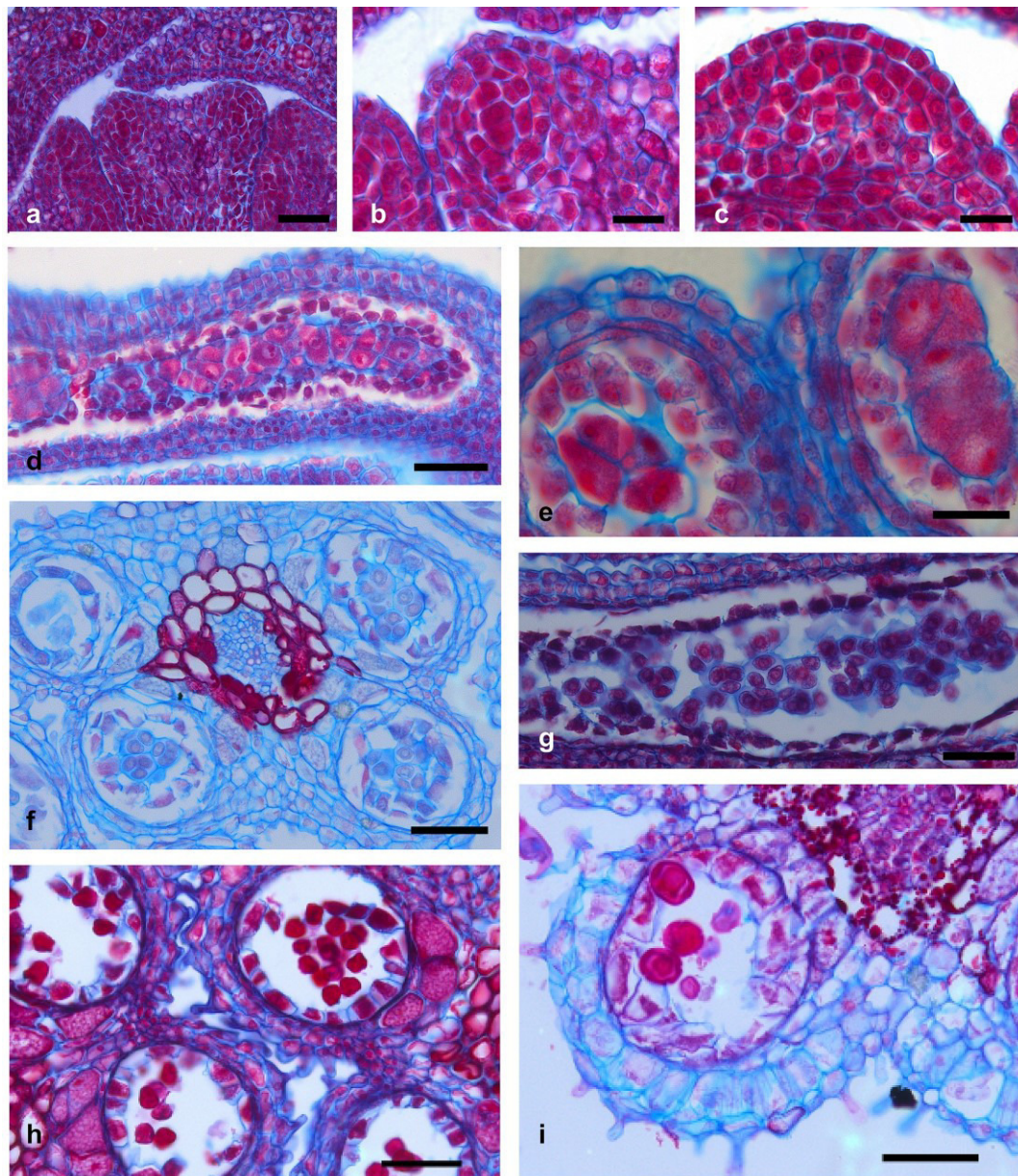


Fig. 3 Microsporogenesis and microgametogenesis in *Trichilia clausenii*. *a*, Beginning of differentiation of the anther. *b*, Formation of the parietal layers of the anther. *c*, Differentiation of the anther tissues: beginning of formation of the parietal layers and the sporogenic tissue. *d* – *e*, Microspore mother cells (longitudinal section). *f* – *g*, Anther showing the microsporangia with tetrads surrounded by callose and secretion from the tapetum (*g* in longitudinal section). *h*, Microspores individualized. *i*, Exine already deposited around each microspore. Bar *c*, *b*, *e* = 20 μ m; *a*, *d*, *f* – *i* = 50 μ m.

Archivesporium cells divide periclinally to form two layers of cells. First, the outer parietal layer, which is located just below the protoderm and will give rise to the endothecium and the middle layer. Subsequently the internal parietal layer is formed, which will give rise to the sporogenic tissue and the tapetum (figs. 3*b*, 3*c*). Cells of the outer layers have large vacuoles and a

dense cytoplasm. The middle layer is composed by two layers of cells. The inner layers present small vacuoles and their cytoplasm appear to be denser.

The sporogenic tissue differentiates and the cells that were previously juxtaposed (adhered by the middle lamella), begin to separate and give rise to the microspore mother cells (MMCs). The MMCs are loose in the locus that begins to form, by the expansion of the anther (figs. 3d, 3e). The locules are filled with the secretion produced by the tapetum. Each MMCs is surrounded by a callose coating. Within this callose wall the mitosis occurs, originating the tetrads of microspores (fig. 3f, 3g). The callose degenerates and the microspores are individualized (fig. 3h).

Microgametogenesis. The exine is deposited around each microspore and gives rise to the sporoderm (figs. 3i, 4a, 4b). The asymmetric division of the cell occurs due to the process of vacuolization pressing the cytoplasm and nucleus to the periphery of the cell. Then vacuoles begin to accumulate starch grains. The microspore is divided by mitosis originating two cells, the generative cell (small) that is encompassed by the vegetative cell (large).

On the bisexual flower morphotype, the generative cell divides by mitosis originating two sperm cells (fig. 4c). In the mature anther, the wall is formed only by epidermis and endothecium, composed of columnar cells with secondary thickening lignified forming trabeculae. The middle layer collapses and the tapetum no longer exists at this phase. The disruption of the stomium promotes the release of the viable pollen grains, which are 4-5-colporate (figs. 4d, 6a - 6c).

On the female flower morphotype, in the mature anther, besides the epidermis and the endothecium, there are also remaining tapetum that start producing a viscous secretion which causes the pollen sterility (figs. 4e, 4f). The pollen grains are pressed to this mass originated from the tapetum, and this prevents their release from the anther (figs. 4g, 4h, 6d, 6e). Tests have shown that pollen grains are alive, but are not viable (weak staining - figs. 2a, 2e).

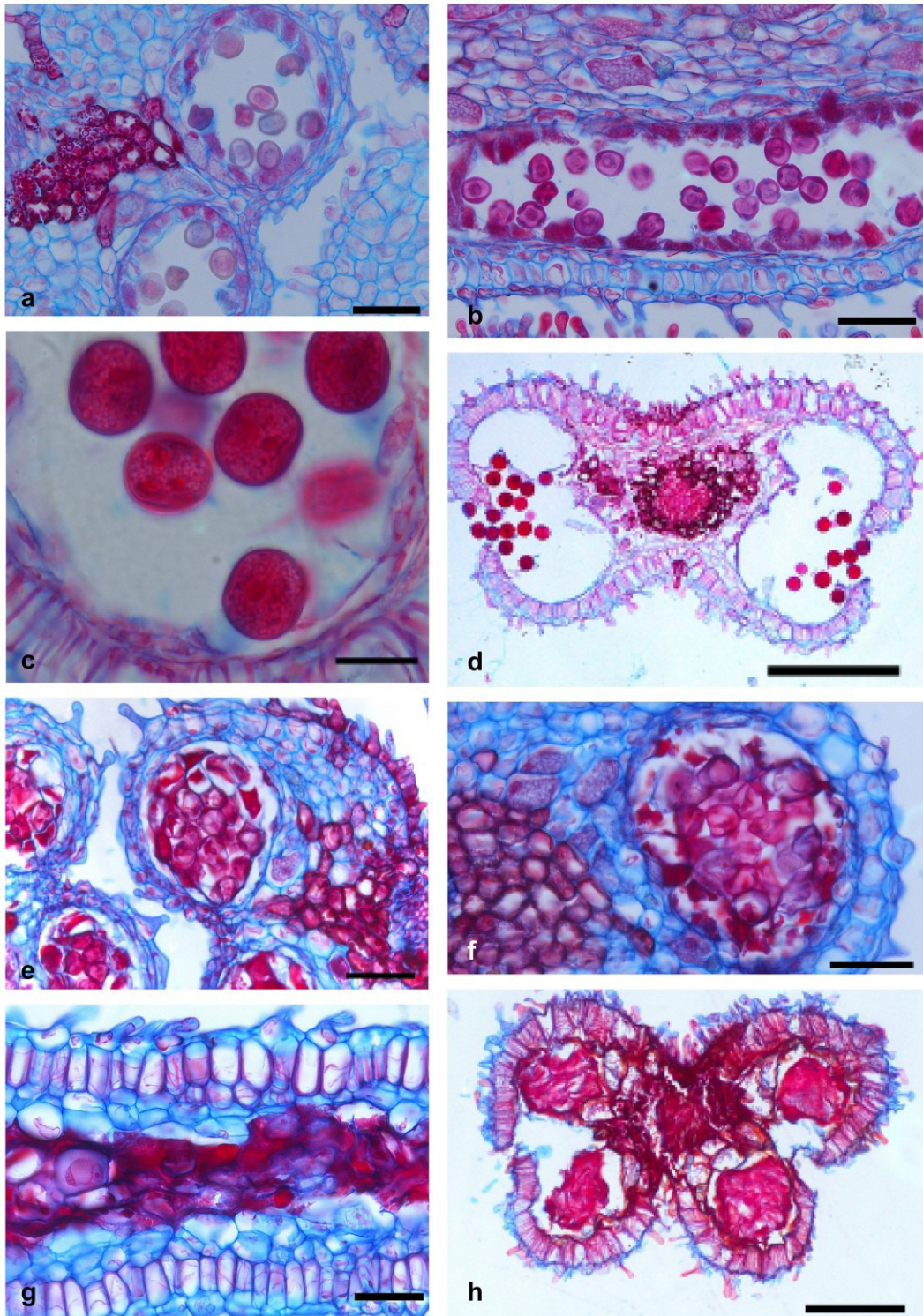


Fig. 4 Microgametogenesis in *Trichilia clausenii*. *a – d*, Anthers of the bisexual floral morphotype. *e – h*, Anthers of the female floral morphotype. *a – b*, Exine deposited around each microspore (*b* in longitudinal section). *c*, Microspore with three cells (two sperm cells and one vegetative cell) at the bisexual floral morphotype. *d*, Anther at anthesis, with pollen grains. *e – f*, Tapetum starting to produce a viscous secretion. *g*, Pollen grains pressed by the mass originated from the tapetum (longitudinal section). *h*, Anther at anthesis with sterile pollen grains pressed at the secretion from the tapetum. Bar *c* = 20 μ m; *a, b, e – g* = 50 μ m; *h* = 100 μ m; *d* = 200 μ m.

Megasporogenesis. Differentiation of the sporogenic tissue that gives rise to the nucellus begins two or three layers below the protoderm. The meristematic tissue of the placenta will form the integuments of the ovule (figs. 5a, 5b). The inner integument is the first to form and consists of three layers of cells. The outer tegument forms later and has four layers thick. The archesporium differentiates into a megaspore mother cell (figs. 5a, 5b). The megaspore mother cell elongates (fig. 5c) and undergoes meiosis to produce four megaspores.

Megagametogenesis. Three of the megaspores above cited degenerate, remaining only one that becomes functional. The expansion of the functional megaspore occurs. In mature gynoecium, there are two ovules per locule. The ovules are arranged in axile placentation, crassinucelate antitropous and collateral (fig. 5e). On them there is a funicular protrusion that will give rise to aryl (fig. 5d).

Megasporogenesis and megagametogenesis follow the same paths in both morphotypes, until forming a viable gametophyte.

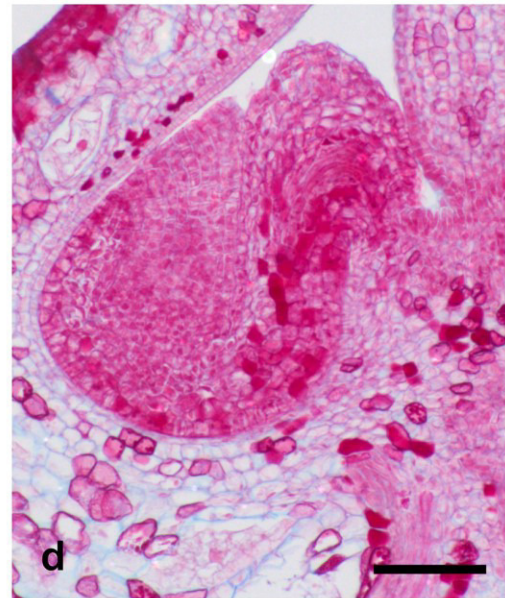
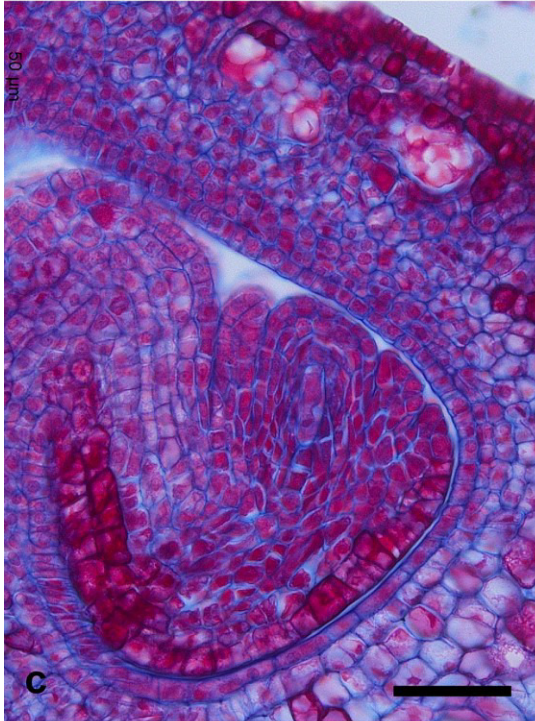
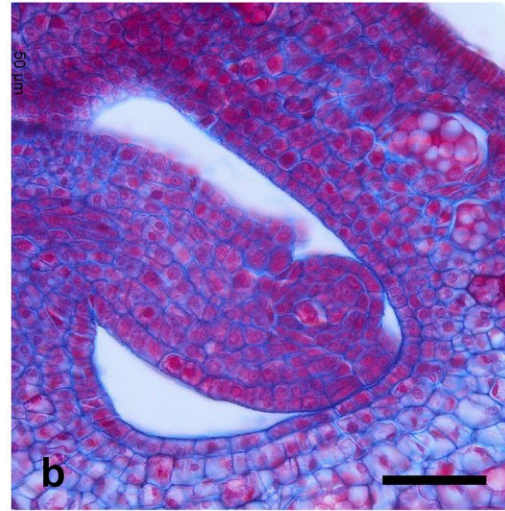


Fig. 5 Megasporogenesis and megagametogenesis in *Trichilia claussenii*. *a – b*, Formation of the ovule with the nucellus (megasporangium) and the initiation of the inner integument. *a*, Differentiated archesporium in the crassinucelate ovule. *b*, tetrad of megaspores, with two nuclei visible. *c*, Extensive functional megaspore (there are at least three nuclei enveloped by a single wall) and onset of megagametogenesis at the same time as the formation of the outer integument. *d*, Funicular protrusion that will give rise to the aril. *e*, Ovary tricarpeolar, trilocular, each locule with two collateral ovules. *f*, aborted seed. Bar *a – c* = 50 μ m; *d* = 100 μ m; *e*, *f* = 500 μ m.

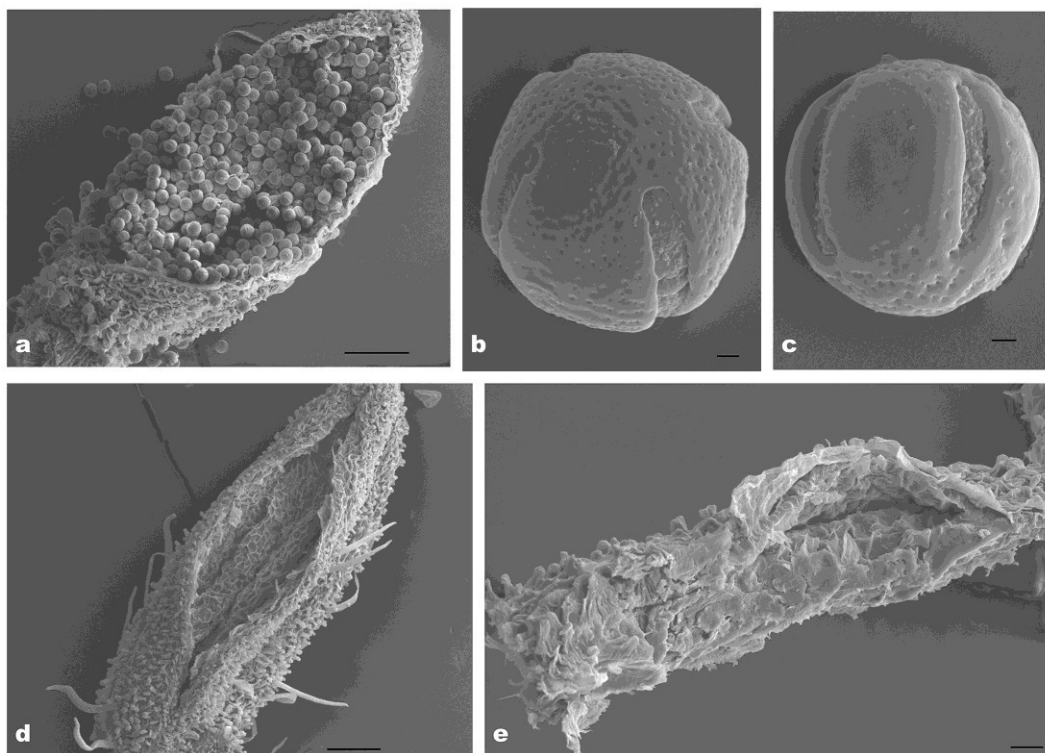


Fig. 6 Scanning electron micrographs of the anther and pollen grain of *Trichilia claussenii*. *a – c*, Morphotype bisexual. *d – e*, Morphotype female. *A*, Anther open longitudinally filled with pollen grains. *b*, Pollen grains 4 colporate. *c*, Pollen grains side view. *d*, Anther open longitudinally with secretion originated from the tapetum. *e*, Secretion of the tapetum isolated (secretion removed from the anther). Bar *b*, *c* = 2 μ m; *e* = 20 μ m; *a*, *d* = 100 μ m.

Discussion

For a long time, the flowers of Meliaceae were considered as bisexual because of their general morphology, but an increasing number of works have shown an impressive diversity of sexual systems within the family, specially related to sexual dimorphism (e.g. Styles 1972; Pennington and Styles 1975). Our findings during the study of *Trichilia classeni* are in general agreement with Pennington et al. (1981) and Pennington (2016), which mention that often the sexual systems in *Trichilia* are dioecious with unisexual flowers, and less frequently constituted by hermaphrodite or

polygamous plants. Likewise, structural analysis made under stereomicroscope by Morellato (2004) suggested that *Trichilia pallida* Swartz could be a dioecious species. However, Schmitt (2013) carried out structural analysis and a controlled pollination experiment, and came to the conclusion that *T. pallida* is either monoecious or gynodioecious. Souza et al. (2001) conducted a morpho-anatomical study on the same species and also on *T. catigua* A.Juss. and *T. elegans* A.Juss., concluding that they are all monoecious. These somewhat conflicting results may be a reflect of distinct accuracy of each methodological approaches, or of effective variations found across populations of widely distributed species.

Concerning to *Trichilia clausenii*, Pirani (1992) reported having found only monoecious individuals in a population at the Serra do Cipó (Minas Gerais, Brazil). Morellato (2004) describes this species as strictly dioecious, based on stereomicroscopic analysis of individuals from Santa Genebra Municipal Reserve, in Campinas (São Paulo, Brazil). Gouvêa et al. (2005) were unable to determine sexual dimorphism in this species, noting the presence of ovules and pollen grains considered morphologically normal. The images presented in the latter work are clearly taken from flowers of the bisexual morphotype.

In the present work it was observed the presence in *T. clausenii* of two floral morphotypes that are macroscopically and microscopically distinct: one with a functional gynoecium associated to stamens that not release viable pollen grains and another with fully functional gynoecium and androecium (figs 1a – 1h). These morphotypes occur in different individuals, thus indicating a likely gynodioecious condition. However, our pollination tests showed that no growth of pollen tubes occurred in any of the pollination types performed in individual 1, and no fruit formation was observed in the same plant along the last two years. Hence, it seems more likely that this species may have a third functionally male morphotype, where the morphoanatomical characters are identical to the bisexual morphotype, but the gynoecium is not functional. This situation was described by Schmitt (2013) as subdioecy, while examining the reproductive system of *Cabralea canjerana* (Vell.) Mart. subsp. *canjerana* and *Trichilia pallens* C.DC., both of

which present individuals with female flowers that form fruits annually, individuals with staminate flowers that never form fruits, and individuals mostly with staminate flowers forming some fruits. The lack of a geitonogamy test in the work by Schmitt (2013) may have led the author to the erroneous conclusion that the staminate plants producing fruits could present some bisexual flowers, in lower quantity. However these flowers may actually be functionally bisexual, with restriction of the female function to the type of pollen that germinates in their stigma (fig. 2h, 2i). Moscheta (1995) also reported the formation of fruits in individuals considered phenotypically male plants, as in *T. catigua*, and classified the species as polygamo-dioecious. Fuzeto et al. (2001) reported the same for *C. canjerana* spp. *polytricha* (A.Juss.) Pennington, classifying the subspecies as dioecious, recognizing no evidence of subdioecy.

The subtle distinctions between flowers of different genders seem to be a widespread condition in Meliaceae, in which a pistillode may be practically indistinguishable from the functional gynoeceum, and the staminodes are only slightly smaller than the stamens (Styles 1972; Pennington and Styles 1975). Subtle sexual floral variations related to organ sterility are also known in several other members of Sapindales. For example, structurally bisexual, but functionally unisexual, flowers occur in *Kirkia* (formerly Simaroubaceae), in representatives of Anacardiaceae and Burseraceae (Bachelier and Endress 2009), and were recently reported for species of *Homalolepis* (as *Simaba*) by Alves et al. (2017), a genus that was so far described as hermaphroditic. The difficulty in adequately interpreting floral sexuality in Sapindales is a reflection of the subtle sexual dimorphism related to morphological differences between floral sexes, and most genera of the order still need to be carefully studied.

According to Ehlers and Bataillon (2007) many studies show that unstable male plants (hermaphrodites) are more prone to produce fruits in resource-rich and moist environments, but remain male in resource-poor settings. This variation suggests that some male genotypes do not allow the development of female expression, while other male genotypes can still produce fruit. These data suggest that subdioecious species may retain a

genetic component for the production of fruits. Delph and Wolf (2005) point out that fruit production in hermaphrodite individuals may vary over the years, depending on environmental conditions prior to flowering. In *T. clausenii*, 30 of the seeds we collected from two bisexual individuals and from two female individuals germinated, showing that 83.3% of the seeds produced are viable.

Barrett and Hough (2013) provide a review of the wide range of morphological, physiological, and life-history traits related to sexual dimorphism in angiosperms. They discuss their ecological consequences, and detail the known genetic and evolutionary processes that drive divergence between distinct sexual phenotypes, stressing that understanding this is still “a central question in evolutionary biology”.

Tests

Our tests indicate that the stigmas of the two morphotypes of this species are receptive. The stigma remains receptive for two days after the anthesis. Carmo (2005) found that although the flowers of *C. canjerana* last to two days, the stigmas are more receptive on the first day of the anthesis. This can be advantageous since the plant spends less energy holding flowers longer than producing new flowers.

According to Munhoz et al. (2008), the tests with acetic carmine and Alexander's stain are inefficient to estimate the pollen viability. But they allow us to make inferences about the integrity of the pollen grains effectively as acetic carmine test indicates the chromosomal integrity red staining of viable pollen grains. Alexander's stain allows distinguishing the aborted grains from the viable grains, since the malachite green blends the cellulose of the pollen wall and the acid fuchsin reacts with the protoplasm. Thus, aborted grains have no nucleus and will only blush green.

The tests confirm the integrity of the pollen grains present in the bisexual morphotype in *T. clausenii*. Even the pollen grains of the female morphotype revealing weaker staining and therefore negative in relation to the grains of the bisexual morphotype, we can observe that the grains are fully formed and alive, but not viable (figs. 2a – 2f). Since, the secretion

released by the carpet interrupts the onset of gametogenesis and therefore the microspore for its development in this stage. It is possible to observe by the form that the microspore is fully formed, with exina.

Contrary to what is most expected in terms of pollination, at the evolutionary and genetic level, xenogamic pollination was not observed among the hermaphrodite individuals. Instead, geitonogamy resulted in the highest number of grains that developed pollen tube, and autogamy had a lower frequency (figs. 2g – 2j; table 1).

Development

The results found for *T. clausenii* are similar to the general patterns reported in the literature on sporogenesis and gametogenesis in angiosperms (e.g. Esau 1974; Weberling 1989; Fahn 1990; Lersten 2004), but some particularities are noted in this species. Its most striking feature is the non-fragmentation of the tapetum, in the mature anther. During the standard development of pollen grains, tapetum collapses before anthesis, a fact that is observed in the anther of the bisexual morphotype, but that does not occur in the anther of the female floral morphotype of *T. clausenii*. The non-fragmentation of the tapetum is a novelty, since there are no similar reports in the literature of the group.

Souza et al. (2001), while studying three species of *Trichilia* (*T. elegans*, *T. catigua* and *T. pallida*), observed the mature anthers of male flowers beginning the process of disintegration of the middle layers and the tapetum. The pollen grains remain attached in tetrahedron type tetrads and the pistillode has a structure similar to a fertile pistil, but with aborted ovules. In female flowers, anthers sterile without pollen also disintegrate the tapetum and the middle layers, and the pistils are developed and contain fully developed ovules. Compared to *T. clausenii*, these three species share similar morphological and developmental features, but with significant differences, such as the androecium of female flower bearing pollen, which is locked into a dense secretion from the persistent tapetum in *T. clausenii*. This species shows a gynoeceum presenting morphologically well-formed

ovules in both morphotypes (figs. 1c, 1d, 5e). The pollen grains are released as monads.

The tapetum is a specialized tissue that nourishes the microspores and later undergoes disintegration as pollen maturation takes place, an event that coincides with the storage of food reserves by the generative cell of the pollen grains, in normal development of the anther. In the female morphotype of *T. clausenii*, some tapetal cells continue secreting even after the formation of the pollen grain, such secretion involves the grains, prevent their release and cause the interruption of gametogenesis, making the pollen grain unfeasible. This inhibition generated by tapetal cells secretion is something new, since it was not previously recorded in any study of the genus.

The secretory-type tapetum (parietal) is the most common to be found in eudicots. Many studies have shown the direct or indirect involvement of this tissue from the development of the microspore mother cell to the germination of pollen (Laser and Lersten 1972; Esau 1974; Fahn 1990; Lersten 2004; Rudall 2007; Karasawa 2009). Vasil (1967) observed or abnormal behavior of the tapetum or degeneration, caused by the deficiency of light, water and mineral nutrients, which generated a failure in the development of pollen. Chauhan and Singh (1968) observed through acid phosphatase activity that the variation in this enzyme caused the tapetum to degenerate into normal anthers as the development of the microspore progresses, and the tapetum persists, becoming hypertrophic and crushing the microorganisms in sterile anthers. In the literature there are reports that non-functional tapetum causes malformation of the microspores, which finish aborting (Laser and Lersten 1972; Fahn 1990; Lersten 2004), but there are no similar reports to that observed in this study. Schmitt (2013) cited the presence of dry mass in the infertile anthers of *C. canjerana* and *T. pallens*, but the lack of a detailed description of this structure prevents an understanding on how that mass was formed.

Another fact, also not expected, concerns the gynoeceum of the bisexual floral morphotype, which being small and morphologically different from gynoeceum of the female morphotype, was previously classified by us

as a supposedly male morphotype, taking into account only the observation of external morphology (figs 1c, 1d). However, throughout the study, gynoecium of the bisexual morphotype proved to be viable, despite the difference in size, also emphasizing the importance of detailed developmental studies.

Conclusion

The data presented herein helps to clarify some uncertainties regarding floral sexuality in *Trichilia*, since they allow to understand that *T. clausenii* exhibits sexual floral dimorphism, presenting at least two functional morphotypes. The pollen viability tests, stigma receptivity, fruit formation, along with the morphoanatomical data, reveals the viability and integrity of the androecium and gynoecium in both floral morphotypes. However in the female morphotype the anther is sterile due to the presence of the secretion of the tapetum that forms a mass that adheres to the pollen, preventing its release and eventually causing its sterility. Additionally, the data obtained through pollination tests have allowed us to characterize the species as gynodioecious. Nevertheless, the possibility that it is in fact subdioecious remains, since studies carried out with other populations found a significant number of functionally male individuals that do not produce fruits.

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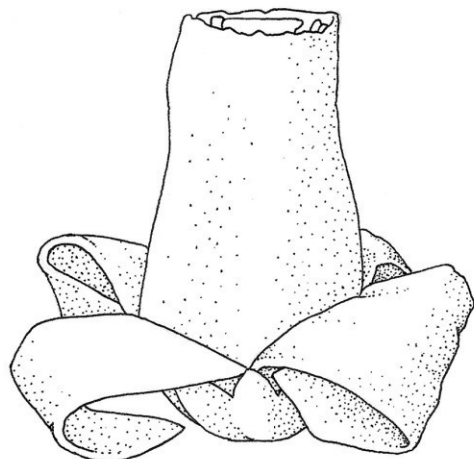
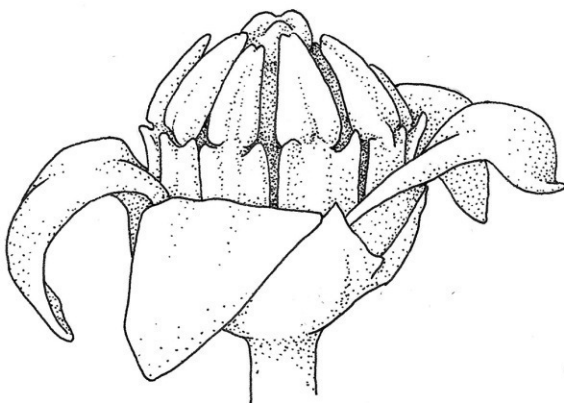
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Chapter II

Structure and development of flowers of
Guarea macrophylla subsp. *tuberculata*
(Vell.) T.D.Penn. and *Trichilia clausenii*
C.DC. (Meliaceae)



ABSTRACT

Premise of the study: The Meliaceae (Sapindales) is a mostly pantropical family composed of c. 575 species. The floral structure and sexual systems in the family are very diverse, often with flowers of a very subtle sexual dimorphism. These variations hamper the determination of floral sexuality in most members of the family, and the structural studies available for a few taxa describe the floral anatomy focusing on histological and embryological aspects. However, there is no detailed study to date regarding the structure and development of internal morphological surfaces of the Meliaceae carpels and androecium. We performed a detailed study of the floral structure of *Trichilia clausenii* C.DC. and *Guarea macrophylla* subsp. *tuberculata* (Vell.) T.D.Penn., which belong to two of the five major genera that composed the family. We aim to contribute to a better knowledge on the morphology and evolution of the family, focusing on the structure and development of the gynoecium and androecium of these two species.

Methods: Flowers of *T. clausenii* and *G. macrophylla* subsp. *tuberculata* in several stages of development were collected, fixed, embedded in Paraplast and sectioned using a rotary microtome. The morphological analysis was done using stereomicroscope and ontogenetic analysis used SEM. Several histochemical tests were performed to investigate the chemical composition of the different exudates.

Key results: We present the data on floral structure of both species under a comparative approach and although several floral structures we found are commonly reported in the literature of the family, we describe some novel structural features, as the ones related to filaments fusion: In *G. macrophylla* subsp. *tuberculata* the filaments show an early fusion, and form a tube that grows behind the anthers which are free from each other. In *T. clausenii* the connate base of the filaments is late, and along most of their length they keep coherent to each other by means of intertwining of non-glandular trichomes present at their margins. Regarding the gynoecium, the main differences between the two species are related to ovule position, stigma shape, presence of an elongated gynophore only in *G. macrophylla*, formation of a compitum only in *T. clausenii*, and histochemical stigma composition. The gynoecia of both species are fully syncarpous (eusyncarpous), since they are synascidiate proximally and symplicate distally. In relation to floral sexuality, the two morphotypes in *T. clausenii* did not differ histochemistry, structural development and vascularization, being differentiated only by the external morphology and the non-release of pollen grains in the female morphotype. In *G. macrophylla* no sexual dimorphism was observed. The features reported here are also compared to other Meliaceae and to other families of Sapindales.

Conclusions: The data presented allowed a comparison with structures of other representatives of Meliaceae, besides an improvement of the knowledge of floral morphology in the group, mainly in relation to the structure and development of the gynoecium and androecium.

Keywords: androecium; diplostemony; floral ontogeny; gynoecium; Sapindales.

INTRODUCTION

The Meliaceae is an essentially pantropical family composed of ca. 575 species, belonging to the order Sapindales. Members of the family are commonly marked by compound leaves and flowers with a staminal tube (Pennington and Styles, 1975). The sexual systems in Meliaceae are very diverse, occurring predominantly unisexual flowers. However, the sexual dimorphism of flowers is very subtle, and structurally every flower exhibits a gynoecium and an androecium, even though functionally one of them may be sterile (pistillode or staminode). Consequently, these variation hampers the determination of floral sexuality in the family (e.g. Pennington and Styles 1975; Styles 1972).

Among the first structural studies of Meliaceae flowers in the 20th century, Wiger (1935) and Narayana (1958a, 1958b) briefly described the floral anatomy of some species, showing the axile placentation and superposed ovules, bitegmic, antitropous to campylotropous, some with micropyle in "zig-zag". Other works focused on how the floral features could contribute to the taxonomy of the family, such as Lal (1994), describing the floral vascularization and other features of *Cedrela* species, as well as Gouvêa et al. (2008a) that characterized the general development of the flower whorls of *Toona* and *Cedrela* and detecting the plesiomorphic and apomorphic characters at the tribal level. In a more detailed study of the structure of the inflorescence and flower of *Switenia macrophylla* King (the mahogany tree), Gouvêa et al. (2008b) established that the morphological differentiation of the floral sexes occurs only in the last stages of floral development, discussing the practical consequences in the conservation strategies of the species.

Other structural works on flowers of Meliaceae include those of Souza et al. (2001, 2002), with *Trichilia* and *Guarea* species, and Moscheta et al. (2002) with *Cabralea canjerana* (Vell.) Mart., all analyzing buds in pre-anthesis and anthesis only. Souza et al. (2002) focused on the histological and embryological aspects of *Guarea macrophylla* Vahl, but did not address the detailed structural aspects of gynoecium. In contrast, Gouvêa's thesis (2005) addressed to the floral development of seven species of Meliaceae,

among them *Trichilia clausenii* C.DC., focusing on the study of the expression of ABC genes related to the development of floral whorls.

According to Pennington and Styles (1975), an important diagnostic feature in Meliaceae is related to the androecium, where it presents from the partial fusion to complete fusion of the filaments, varying widely among their genera.

Although carpels of the family are usually described as syncarpous (Harms 1940; Gouvêa 2008a, b), there is no detailed study to date on the development and mode of closure of carpels (Bachelier and Endress, 2008), understand the floral evolution, as several articles have already demonstrated (Endress, 2011, 2015). The lack of such information also hampers an accurate comparison with other representatives of Sapindales previously reported.

In the present work we studied one species of *Trichilia* P.Browne and one of *Guarea* Allamand ex L., which are two out of the five major genera that compose the family, both belonging to the subfamily Melioideae and to tribes Trichilieae and Guareeae, respectively (Pennington and Styles, 1975). The molecular phylogeny available for the family indicates that the Trichilieae tribe is not monophyletic, and that the Guareeae tribe presents inconclusive data on its monophyly (Muellner, 2003). *Trichilia* is represented by 81 species in Tropical America (from Mexico to Argentina), around 20 species in Africa, and two species in Madagascar (Pennington, 2016); *Guarea* is represented by 69 species, occurring predominantly in Tropical America in plains below 1000 m altitude (Pennington and Clarkson, 2013).

Both genera are composed of trees or treelets, which apparently present syncarpous carpels, 1-2 ovules per carpel and loculicidal capsules.

We performed a detailed study of the floral structure of *Trichilia clausenii* C.DC. and *Guarea macrophylla* subsp. *tuberculata* (Vell.) T.D.Penn., aiming to contribute to a better knowledge to the floral morphology and evolution of the family. We focused mainly on the structure and development of gynoecium and androecium, aiming to detect the possible variations on their development and thus, understand the sexual systems of these species and to allow an accurate comparison with the

carpel structure of other representatives of Sapindales, for which these data are already available, and also provide data on androecium.

MATERIALS AND METHODS

Flowers of *Trichilia clausenii* and *Guarea macrophylla* subsp. *tuberculata* in several stages of development were collected at the Reserva Florestal da Cidade Universitária “Armando Salles Oliveira” (Reserva CUASO - 46° 43' 47,42" W e 23° 33' 56,64" S) and Horto Oswaldo Cruz in Instituto Butantan (23° 34' 1,22S 46°43'2, 74"O) in São Paulo, Brazil (Rossi, 1994; Zanelli, 2008). Vouchers of both species are deposited at the Herbarium SPF of the Department of Botany at USP.

The material were fixed in FAA (formaldehyde, glacial acetic acid, 50% ethanol, 1:1:18 v/v) for 24 hours, dehydrated in an ethyl series and storage in 70% ethanol for morphological and anatomical studies (Johansen, 1940). For the histochemical studies, the flowers were fixed in BNF (buffered neutral formalin) for 48 hours (Lillie, 1965).

Observations of general aspects of flower morphology were made using a Leica M125 stereomicroscope, specially while searching for occurrence of unisexual flowers in both species, and with measurements of floral parts made with a digital caliper Digimes model LR44. For the anatomical and histochemical analyses, the material was dehydrated through a *terc*-butyl series and embedded in Paraplast (Johansen, 1940). The embedded material was longitudinally and transversally sectioned at 8 -14µm for *T. clausenii* and at 10 - 18µm for *G. macrophylla tuberculata*, using a Leica RM2145 rotary microtome (Leica Microsystems, Wetzlar, Germany).

The sections for the anatomical analysis were stained with astra blue and safranin (Gerlach, 1984), and the slides were mounted in Permount resin (Fisher Scientific, Pittsburg, PA). The material was analyzed using a Leica DM 400B light microscope (Wetzlar, Germany) coupled with a digital camera.

For ontogenetic analysis, the flower buds at different developmental stages were dissected under stereomicroscope, dehydrated through an

ethanol series and critical point dried. The material was mounted on aluminum stubs and coated with gold and examined in a Zeiss Sigma VP scanning electron microscope. For the histochemical analysis, the tests described in table 1 were performed according to Demarco (2017).

TABLE 1 - List of histochemical tests performed, chemical classes detected and their references.

Staining	Composition	References
Ruthenium red	Acidic mucilage	Gregory and Baas (1989)
PAS reaction (Periodic Acid: Schiff's reagent)	Carbohydrates	McManus (1948)
Aniline blue black	Proteins	Fisher (1968)
Sudan black B	Lipids	Pearse (1985)
Sudan IV	Lipids	Pearse (1985)
Nile blue	Acidic and neutral lipids	Cain (1947)
Copper acetate and rubeanic acid	Fatty acids	Ganter and Jollés (1969, 1970)
Ferric chloride	Phenolic compounds	Johansen (1940)
Wagner's reagent	Alkaloids	Furr and Mahlberg (1981)

RESULTS

Morphology – Flowers of *Guarea macrophylla* subsp. *tuberculata* are dichlamydeous (heterochlamydeous) and bisexual. They are produced in axillary or lateral, cauliflorous thyrses. The perianth is tetramerous, occasionally pentamerous or hexamerous. The calyx is gamosepalous, patelliform and cyathiform (Fig. 1F). Sepals are brown, 2.5 – 3 mm long, fused on 2/3 of their length with valvate aestivation. The corolla is actinomorphic, mostly with four or five free petals, which are valvate or slightly imbricate in bud. Petals are rosefish-cream and 8.5 – 9 mm long. The androecium is composed of eight stamens (or 10 or seldom 12). The staminal tube is formed by the connation of the filaments (Figs. 1 F – 1I). The anthers are bithecal, tetrasporangiate, inserted by the connective at the upper third of the inner face of the staminal tube; they are latero-introrse, with longitudinally dehiscence (Figs. 1G – 1 J, 9G). The gynoecium present four antesealous carpels, syncarpous and loculi with two ovules superposed. The style is stout, pilose at the lower half, and the stigma is discoid (Figs. 1G – 1I). The ovary is superior, formed at the apex of the terete gynophore. The nectary is located at the expanded distal portion of the gynophore (Figs. 1G, 1I). Flowers of *Trichilia claussenii* are dichlamydeous (heterochlamydeous) and functionally can be unisexual or bisexual. Inflorescences are usually axillary elongated thyrses. The perianth is pentamerous (Fig. 11A, 12G). The calyx is gamosepalous, patelliform or cyathiform (Fig. 1A). Sepals are greenish-cream, 1–2 mm long, fused 1/3 their length, with valvate or slightly imbricate aestivation. The corolla is actinomorphic, with five free petals, which have a cochlear imbricate aestivation. Petals are yellowish-cream and 2–3 mm long. The androecium is composed of ten stamens (Fig. 1C). The filaments are distally coherent to each other by the intertwining of trichomes, forming a staminal tube (a pseudotube), but they are connate near to the base (Fig. 1A).

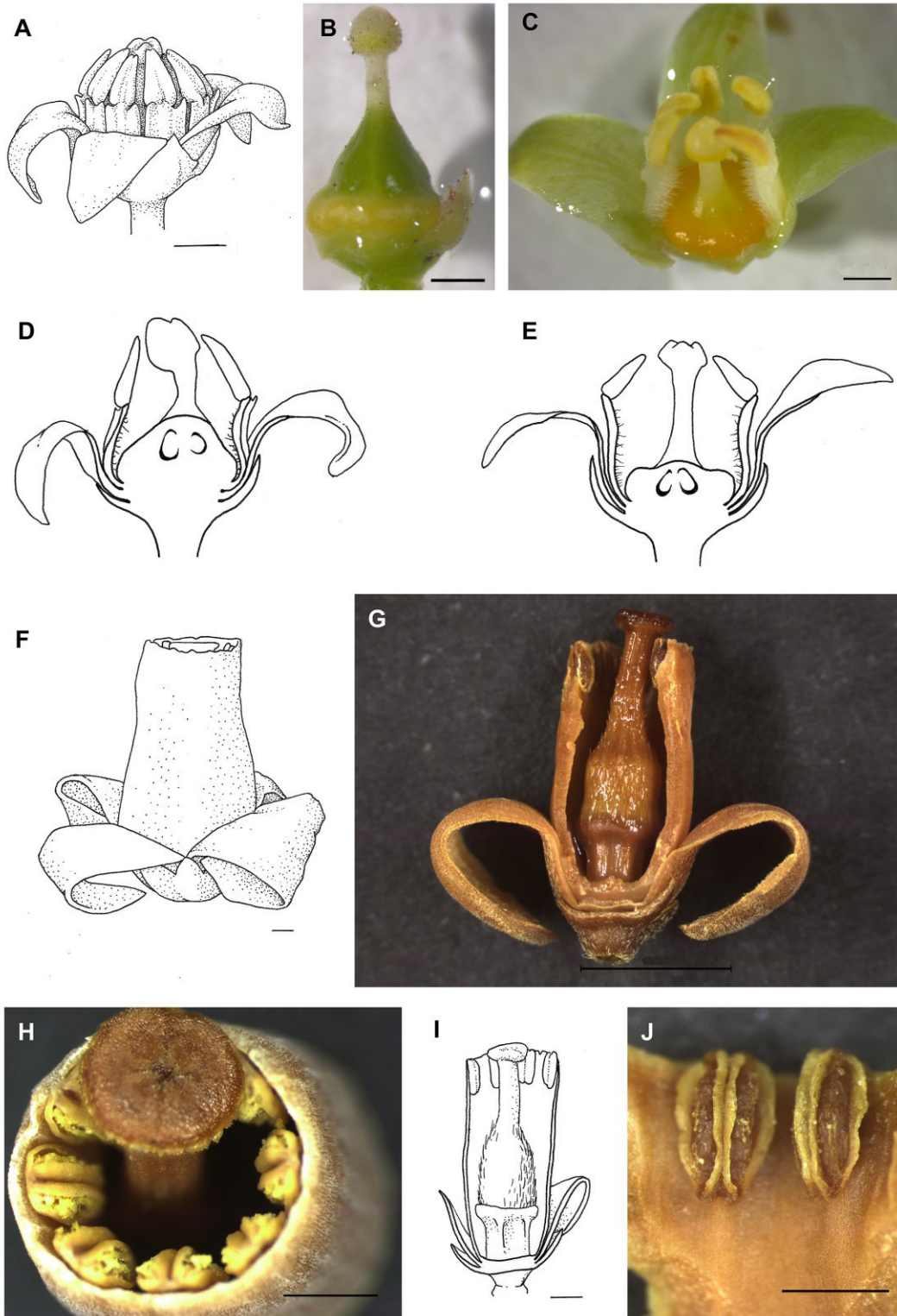


Figure 1. Structure of the flowers of *Trichilia clausenii* (A – E) and *Guarea macrophylla* subsp. *tuberculata* (F- J). (A) A pentamerous flower at anthesis. (B) Gynoecium of the female morphotype. (C) Flower of the bisexual morphotype (part of the androecium and perianth were removed to show the style shape and the bright-orange nectary). (D) Longitudinal view of the flower (part of the gynoecium, androecium and perianth were removed to show the ovules) showing the conical-pyramidal gynoecium. (E) Longitudinal view of the flower (part of the gynoecium, androecium and perianth were removed to show the small ovule) showing the conical gynoecium immersed in the annular nectary. (F) Side view of the flower at anthesis, showing fused sepals, free petals, staminal tube and part of the stigma. (G) The flower (part of the androecium and perianth were removed to show style shape, insertion position of the anthers in the staminal tube and gynophore. (H) Detail on top view of the staminal tube and discoid stigma; note anthers full of pollen grains. (I) The flower (part of the androecium and perianth were removed to show style shape, insertion position of the anthers in the staminal tube and gynophore with trichomes. (J) Anthers latero-introrse, with longitudinally dehiscence inserted at the upper third of the inner face of the staminal tube. Bar B, C= 200µm; A, D – F, I = 1 mm; H, J = 2 mm; G = 5 mm.

The anthers are longitudinally dehiscent (latrorse) and tetrasporangiate. The bisexual morphotype has a gynoecium with an cylindrical shape at the base, conical in apex (Figs. 1C, 1E). The female morphotype presents gynoecium is conical-pyramidal (Figs. 1B, 1D). The gynoecium of both morphotypes lack a gynophore and is syncarpous with three antesealous carpels, each locule with two collateral ovules (Figs. 10 E, 11H). The style is short, stout and glabrous. The stigma is trilobed at the apex (Figs. 11D, 12 A, 12 C, 12G). The ovary is superior and is surrounded at the base by a conspicuous intrastaminal nectary disc (Figs, 1B – 1E, 12B, 12C, 12I).

Histology – The sepals, petals and the filament are formed by an uniseriate epidermis. And these last two present epidermic cells more elongated (papillose) in both faces, and present more trichomes than the sepals in the abaxial face in both species. The filaments of *Trichilia* bear tectonic trichomes on both faces, however they are longer and lignified on the adaxial face. The mesophyll present chlorophyll parenchyma cells in the sepals, while in the remaining organs the parenchyma is fundamental. The mature anther is formed by an uniseriate epidermis and an endothecium composed of columnar cells with lignified trabeculae (Figs 2H, 2L). In *Trichilia*, the anthers are well developed in both morphotypes, but the bisexual present viable pollen grains that are released. Already in female morphotype the pollen grains is inviable, because they are trapped in an amorphous mass. Pollen grains are 4-5-colporate (Fig. 9G, 9H, 12H).

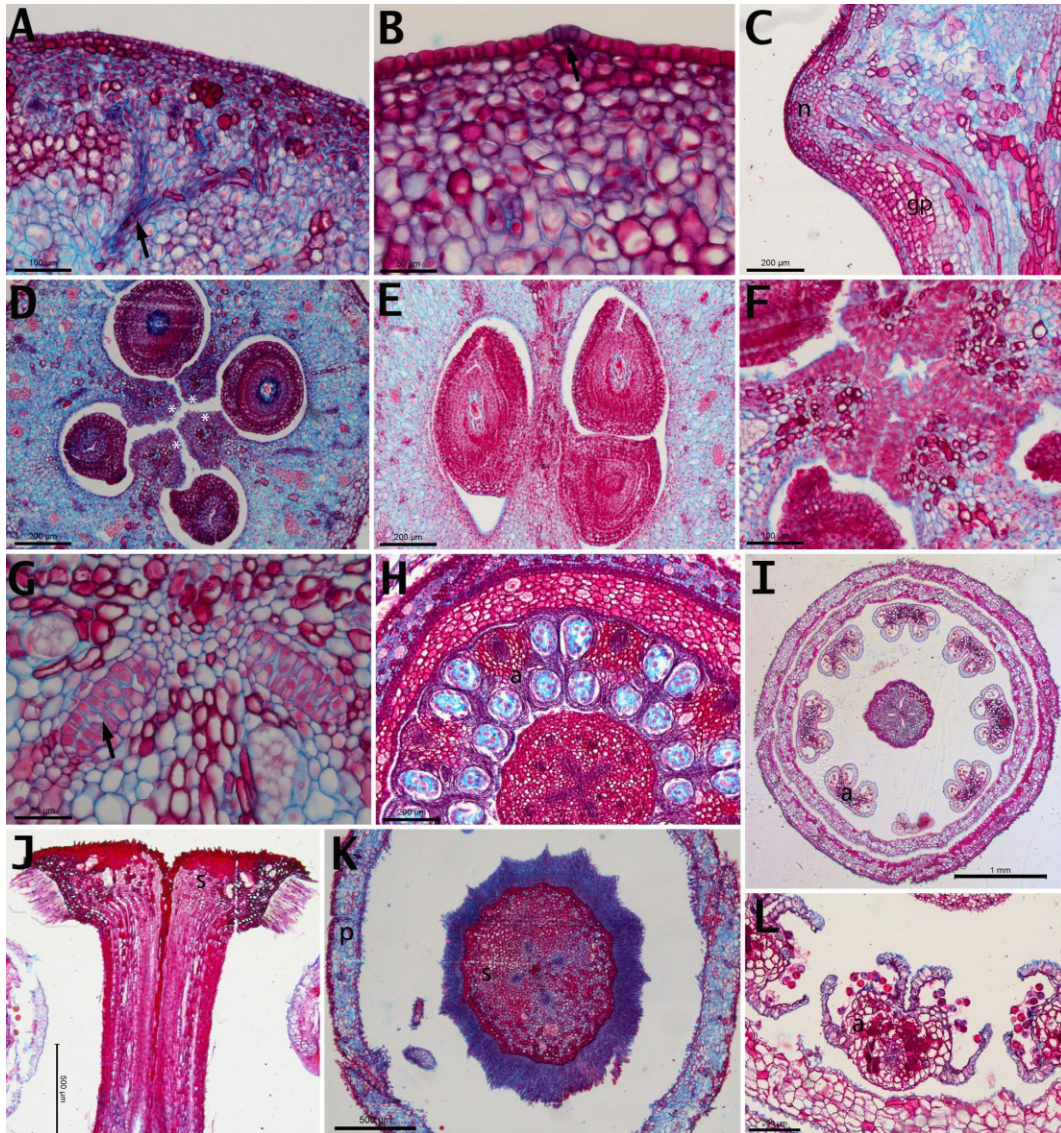


Figure 2. Flowers the of *Guarea macrophylla* subsp. *tuberculata*. (A, B, D, F – I, K, L) Transverse sections. (C, E, J) Longitudinal sections. (A) Nectary vascularized by phloem (arrow). (B) Nectariferous tissue with stomata at the epidermis (arrow). (C) Detail of the nectariferous tissue present only in the apical portion of the gynophore. (D) Unilocular region of the carpel with the presence of placental obturators in the septal region (asterisk). (E) Ovules bitegmic, superposed. (F) Detail of the placental obturator. (G) Fusion of the central portion between the narrow stilar canals (arrow). (H) Transection showing the tetrasporangiate anthers with pollen grains forming; congenitally fused staminal tube and stigma with four closed stilar canals. (I) Fully developed anthers with pollen grains; congenitally fused staminal tube, stigma with four open stilar channels and free imbricate petals. (J) The receptive region of stigma with secretory trichomes. (K) Apical region of stigma with four closed stilar canals and receptive region in purple-blue. (L) Anthers opened, latero-introrse, releasing pollen. a, anther; gp, gynophores; p, petal; n, nectary;; s, stigma.

Secretory idioblasts are present in all floral organs, in different quantities, and it is possible to differentiate two types, by size and appearance of secretion. The predominant idioblasts are the standart ones

that produce phenolic compounds, whereas the wide idioblasts produce oil and are rarer (Table 2 - Figs. 4H, 4K). The nectary of the *Guarea* is located at the expanded distal portion of the gynophore, is composed by a nectariferous parenchyma vascularized only by phloem which release the nectar through stomata (Figs. 2A – 2C, 9F).

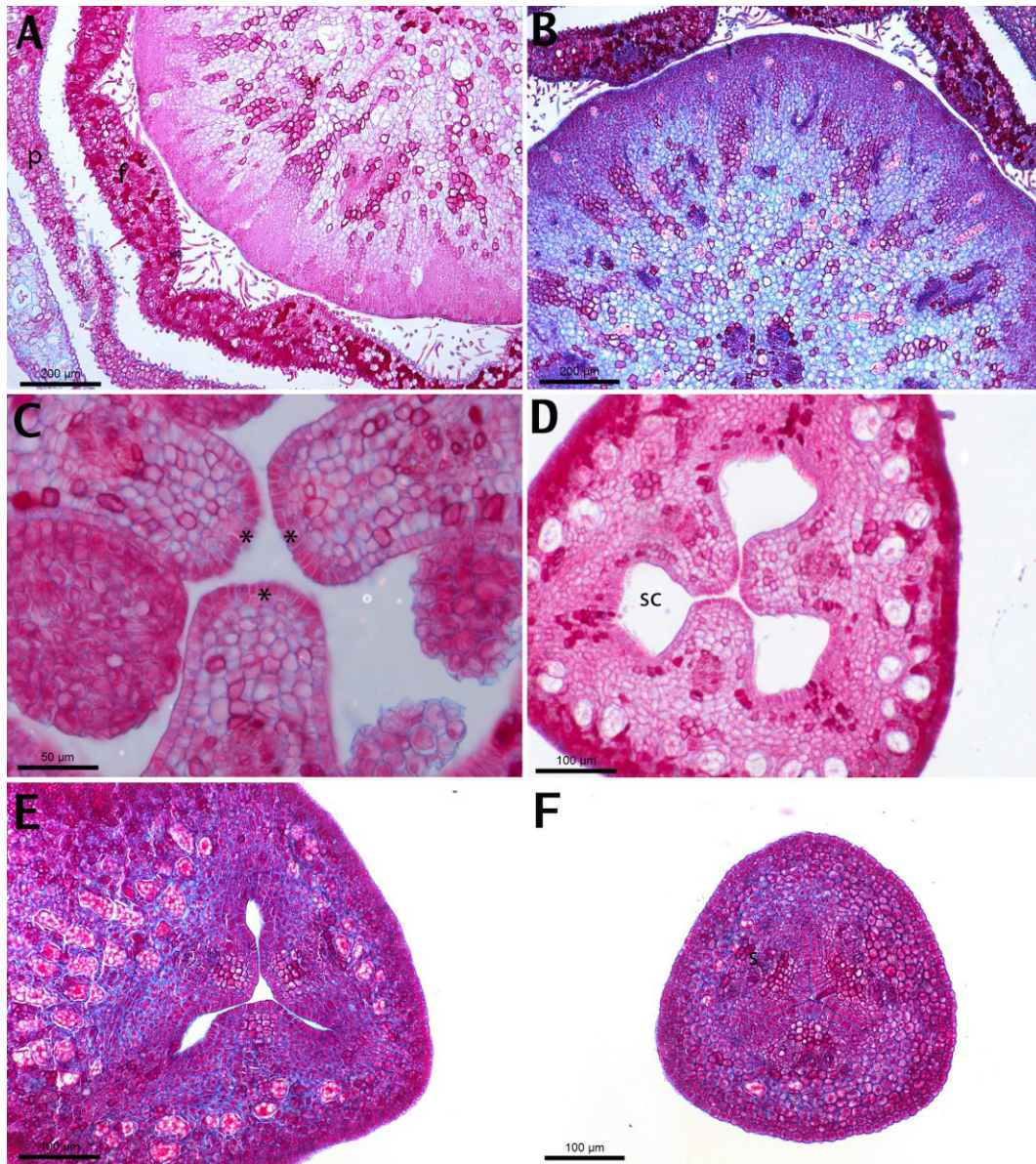


Figure 3. Flowers the of *Trichilia clausenii* in transverse sections. (A) Free imbricate petals, filaments connate at the base. (B) Nectary vascularized by phloem. (C) Detail of the unilocular region of the carpel with placental obturators in the septal region (asterisk). (D) The unilocular region of the carpel with placental obturators in the septal region and wide idioblast. (E) Apical region of the ovary with compitum and wide idioblast. (F) Apical region apical of the style with narrow styler canal and compitum. f, filaments; p, petal; sc, styler canal; s, stigma.

Flowers of *Guarea* have ovules with axile placentation, crassinucelate, bitegmic, campylotropous (Figs. 2D, 2E). The outer integument is formed by 4-5 layers of cells and the inner integument by 3-4 layers which has a distinctive endothelium. The micropyle is formed by both integuments (Fig. 2E). The style has four styler canals and, although there is no styler compitum, the placental obturator present in the unilocular region at the top of the ovary allows the pollen tubes to reach any of the locules (Figs. 2F – 2I, 2K). The discoid stigma has secretory trichomes which produce mucilage, proteins, fatty acids and phenolic compounds (Table 2 – Figs. 4A – 4F).

The gynoecium of *Trichilia* has epidermis glabrate and papillose, and the ovules with axile placentation, crassinucelate, bitegmic, anatropous and antitropous. The outer integument is formed by four layers of cells and the inner integument by three layers. The ovules present a protrusion in the funiculus that will subsequently give rise to an aril. The micropyle is formed by both integuments. The nectariferous disk is located around the ovary base and is composed of secretory parenchyma vascularized only by phloem (Figs. 3A, 3B). The nectar is released through stomata. The style has a central canal with triangular shape, forming a compitum that extends from the style to the ovary and allow pollen tubes reach any locule (Figs. 3D – 3F). The compitum is composed of the secretion produced by the placental obturator present there. The secretory cells of the obturator are less evident and elongated than those present in the *Guarea* obturator (Figs. 2F, 3C, 3D). The stigma has the receptive surface in a subterminal region (Figs. 1B, 1C, 11 G, 11K) with secretory trichomes which produce acidic lipids and phenolic compounds, and some idioblasts containing acidic lipids (Table 2 – Figs. 4G – 4L).

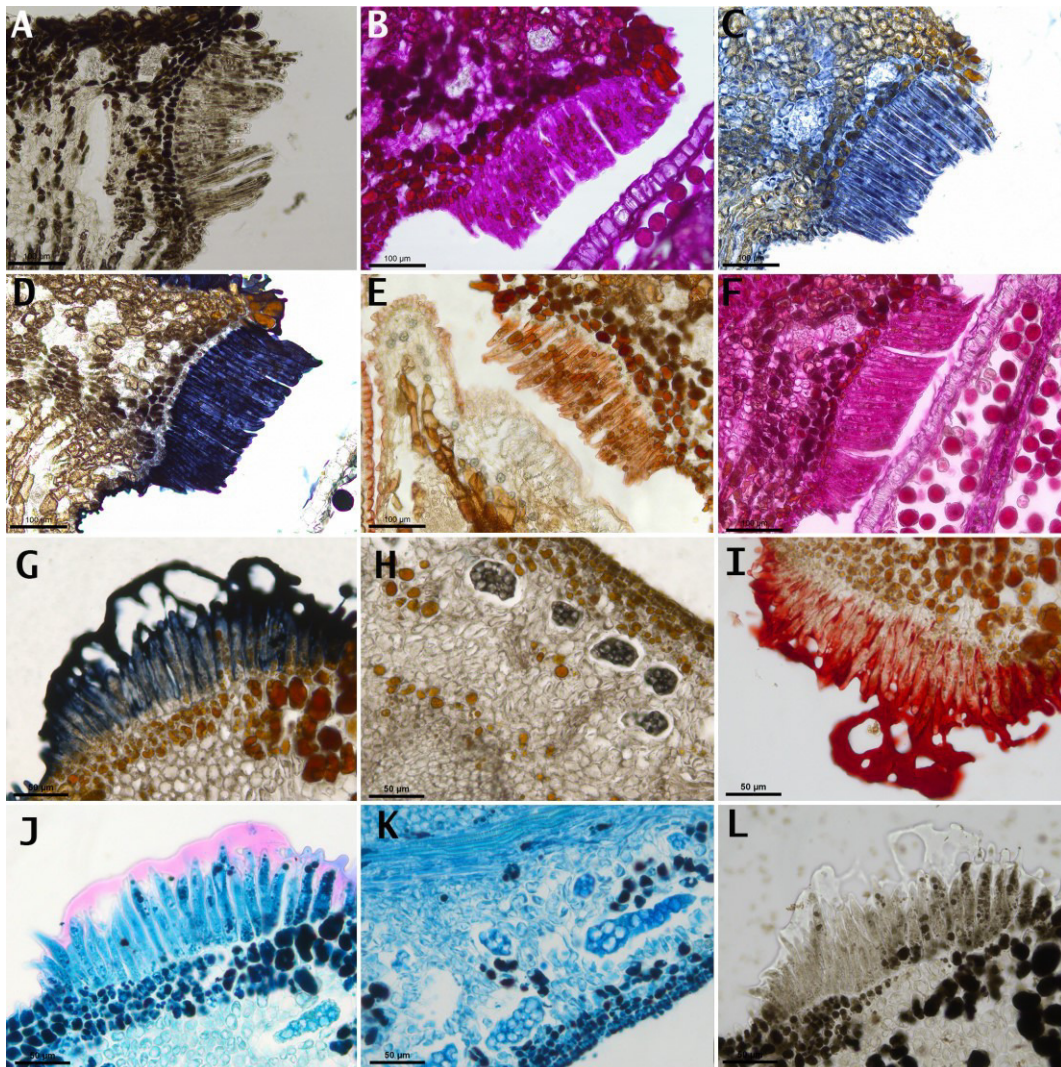


Figure 4. Positive results for the histochemical tests performed on floral glands of *Guarea macrophylla* subsp. *tuberculata* (A – F) and *T. clausenii* (G – L). (A) Staining with ferric chloride in region of the stigma indicating the presence of phenolic compounds. (B) Staining with PAS reaction (Periodic acid Schiff's reagent) in region of the stigma indicating the presence of carbohydrates. (C) Staining with Aniline blue Black in region of the stigma indicating the presence of proteins. (D) Staining with Sudan black B in region of the stigma indicating the presence of lipids. (E) Staining with Sudan IV in region of the stigma indicating the presence of lipids. (F) Staining with Ruthenium red in region of the stigma indicating the presence of acidic mucilage. (G) Staining with Sudan black B in region of the stigma indicating the presence of lipids. (H) Staining with Sudan black B at the wide idioblast indicating the presence of lipids. (I) Staining with Sudan IV in region of the stigma indicating the presence of lipids. (J) Staining with Nile blue in region of the stigma indicating the presence of acidic lipids. (K) Staining with Nile blue at the wide idioblast indicating the presence of acidic lipids. (L) Staining with Ferric chloride in region of the stigma indicating the presence of phenolic compounds.

TABLE 2 - List of histochemical tests performed in *G. macrophylla* subsp. *tuberculata* and *T. clausenii* for the floral glands.

Staining	<i>Guarea macrophylla</i> subsp. <i>tuberculata</i>			<i>Trichilia clausenii</i>		
	Stigma	Standard idioblast	Wide idioblast	Stigma	Standard idioblast	Wide idioblast
Ruthenium red	+	-	-	-	-	-
PAS reaction (Periodic acid Schiff's reagent)	+	-	-	-	-	-
Aniline blue black	+	-	-	-	-	-
Sudan black B	+	-	+	+	-	+
Sudan IV	+	-	-	+	-	+*
Nile blue	-	-	+*	+	-	+
Copper acetate and rubeanic acid	+*	-	+*	-	-	+*
Ferric chloride	+	+	-	+	+	-
Wagner's reagent	-	-	-	-	-	-

Positive (+); Negative (-); Weak positive (+*).

Vascularization (Figs. 5A, 7A) – At the base of the receptacle in the level of the calyx of *Guarea*, one trace diverges from the stele and branches into five traces that will supply each sepal (Fig. 5B). Besides the four initial traces, two extra traces diverge from the stele to supply only two sepals (Figs 5C – 5E). Above this level, the petal vascular traces are observed: one trace diverges from the stele, branching into four traces that will supply each petal (Figs 5F, 5G).

Subsequently, the staminal traces are observed, the outer traces will supply the antesealous stamens, and the inner traces will supply the antepetalous stamens (Figs. 5H – 5J). Each stamen is vascularized by a single bundle. In the gynophore the fundamental bundles remain in the center, and in the distal region some phloem strands enter the nectary (Figs. 6A – 6C). At the apex of the gynophore, a dorsal trace that branches into many smaller traces and two ventral traces vascularize each carpel (Figs. 6D – 6F). Other gynoecium vascular traces differentiate into one trace septa (one for each septum) and that later it branches in two (Figs. 6E – 6H). In the style, the twelve traces are maintained (derived from the septa and from the dorsal of each carpel – Figs. 6I, 6J). The stigma is not vascularized (Fig. 6K). The gynoecium is synascidiate proximally (from the base to almost the top of the ovary) and symplicate distally (from the top of the ovary to the stigma).

In the receptacle of *Trichilia*, in the level of the calyx, one trace diverges from the stele and branches into three traces that will supply each sepal (Figs. 7B – 7F). In the second perianth whorl (corolla), the divergence of one trace is observed in the stele, branching into three traces that will supply each petal (Figs. 7D – 7F). Above the level of divergence of the petal traces, traces of the two androecium whorls are observed with one trace per stamen (Figs. 7G, 7H). The outer traces will supply the antesealous stamens and the internal traces will vascularize the antepetalous stamens (Figs. 7H – 7J). In the receptacle it is also possible to observe the base of the nectary and its vascular traces diverging from the androecium (Fig. 7J). At a higher level it is observed the divergence of the traces of the gynoecium: three dorsal traces and six ventral traces, which branch out and fuse laterally (one from each adjacent carpels) to form the septum bundle (Figs. 7I – 7N).

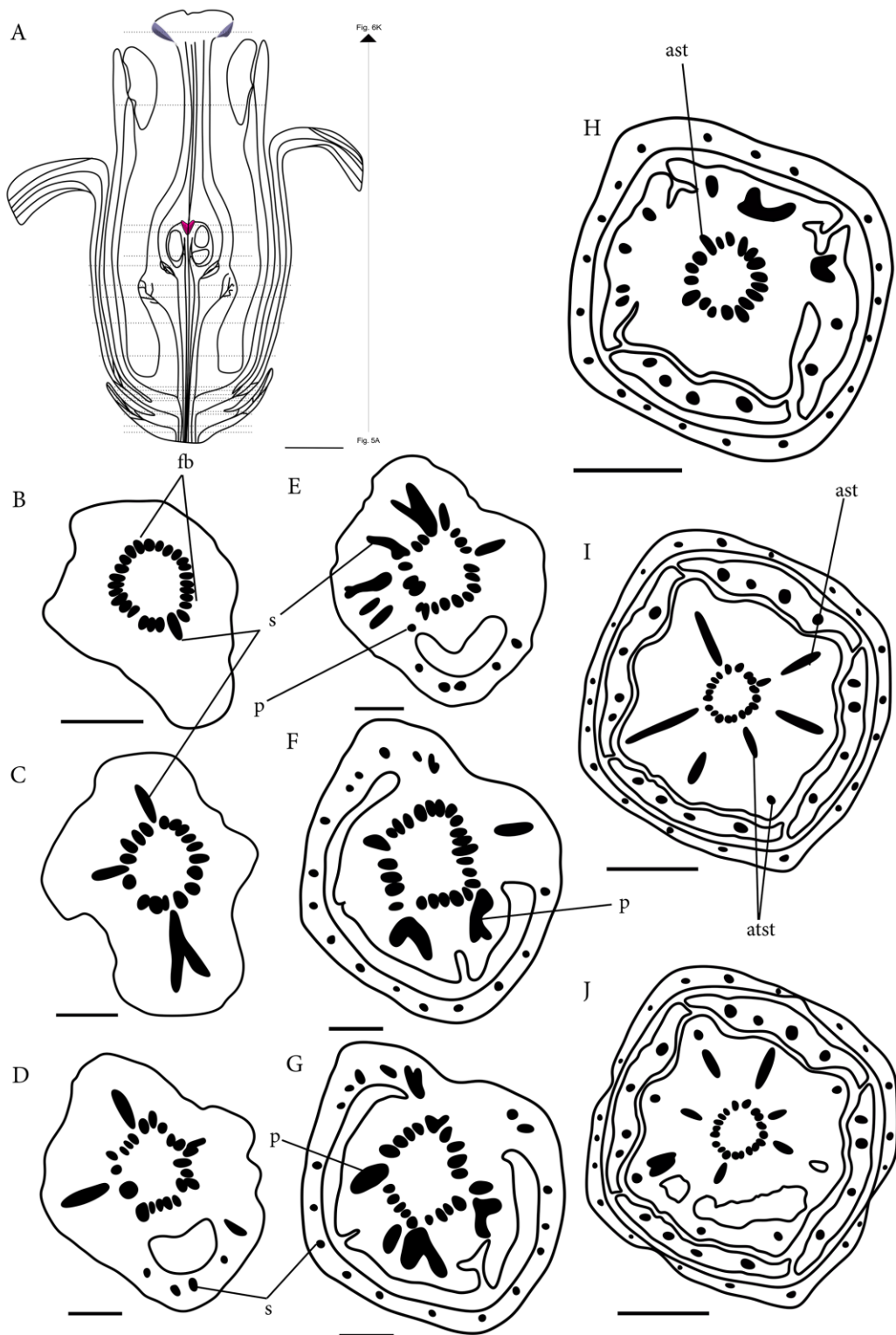


Figure 5. Diagrams of sections of the floral bud of *Guarea macrophylla* subsp. *tuberculata* from the base of bud up to the base of the gynophore. Dark shading indicates vascular tissues, including stele, traces and vascular bundles. (A) Longitudinal section. (B – J) Transverse sections. (B) Floral stele with one trace of the sepal diverging. (C) Divergence of the sepals. (D) Sepals traces. (E) Divergence of the petals. (F – G) Petals traces. (H) Divergence of the antesepalous staminal traces. (I) Antesepalous staminal traces and divergence of the antepetalous stamens. (J) Staminal traces. ast, antesepalous staminal trace; atst, antepetalous stamens trace; fb, fundamental bundles; p, petal trace; s, sepal trace. Bar B-G =500 μ m; H-J=1mm.

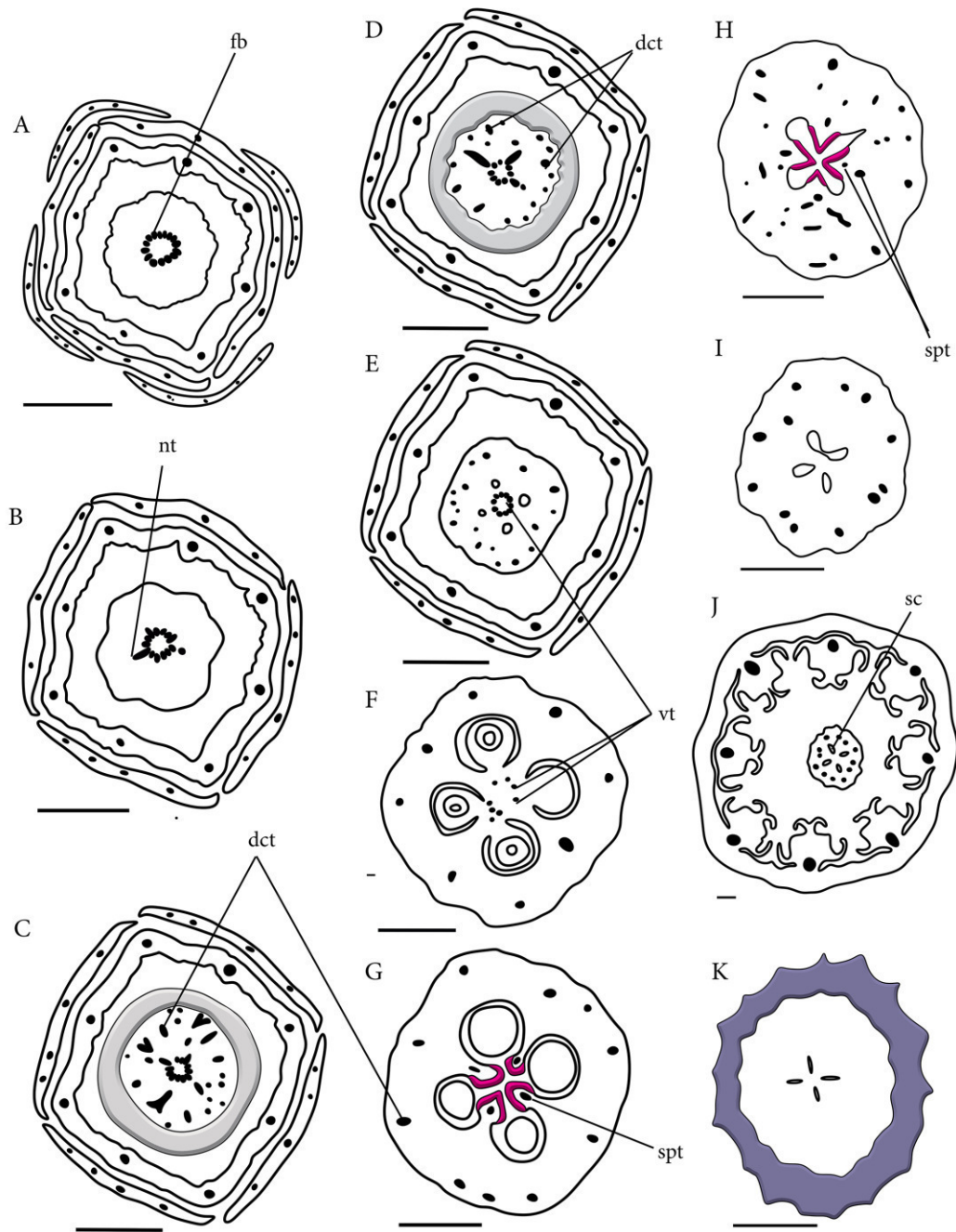


Figure 6. Diagrams of transverse sections of the floral buds of *Guarea macrophylla* subsp. *tuberculata* from the base of the gynophore up to the bud apex. Dark shading indicates vascular tissues, including stele, traces and vascular bundles; grey shading indicates nectary tissue (nectar bundles not represented); purple shading indicates the receptive region of stigma and pink the placental obturator. (A) Detail of the gynophore, the fundamental bundles remain in the center. (B) Divergence vascular traces of the nectary. (C) Divergence of the dorsal carpellary traces from the receptacular bundles. (D) Dorsal trace that branches into many smaller traces. (E) Divergence of the ventral traces. (F) Two ventral traces vascularizing each carpel. (G) Divergence of the septal trace and detail of the placental obturator. (H) Branches in two traces of septal at the unilocular region at the ovary apex. (I - J) Twelve traces derived from the septa and from the dorsal of each carpel and four styler canals. (K) Mid region of stigma with the receptive region and styler canals fused. dct, dorsal carpellary traces; fb, fundamental bundles; nt, nectary trace; pt, petal trace; s, sepal trace; sc, styler canal; spt, septal trace; st, staminal trace; vt, ventral trace. Bar J=200µm; F-I, K=500µm; A-E =1mm.

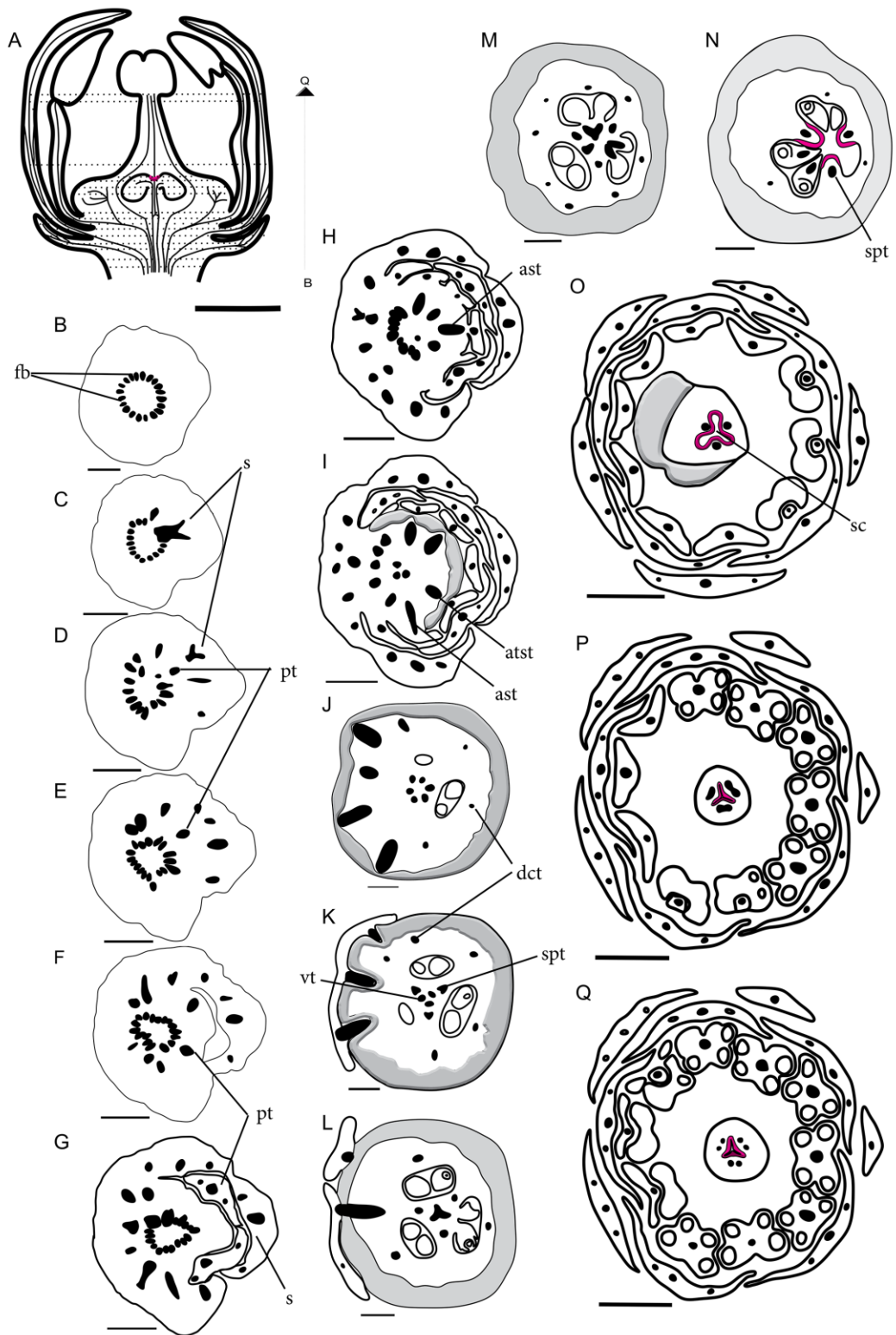


Figure 7. Diagram of sections of the floral bud of *Trichilia clausenii* from base to apex. Dark shading indicates vascular tissues, including stele, traces and vascular bundles; grey shading indicates nectary issue (nectar bundles not represented); pink the placental obturator and compitum at the style region. (A) Longitudinal section. (B – K) Transverse sections. (B) Fundamental bundles. (C) Floral stele with trace of the sepal diverging. (D - E) Divergence of the sepals and petals. (F) Divergence of the petals. (G) Petals traces. (H) Divergence of the antesepalous staminal traces. (I) Antesepalous staminal traces, divergence of the antepetalous stamens and the ventral traces. (J) Staminal traces, the base of the nectar with its vascular traces diverging from the androecium, branches of the ventral traces and divergence of the dorsal carpellary traces. (K) Ventral traces branch out and fuse laterally (one from each adjacent carpels) forming the septum bundle. (L - M) Ventral traces vascularizing each carpel. (N) Detail of placental obturator in the unilocular region. (O) Basal region of style with the compitum (pink). (P) Basal region of stigma with the compitum (pink) and anthers tetrasporangiate. (Q) Mid of the stigma. ast, antesepalous staminal trace; atst, antepetalous stamens trace; dct, dorsal carpellary traces; fb, fundamental bundles; pt, petal trace; s, sepal trace; sc, stylar canal; spt, septal trace; st, staminal trace; vt, ventral trace. Bar B, J-N=200µm; C- I, O-Q=500µm; A=1mm.

In the style, vascularization is provided by three lateral bundles, derived from the septum bundle and three central bundles derived from the dorsal bundles, which are greatly reduced (Figs. 7O – 7Q). The gynoecium of *T. clausenii* is synascidiate proximally (from the base to almost the top of the ovary) and symplicate distally (from the top of the ovary to the stigma).

Ontogenesis – The sepals primordia of *Guarea* appear as four protuberances beginning in the anterior position, then in posterior and finally in lateral position (Figs. 8A, 8B). Subsequently, the petals primordia appear simultaneously, alternating the sepals (Fig. 8C). Following the petal primordia development, the androecium begins its development with the antesepalous stamens (outer whorl) and then the antepetalous one (internal whorl), developing almost simultaneously clockwise (Fig. 8D).

During development of the stamens, the filament column elongates as a single structure (early connation), so the filaments are already united from the base and only the anthers are separated from each other (Fig. 8E). In intermediate stages of the flower development, the filament column grows behind the anthers, surpassing and leaving them inserted on the inner side of the staminal tube, all at the same height (Figs. 8F, 8I, 9A, 9B).

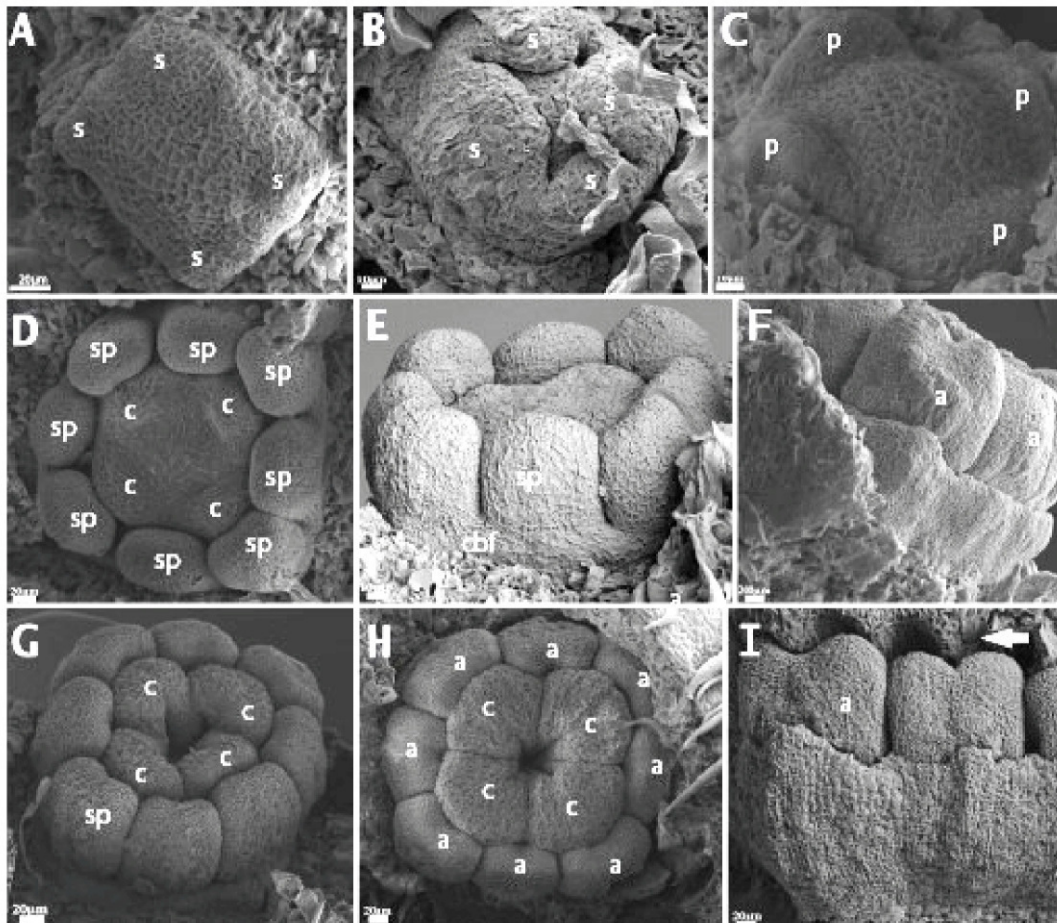


Figure 8. Scanning electron micrographs of the flowers of *Guarea macrophylla* subsp. *tuberculata* in early stages of development. (A) Tetragonal dome with initiation of sepal primordial. (B) Development of primordial sepal showing congenitally fused. (C) Initiation of the petals primordia free (removed sepals). (D) Antesepalous stamens primordia development in outer whorl and then the antepetalous in internal whorl, almost simultaneously clockwise and the formation of four protuberances of the carpels (petals and sepals form removed). (E) Side view of filaments primordia already united from the base. (F) Side view of filaments primordia already united from the base and only the anthers are separated from each other. (G) Initiation of synascidiate region growth as a tube, and the distal portion of the carpels elongate freely. (H) Superior view of the carpels and androecium just before the stigma differentiation. (I) Detail showing the filament column grows behind the anthers and lateral recesses formed in at the beginning of stigma differentiation by pressure of the anthers (arrow). a, anther; c, carpel; cbf, connate base of filaments; p, petal; s, sepal; sp, stamen primordial

The gynoecium originates with the formation of four antesepalous protuberances (Fig. 8D). Carpels primordia are partially free. The synascidiate region grows as a tube, and the distal portion of the carpels elongate freely and fuse postgenitally (intercarpelar symplicate region), originating a syncarpous gynoecium (Figs. 8G, 8H). The fusion of the carpels in the symplicate region results in a unilocular apical region, where the placental obturator will subsequently develop. At the top of the ovary,

the style folds and fuse forming the four styler canals. During the origin of the stigma, lateral recesses are formed due to spatial pressure of the anthers (Figs. 8I, 9A, 9E). In its lateral region, the stigmatic papillae differentiate in the receptive surface (Figs. 2J, 9D, 9E).

Close to the pre-anthesis, the gynophore elongates and the nectary appears as a distal annular protuberance (Figs. 9B, 9C). The ontogeny of *T. clausenii* begins with the formation of the perianth as a pentagonal dome (Fig. 10A). The first primordium originates in anterior position and the other sepal primordia arise clockwise. The petals primordia appear simultaneously, alternating the sepals (Figs. 10A – 10C). Inner to the petals primordia, the stamens primordia develop almost simultaneously counterclockwise in both whorls (Fig. 10D). The anthers are inserted in the apex of the filaments (Figs. 11C, 11F, 11J, 12D, 12E). The filaments elongates free from each other (Figs. 10D – 10F, 10I). The connate base of the filaments emerges late (Fig. 10K). Along most of the filaments length the coherence between them is given by non-glandular trichomes (Figs. 11F, 11I, 12E, 12F). The gynoecium primordial starts as a quadrangular dome; however, generally only three protuberances develop, giving rise to three carpels (Figs. 10E, 10G). Two carpels are antesealous and the third carpel is located between the stamens from the first and second whorls (Fig. 10E).

Ovary composed of four carpels was found (Fig. 10L). The development of carpels is similar to that found in *Guarea* (Figs. 10G, 10H, 10J, 11B,). However, the folding and fusion of the style tissues form the styler canal with a compitum and during the differentiation of the stigma of *Trichilia* (Fig. 10J), is formed a stigma-head with three expanded lobes and a "collar" in the receptive surface (basolateral portion of the stigma) (Figs. 11B, 11E, 11K). The nectary develops as an intrastaminal disk around the base of the ovary at the intermediate developmental stages (Figs. 11B, 11D, 11 E, 12A, 12B, 12I).

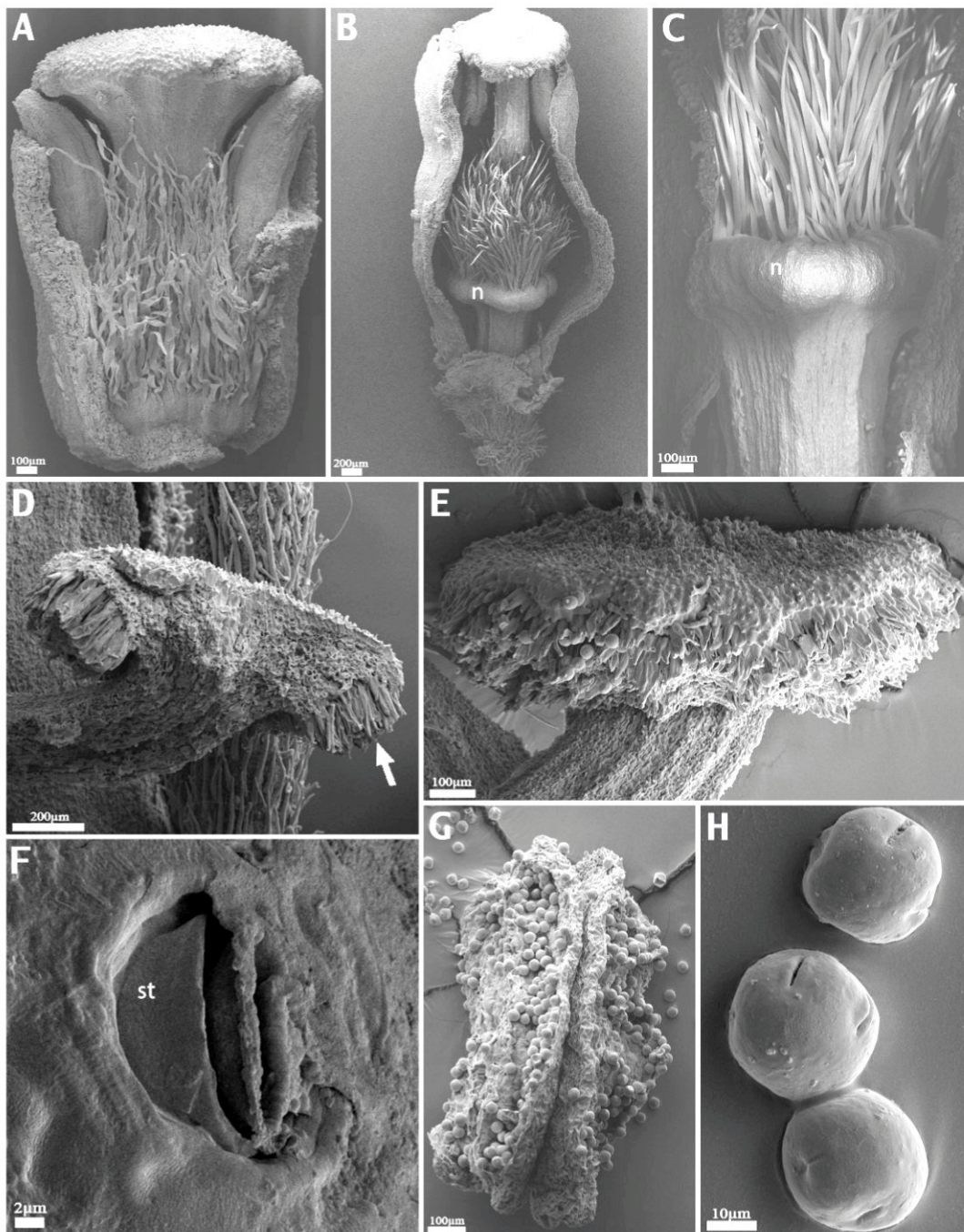


Figure 9. Scanning electron micrographs of the flowers the of *Guarea macrophylla* subsp. *tuberculata* at different developmental stages. (A) Side view showing the already differentiated style and stigma, and start of the development of the gynophore. (B) Side view of the gynoecium mature with elongated gynophore. (C) Detail of the region of the gynophores with nectary annular no apex and trichomes of the ovary and style region. (D) Detail discoid stigma cut longitudinally, showing the receptive region (arrows). (E) Detail of the receptive region of discoid stigma with pollen grains at the secretory trichomes. (F) Detail of stomata present on the surface of the nectary. (G) Mature anther releasing the pollen grains. (H) Pollen grains 4-5-colporate. n, nectary; st, stomata.

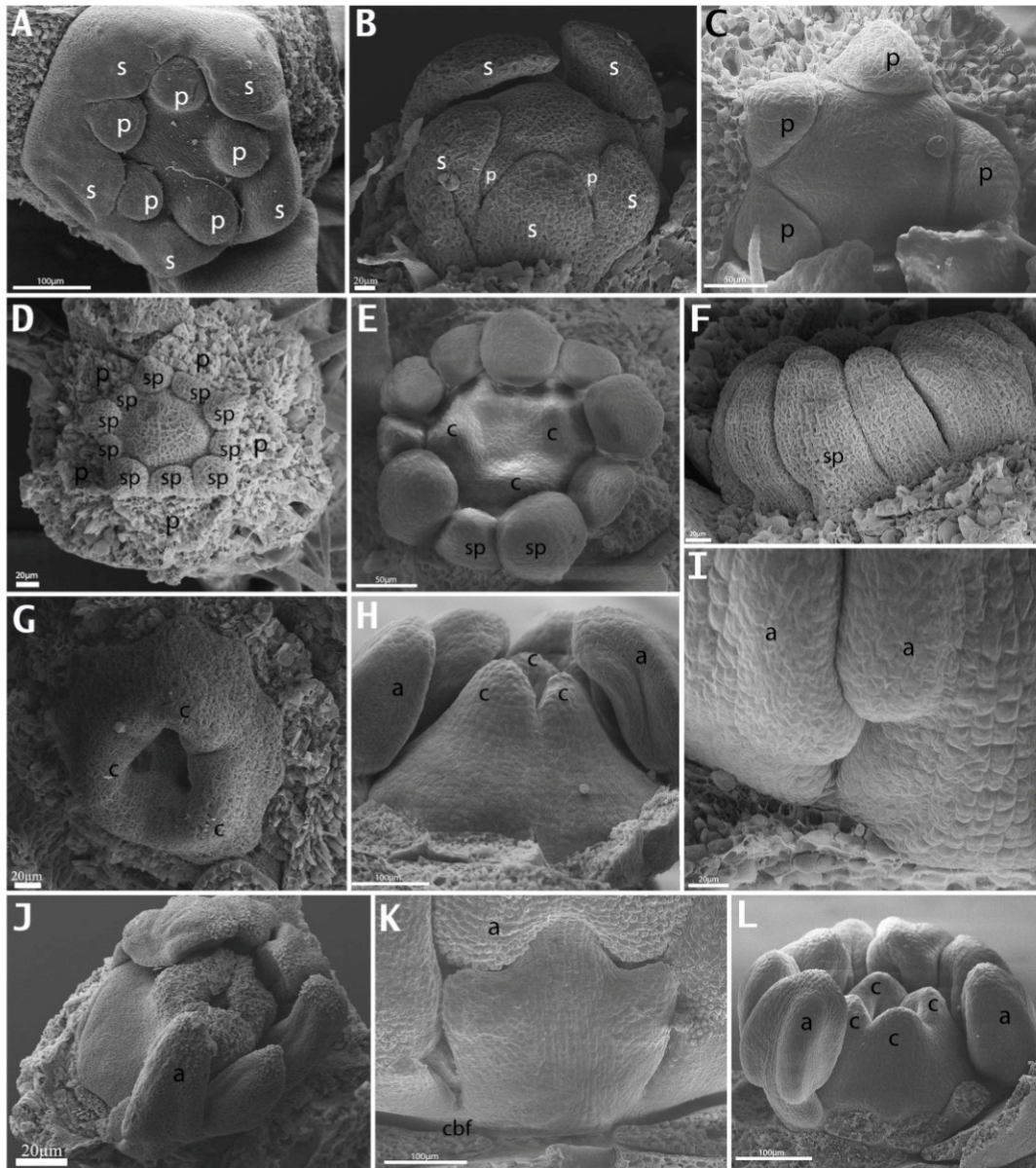


Figure 10. Scanning electron micrographs of the flowers of *Trichilia clausenii* in early stages of development. (A) Pentagonal dome with sepal primordia arise clockwise and initiation of the petals primordia. (B) Side view of floral bud, showing congenitally fused sepals and petals primordia. (C) Petals primordia free, alternating the sepals. (D) Antesepalous stamens primordia development in outer whorl and then the antepetalous in internal whorl, almost simultaneously counterclockwise (petals and sepals form removed). (E) Androecium a little more developed and the formation of three protuberances of the carpels. (F) Side view of the filaments elongates free from each other. (G) Initiation of synasciade region growth as a tube, and the distal portion of the carpels elongate freely. (H) Side view of the carpels with the distal portion freely. (I) Detail showing the base of the filaments still free during the differentiation of the anthers. (J) Stage of the development intermediate of the gynoecium and androecium. (K) Detail showing the base the connate filaments late and the region of insertion of the anther in the filament. (L) Gynoecium composed of four carpels. a, anther; c, carpel; cbf, connate base of filaments; p, petal; s, sepal; sp, stamen primordium.

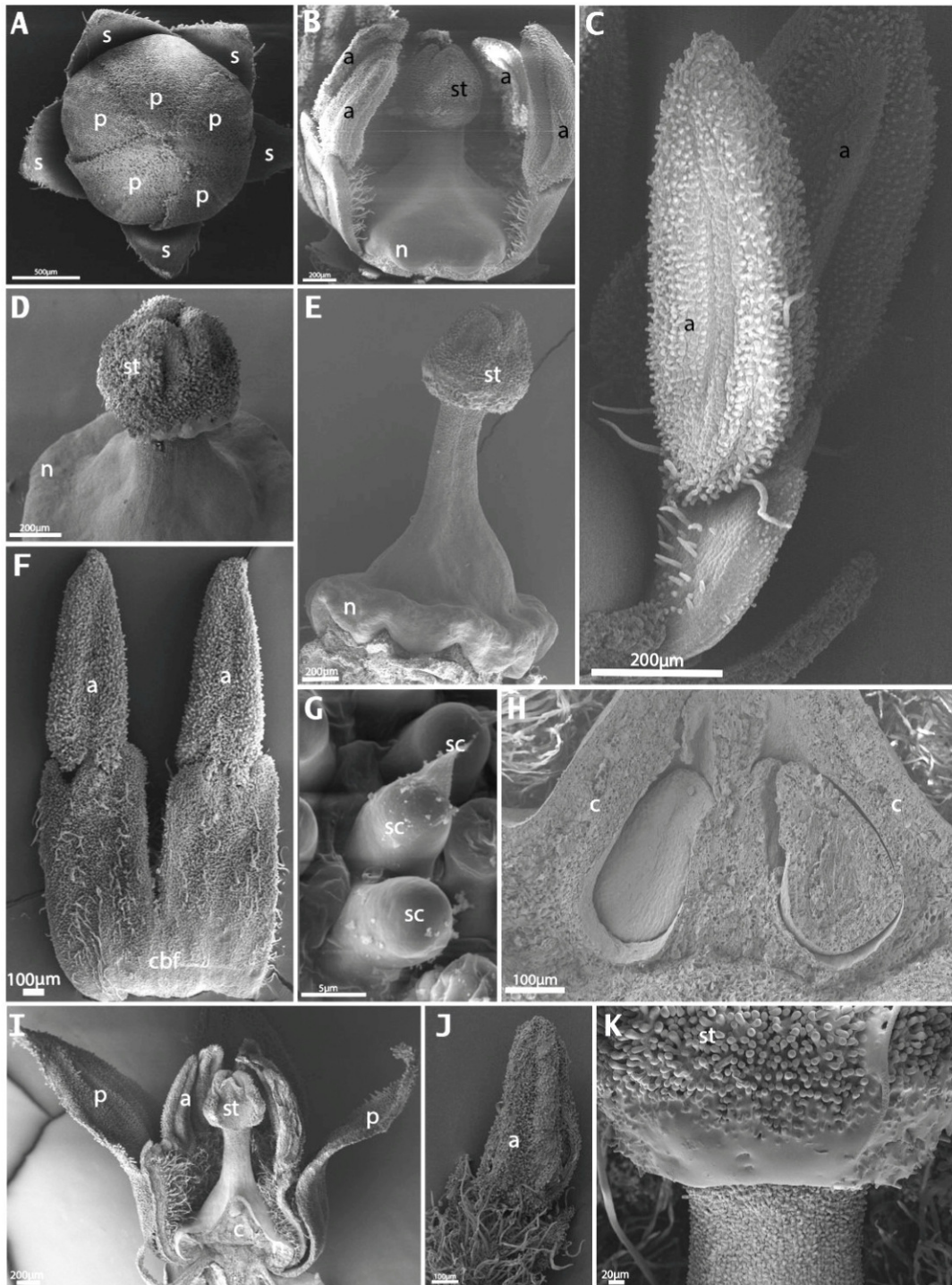


Figure 11. Scanning electron micrographs of the flowers mature the of morphotype female of *Trichilia clausenii* at different developmental stages. (A) Floral bud at intermediate stage of development. (B) Side view showing the already differentiated style and stigma, anthers developed with longitudinal dehiscence and presence of elongated trichomes on the adaxial face. (C) Detail side of the anther. (D) Detail of the stigma trilobed. (E) Side view of the gynoecium mature. (F) The base the connate filaments and apex free. (G) Detail the secretory cells of the receptive region of the stigma. (H) Longitudinal section of the gynoecium showing the locules with ovules. (I) Flower in anthesis (part of the perianth, androecium and gynoecium were removed to expose the locules). (J) Detail of strand trichomes and anther opening longitudinally. (K) Detail of the receptive region "collar" of stigma covered with secretion. a, anther; cbf, connate base of filaments; n, nectary; p, petal; s, sepal; sc, secretory cell; st, stigma.

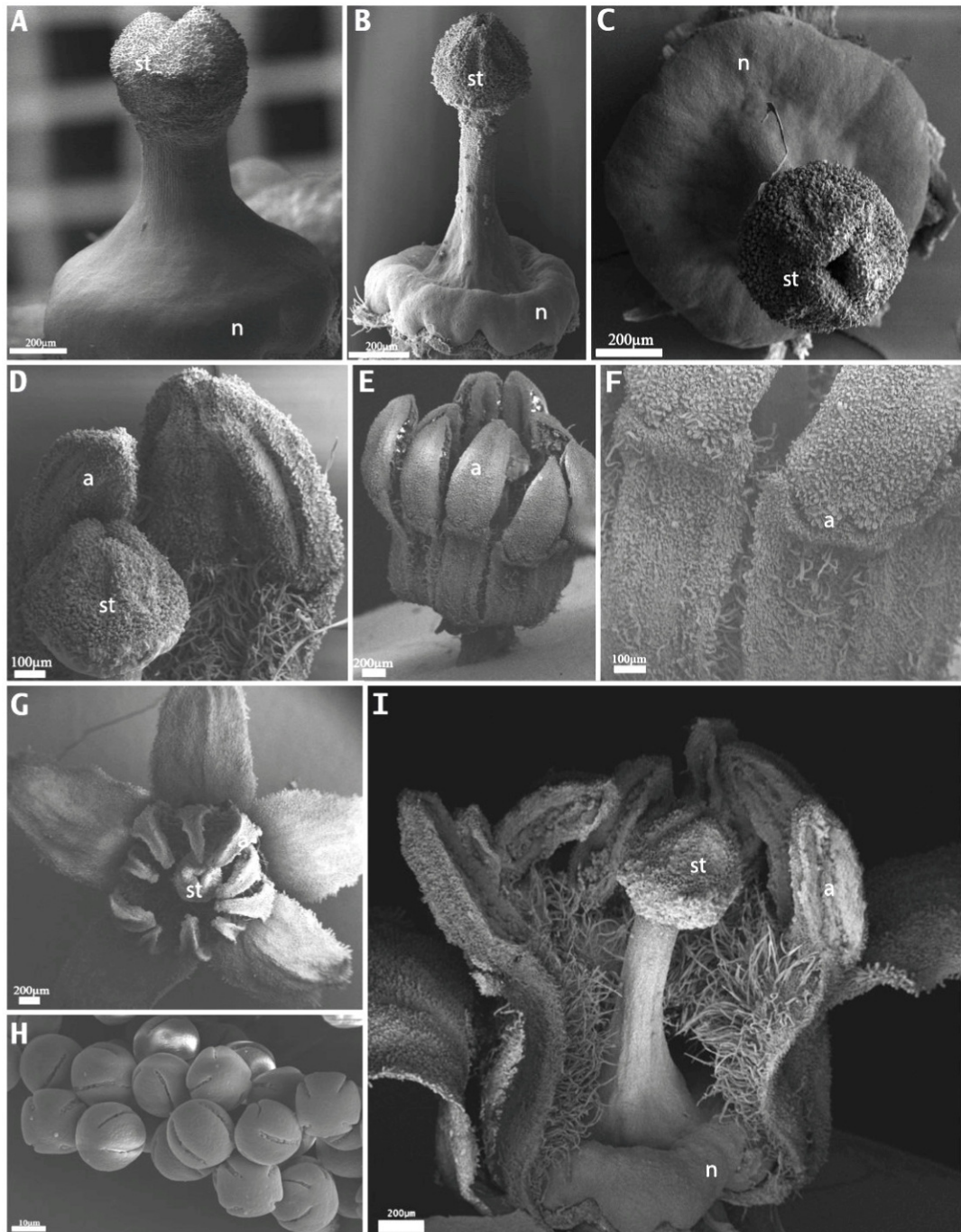


Figure 12. Scanning electron micrographs of the flowers mature the of morphotype bisexual of *Trichilia clausenii* at different developmental stages. (A) Side view showing the already differentiated style and stigma. (B) Side view of the gynoecium mature. (C) Detail of the stigma trilobed. (D) Detail side of the anther and stigma. (E) Pseudotube with filaments distally coherent to each other by the intertwining of trichomes, but connate in the base. (F) Detail of intertwining by trichomes of the filaments. (G) Top view of the flower in anthesis. (H) Pollen grains 4-5-colporate. (I) Flower in anthesis (part of the perianth, androecium and gynoecium were removed). a, anther; n, nectary; p, petal; st, stigma.

DISCUSSION

Morphology – Flowers of *Trichilia* and *Guarea* share several morphological and anatomical features; the main differences observed are related to the number of flower parts, degree of union of filaments and anther position, ovule position, style pubescence, stigma shape, presence or absence of gynophore and of a styler compitum, and stigma secretion composition. Some other distinctions are minor variations, such as: the distribution of trichomes; fusion to a greater or smaller extent of the sepals; cells more elongated and prominent in *Guarea* placental obturator.

Regarding the anatomy of the perianth, androecium and gynoecium, our results are similar to those found by Souza et al. (2002), with some exceptions: we found no laticiferous idioblasts; we did not observe the hypostase at the chalaza and defined the ovule as being campylotropous and not hemianatropous; moreover we observed the presence of a placental obturator. The nectary of the species of the *Trichilia* studied by Souza et al. (2001) presented a secretory parenchyma where the secretory cells were prominent, while in *Trichilia* and *Guarea* the parenchyma is fundamental, not secretory and the nectar is released via stomata.

Dahlgren (1991) reported the presence of a placental obturator for several families of eudicots, but Meliaceae was not included. Moscheta (1995) described the presence of a placental obturator over the ovule of *Trichilia catigua* A.Juss., but did not mention its presence in *G. kunthiana* A.Juss. Souza et al. (2002) reported the absence of an obturator in *G. kunthiana* and in *G. macrophylla*, despite referring to the presence of papillose cells in the placental region. We report here the presence of a placental obturator in both studied species.

Trichilia clausenii shares more similarities with *T. elegans*, than with the other species studied by Souza et al. (2002). Both present a pentamerous perianth and petals has the same pattern of vascularization. A nectariferous disk is present and the gynoecium epidermis is glabrate and papillose. However, they differ in relation to the style, which is provided with

a central canal that narrows at the apex in *T. clausenii* and, while in *T. elegans*, the narrowing is solid and occurs at the base and at the apex.

Histochemistry – The epidermis of the apical portion of the stigma of both *Trichilia* and *Guarea* presents a large amount of phenolic compounds, crystals, idioblasts and due to its composition is not a receptive region. In *Trichilia* the receptive region is located at the subterminal part of the conical stigma, and in *Guarea* it is located at the lateral part of the discoid stigma. Idioblasts, mostly phenolic and more rarely oleiferous are present in all floral organs. Souza et al. (2002) described the presence of laticiferous idioblasts in two species of *Guarea*, but there are no specific studies that describe the chemical composition of the idioblasts of Meliaceae. The specialized cell (or a row of these cells) containing latex is classified as laticifers (Fahn, 1979). According to Prado and Demarco (2018) some secretory structures are incorrectly classified as laticifers due to an erroneous interpretation based often on the color and appearance of secretion. No latex was observed in *Guarea* cells. Therefore, studies to understand the chemical composition are still necessary in this group.

Sexual dimorphism – Gama et al. (in prep., see Chapter 1) compared *T. clausenii* with other three species of the genus studied by Souza et al. (2001) (*T. elegans* A.Juss., *T. catigua* and *T. pallida* Sw.), and reported that the four species share similar morphological and developmental features, but pointed the presence of pollen in the female flower in *T. clausenii*, which nevertheless is blocked by a dense secretion of the persistent tapetum. The pollen grains are released in monads and not in tetrahedron type tetrads. And this species presents a gynoeceium with morphologically well-formed ovules in both morphotypes. In addition, we also observed that the *Trichilia* ovules integuments are formed by multiple layers with a smaller variation in number. The morphological results obtained for *Guarea* are in general accordance with those described by Souza et al. (2002). However, those authors describe sexual dimorphism, with the presence of pistillodes in the male flower and pollen-free

staminodes in the female flower, while only one morphotype (functionally bisexual) was observed in the populations sampled in the present study. It is possible that there may be variations across populations of different regions along the wide range of this species, but it is also likely that populations in smaller areas as the one studied by us may show a strongly biased sex ratio.

Vascularization – In *Trichilia* we observed that each sepal has one median and two lateral vascular traces, a feature reported to most rosids and to some Meliaceae (Narayana, 1958a, 1958b; Bachelier and Endress, 2009). However in *Guarea*, one trace per sepal diverges from the stele and each trace branches into other five traces that will supply each of the four sepals. The reduction in the number of sepal traces is described for *Aglaia roxburghiana* (Wight and Arn.) Mig., but its increase have not been reported in the family so far (Nair, 1963). The petals of *Trichilia* and *Guarea* receive a single trace which divides into three or more bundles. The androecium consists of two whorls of stamens forming a tube and their traces arise independently from the main stele. The subtle divergence of the antisepalous traces occurs a little earlier than the others, a fact also reported for *Chloroxylon* (Narayana, 1958 b).

According to Murty and Gupta (1978), the nectariferous disk is considered receptacular in the species of Meliaceae studied by them. Weberling (1989) also describes that in several angiosperm groups nectariferous disks usually develop from the floral receptacle, but some studies have shown that nectaries in basal eudicots may have different positions in relation to carpels or petals (Endress, 2010; 2011), or either that nectaries may result from a floral structure that has become nectariferous, such as the gynophore in *Homalolepis* (as *Simaba*, Simaroubaceae; Alves et al., 2017). In *Trichilia*, the traces of the nectariferous disk originate from the staminal traces, as described by Narayana (1958a, 1958b), for other species of the family, while in *Guarea* the fundamental bundles go up through the gynophore and vascularize the nectary which is located at its apical region. Nair (1963) reported that in some Meliaceae the nectariferous

disk does not receive any vascular supply, while in other species of the family it can be supplied by branches of the staminal traces. The vascular tissue of the disk is composed only by phloematic strands.

The peculiar contrast between the number of parts of the perianth and gynoecium observed in flowers of *Trichilia* (provided with a pentamerous perianth and a tricarpeolar gynoecium, sometimes tetracarpeolar) fits the “trend” reported by Narayana (1958a, 1958b) in the Meliaceae towards reduction in the number of stamens and carpels as well as the traces that supply them. Nair (1963) attributes this reduction to the loss of a whorl or both, in unisexual flowers.

In *Trichilia* the septal trace originates from the ventral traces of the carpel that branch out and fuse in the region of the septum, while *Guarea* presents a different origin of the septal traces, which derive from the stele.

In other genera of the family, as *Melia*, *Swietenia*, *Cedrela* and *Cipadessa*, the ventral bundles continue to the top of the style, after supplying the ovule, and divide into two or more branches. However, in *Walsura* and *Aglaia*, they are consumed in the ovary supply, nourishing the placenta (Narayana, 1958b), a condition also observed in *Trichilia* and *Guarea*, where the dorsal and septal traces enter the style. Usually the dorsal traces and secondary marginal traces are suppressed, but in *Melia* and *Cedrela* also bear dorsal traces in the style (Narayana 1958 a, 1958b; Nair 1963). The style has a narrow stylar canal with branches, corresponding to the number of carpels (Nair, 1963). In the case of *Trichilia* the stylar canal presents three branches, whereas in *Guarea* is composed of by four canal and not just a stylar canal.

Ontogenesis – Obdiplostemony is an uncommon phenomenon in the majority of angiosperms but is commonly found in families of the Sapindales order (Stevens, 2001; Bachelier and Endress, 2008; Pirani et al., 2010). Obdiplostemony is considered a simple condition derived from the diplostemony, where it results from the successive alternation of whorls and the stamens of the external whorl are antepetalous (Weberling, 1989; Alves et al., 2017). Our study of flowers of *Trichilia* and *Guarea* reveal that

the androecium of both species presents antesepalous stamens (outer whorl) and antepetalous stamens (inner whorl). Due to the maintenance of the whorls positions throughout the development, we classified these flowers as diplostemonous. This situation differs from the findings of Alves et al. (2017) for *Homalolepis* (as *Simaba*, Simaroubaceae, Sapindales), where the whorl of the antesepalous stamens differentiate before the antepetalous whorl, but throughout the development these whorls change positions and the external whorl of stamens becomes antepetalous.

Harms (1940) reported that Meliaceae flowers are mostly obdiplostemonous, rarely diplostemonous with alternipetalous carpels. However, the results from our ontogenetic and vascular studies reveals that the four carpels are antesepalous in *Guarea*, while in *Trichilia* two carpels are antesepalous and the third carpel is located between stamens from the first and second whorls, but the three traces of sepals are aligned with the dorsal carpellary bundles.

The sepals are congenitally fused at the base and the apical region is free throughout the development, giving rise to gamosepalous flowers that present different extensions of their fusions in *Trichilia* and *Guarea*. According to Stevens (2001), sepals in Meliaceae are often connate, with a single vascular trace. The petals are free throughout their development, a feature maintained by most of the rosids (e.g. Stevens, 2001).

The filaments have a late union at the base, and along their length, are coherent by the intertwining of non-glandular trichomes in *Trichilia*, while in *Guarea* the filaments are entirely connate and only the anthers are separated from each other. Both conditions are in accordance to Pennington and Styles (1975), who emphasize the importance of the androecium tube as a diagnostic characteristic of Meliaceae, where a wide array of partial to complete fusion of the filaments can be observed among its genera. An androecium with connate filaments is ascribed as a synapomorphy of the family by Stevens (2001), and this has been also recovered in a recent evolutive study by Gama et al. (in prep. see Chapter 3). Several authors (e.g. Stevens, 2001) have also emphasized that in most members of the family the mouth of the staminal tube is blocked by a large stigma, as here

observed in *Guarea*, whose stigma is discoid. We believe that secondary pollen presentation is likely in this species. Further studies are necessary to clarify this. So far, within the Meliaceae secondary pollen presentation has only been reported for the genus *Turraea* (White and Styles, 1963; Yeo, 1993).

Bachelier and Endress (2008) reported that even though the Meliaceae gynoecium is generally described as syncarpous, it remains uncertain whether the style is actually syncarpous or whether it results from a postgenital union of the carpels. According to the classification of Leinfellner (1950), we may classify the gynoecium of both studied species as fully syncarpous (eusyncarpous), since the gynoecium is synascidiate proximally and symplicate distally. However in the central region of the style of *Guarea*, the tissues fuse leaving only four styler canals. On the other hand, in *Trichilia* the central portion of the style does not merge, forming a central canal (compitum) and its lumen is directly connected to the ovary locule.

The general aspects of the floral morphology of *T. clausenii* and *G. macrophylla* subsp. *tuberculata* reported here mostly corroborate the descriptions presented in the available literature for both species. However, we bring some new information such as the *Guarea* ovule being campylotropous and presenting a placental obturator; the style of *Trichilia* has a central canal that narrows only subtly at the apex differing from another species, whereas in *Guarea* the style has four central channels; both releasing the nectar via stomata; have nectary receiving vascular supplement composed only of phloem strands; to be diplostemony flowers. In addition, comparing *Trichilia* and *Guarea* showed that they share several morphological, anatomical and histochemical features, but differ in some features of both the gynoecium as the androecium.

These data allowed a comparison between the structures of other representatives both within the genera itself and the order itself. The knowledge of the flowers of Meliaceae was improved, mainly in relation to the structure and development of the gynoecium and androecium. In relation to floral sexuality, the two morphotypes described for *Trichilia* (Gama et al.,

in prep., see Chapter 1) did not differ in histochemistry, basic structural development and vascularization. Being differentiated only by the external morphology and the non-release of pollen grains in the female morphotype. In *Guarea* there are descriptions of sexual dimorphism, but the population analyzed showed flowers with well-developed ovules and well-formed pollen grains. And all individuals presented abundant fruit production during the study period, but detailed studies of pollen viability and reproductive biology are still necessary.

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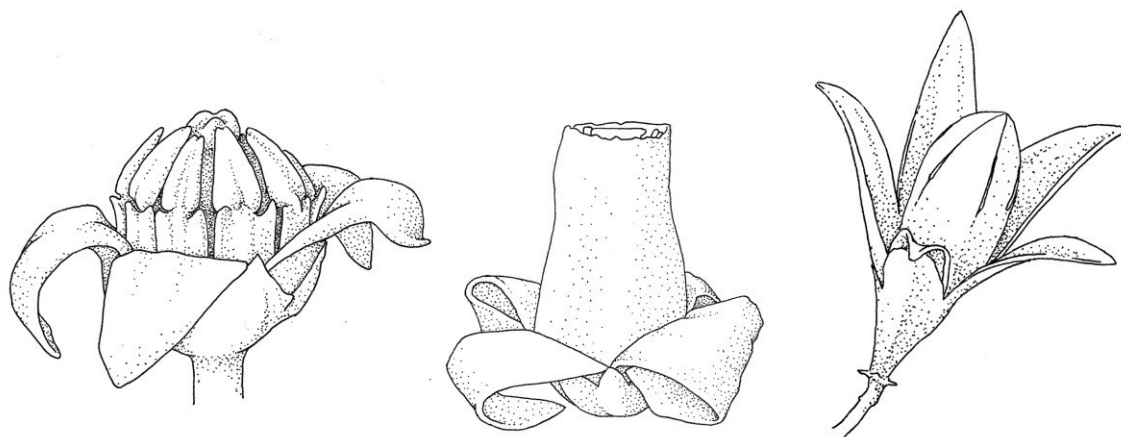
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Chapter III

Insights on evolution of reproductive
structures in the mahogany family (Meliaceae)
under a phylogenetic framework



ABSTRACT

Meliaceae are a mostly pantropical family of the order Sapindales, closely related to Rutaceae and Simaroubaceae. The flowers of Meliaceae are typically provided with a staminal tube, formed by filaments that are fused partially or totally, but several genera of the Cedreloideae subfamily have free stamens, which may be adnate to an androgynophore in some taxa. The fact that the family presents a wide diversity of floral and fruit features, as well as of sexual systems and pollination biology, pose interesting questions on the evolutionary processes that might have taken place during its history. In this study we aim to analyse the main morphological characters related to the reproductive structures of Meliaceae, upon a recent molecular phylogenetic framework, in order to look for putative synapomorphies for some clades within the family, and to evaluate hypotheses on floral evolution and sexual systems in the group. We selected and encoded 20 reproductive morphological characters, and used 31 Meliaceae terminals from its two main clades (Cedreloideae and Melioideae), and six terminals of Simaroubaceae as an outgroup. Our reconstruction of the sexual systems evolution suggests that the ancestor of Meliaceae possibly was provided with united stamens and unisexual flowers in dioecious individuals; subsequently in the ancestor of the Cedreloideae lineage there was a change free stamens and to monoecy. Most characters studied show some degree of homoplasy, but the isostemonous androecium and an androgynophore with petals adnate to it were reconstructed as unique synapomorphies helping to define the *Cedrela-Toona* clade. In addition, the fused cotyledons and the capsule with a rudimentary columella emerge as synapomorphies of the *Carapa-Xylocarpus* clade. The comparative approach of our study and the evolutionary hypotheses generated herein reveal several aspects demanding detailed further structural investigation, and possible pathways of the reproductive structures along the lineages diversification, mostly related to specialization on floral biology and dispersal strategies.

Keywords: ancestral state reconstruction; floral evolution, rosids, Sapindales, sexual systems.

INTRODUCTION

The Meliaceae is a mostly pantropical family composed of about 575 species distributed in 50 genera (Mabberley, 2011; Stefano *et al.*, 2015). The available phylogenies of the order Sapindales point to a close relationship of Meliaceae to Rutaceae and Simaroubaceae (e.g. Gadek *et al.* 1996, APG IV 2016), and only recently a moderate support for its position as sister to the latter emerged (Muellner-Riehl *et al.*, 2016).

The representatives of Meliaceae are trees or shrubs with predominantly alternating and pinnate leaves, but these can be also trifoliolate or unifoliolate (Pennington & Styles, 1975; Mabberley, 2011). The inflorescences may be terminal, axillary or cauliflorous, and are usually thyrse. The flowers are usually tetramerous to hexamerous, with a truncated calyx or free sepals; the petals are generally free, longer than the sepals. The most striking morphological feature of the family is the presence of connated filaments, forming a staminal tube, the mouth of which is blocked by a large stigma. In some genera as *Cedrela*, the filaments are free and adnate to an androgynophore. An intrastaminal disk is usually present and varies in size and form among genera. The ovary is superior or semi-inferior, generally 3-5-locular, with one or two ovules per locule. Fruits are mainly capsules or drupes, with often arillate or winged seeds (Pennington & Styles 1975; Pennington *et al.*, 1981; Stevens, 2001). General aspects of the morphological heterogeneity in flower and fruits of selected genera of Meliaceae are depicted in Figure 1.

The variety of sexual systems in Meliaceae is remarkable. Although numerous taxonomic, floristic and monographic works from the first half of the 20th century used to state that plants in the family were hermaphroditic (with bisexual flowers), White & Styles (1963) were the first to note that most of the African species examined had functionally unisexual flowers, but each male or female flower had well-formed though sterile rudiments of organs of the opposite sex. Subsequent works have shown that the flowers are predominantly unisexual, in dioecious, monoecious or polygamous plants, but with a very subtle sexual dimorphism of flowers (e.g. Styles, 1972,

Pennington & Styles, 1975; Pirani, 1992; Mabberley, 2011). Styles (1972) presented the sex distribution of flowers across 17 genera of Meliaceae, describing the subtle distinctions between the floral types, and the various sexual systems, and they also investigated secondary sexual characters. In the large genus *Aglaia*, with around 120 species, Pannell (1980) reported that all the studied taxa were strictly dioecious, with the female does not produce pollen and the male does not produce fruit. In *Cabralea*, a study on reproductive biology showed that male and female plants have morphologically similar flowers, but the former have larger inflorescences with more flowers those of female plants; the latter remain open and receptive for two days, whereas male flowers last only one day, and this seems to compensate for the imbalance of the number of flowers per plant between the two sexes (Franceschinelli *et al.* 2015). In *Trichilia*, although De Wilde (1968) stated that all African species were strictly dioecious, the Neotropical *Trichilia clausenii* is reported as gynodioecious, with infertile pollen produced by female flowers and bisexual flowers with fertile ovule and pollen (Gama *et al.*, in prep., see Chapter 1). In a study on floral development of *Cedrela* and *Toona*, functionally either male or female flowers were considered plesiomorphic traits within the family (Gouvêa *et al.*, 2008), but a hypothesis like this remain to be rigorously tested under a phylogenetic framework.

Yet, for such a well-known group of plants, with many commercially important hardwoods, as mahogany (*Swietenia*), it is remarkable how little is known about reproductive biology and pollination. There are reported cases of pollination by moths, flies and bees. For *Cedrela* it was reported visitation of night-flying moths and recent studies show that stingless bees, as *Tetragonisca* collect pollen from *C. odorata* L. Bees and moths are also believed to pollinate some species, such as *Trichilia*, where small *Meliponinae* bees were seen pollinating their flowers in Costa Rican forests (Bawa & Opler, 1975). Yeo (1993) reports that several species of *Turraea* have flowers with features typical of moth pollination, and he also describes secondary pollen presentation in the genus. Morellato (1991) reported the pollination of species of *Cabralea*, *Cedrela*, *Guarea* by moths, and *Trichilia*

by flies and hemiptera. Moths of five families were observed pollinating *Cabralea canjerana* (Vell.) Mart. subsp. *canjerana* (Carmo, 2005; Franceschinelli *et al.*, 2015). Moths were also observed visiting flowers of *Guarea* spp. (Styles & Khosla, 1976). Stingless bees from the genus *Trigona* were captured visiting four species of *Aglaia*, as well as *Hymenoptera* (e.g. Eumeninae wasps), some species of *Diptera* from the families Syrphidae, Drosophilidae, Cecidomyiidae and Lauxaniidae were captured visiting male flowers of Meliaceae (Pannell, 1992). Further studies in this area are necessary before we can achieve a better understanding of the relations between the evolution of sexual systems and the pollination biology.

Finally, detailed data from floral morphological analyzes may be very helpful in these evolutionary issues among the Meliaceae. In other families of Sapindales studies have been done in a comparative context and mostly contributed to the systematics of the groups (e.g. Bachelier & Endress 2008, 2009; Pirani *et al.*, 2010; Bachelier *et al.*, 2011; El Ottra *et al.*, 2013; El Ottra, 2014, Alves *et al.*, 2017). Thus, this moment is conducive to a study with flowers of Meliaceae, in a comparative context, aiming to provide subsidies to the macrosystematics and macroevolution of the family.

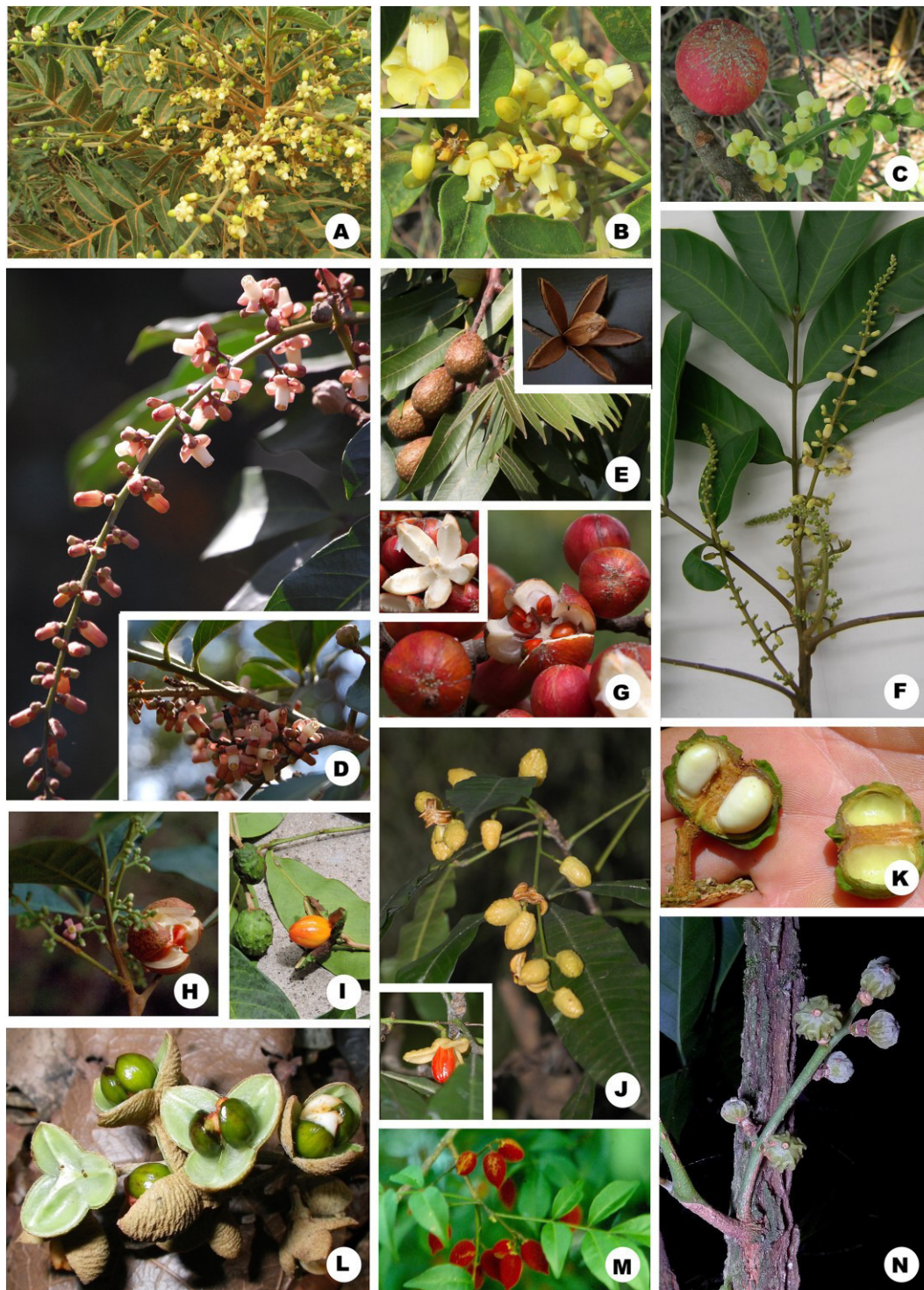
The objective of this study is to analyse the main morphological characters of the flowers in Meliaceae, upon the current phylogenetic framework, aiming to find putative synapomorphies for some clades within the family, and to evaluate hypotheses on floral evolution and sexual systems in the group.

MATERIALS AND METHODS

To identify potential synapomorphies for each clade and examine patterns of morphological homoplasies, we selected 20 floral morphological characters. The characters were encoded from our original data and from the literature (Pennington & Styles, 1975; Mabberley, 2011; Clayton, 2011; Devecchi *et al.* 2018). In cases of genera with polymorphic character, all applicable character states were scored. Only the 31 genera of Meliaceae included in the phylogenetic study by Muellner-Riehl *et al.* (2016) were here

analyzed. Morphological concepts and terminology follow Pennington & Styles (1975), Bawa & Beach (1981), Weberling (1989) and Sakai & Weller (1999). Regarding the androecium, the characterization of filaments coherent by intertwining trichomes as a “pseudotube” follow Devecchi *et al.* (2018). Due to the current lack of anatomical evidence for most taxa, we encoded as “indistinct” all cases where a nectary is not morphologically visible with the naked eye or with a stereomicroscope. *Receptaculum pollinis* is a special type of stigma that is related to secondary presentation of pollen in some Meliaceae (Pennington & Styles, 1975; Yeo, 1993). Even though Pannel (1992) suggests the any type of colored seed coat or seed outgrowths in Meliaceae should be called as “aril”, we follow Pennington & Styles (1975), according to whom sarcotesta is the fleshy part that appears as a localized or general swelling of the seed coat, and arillode is the fleshy outgrowth that forms partially or completely free flap of tissue involving some or all of the seed. For the evolutionary analysis, we used the Meliaceae clade and some terminals of Simaroubaceae as external group from the phylogenetic tree of Muellner-Riehl *et al.* (2016), which was based in three molecular markers: plastidial regions *rbcL* and *atpB*, and the *trnL-trnLF* spacer region, which produced congruent phylogenies using maximum likelihood and Bayesian methods. We used the reconstruction of the Ancestral State of Parsimony (Maddison & Maddison, 2005) in Mesquite v.3.4 (Maddison & Maddison, 2018).

Figure 1. General morphology of inflorescences, flowers and fruits of Meliaceae. A, Inflorescence ♂ of *Cabralea canjerana* subsp. *polytricha*. B, Inflorescence ♀ (a thyse) of *Cabralea canjerana* subsp. *polytricha*; flower (top left). C, Inflorescence ♀ and fruit (a fleshy capsule) of *Cabralea canjerana* subsp. *polytricha*. D, Inflorescence (a thyse) of *Guarea macrophylla* subsp. *tuberculata*; flowers and fruit (lower right). E, Fruits (septifragal 5-carpellate capsules) of *Cedreia fissilis*; fruit open (lower right). F, Shoot with axile inflorescences (thyres) of *Guarea guidonia*. G, Fruits (4-5-carpellate fleshy loculicidal capsules) of *Cabralea canjerana* subsp. *polytricha*; open capsule without seeds (top left). H, Inflorescence and 4-carpellate fleshy loculicidal capsule of *Guarea kunthiana*, note seeds with shiny red sarcotesta. I, Fruits (muricate, 3-carpellate loculicidal capsules) of *Trichilia cf. pallens*. J, Shoot with fruits of *Trichilia clausenii*; open 3-carpellate loculicidal capsule with sarcotestal seed (lower left). K, Fruit in longisection of *Guarea suberosa*, note immature seeds with white sarcotesta. L, Fruit (a striate tricarpellate loculicidal capsule), of *Trichilia hirta*, showing 2-3 seeds with a green sarcotesta and a white aril. M, Shoot with fruits (red 3-carpellate capsules) of *Trichilia elegans*. N, Lateral infructescence of *Guarea suberosa* with young costate capsules. Photographs (A – C and G) by R. Mello-Silva; (D, F, J) by D. Gomes; (E) H. Moreira and R. L.Gama; (H and M) by J. R. Pirani; (I) by A. S. Castro; (K and N) by A. Popovkin; (L) by L. Nusbaumer.



RESULTS

A list of the 20 character descriptions and coding is given in Table 1, and the expression of these character states for each species is provided as a data matrix in Table 2. Figures 2 and 3 depict the diversity of floral and fruit characters studied here.

Results of the reconstruction of ancestral state characters are presented in Figs. 4-8. In Fig. 4A, sexual systems are plotted. We used the parsimony criterion to discuss the evolutionary hypothesis.

Table 1. List of morphological characters and their character state coding used for the ancestral state reconstruction in selected taxa from Meliaceae and Simaroubaceae.

1. **Sexual system:** unisexual flowers in the same individual (monoecious plants) (0); unisexual flowers in distinct individuals (dioecious plants) (1); unisexual and bisexual flowers (polygamous plants) (2); only bisexual flowers (hermaphrodite plants) (3).
2. **Fusion of sepals:** free (0); partially connate (1); completely connate (2). Figs. 2A, B.
3. **Corolla aestivation:** imbricate (0); valvate (1); contorted (2).
4. **Number of petals:** three (0); four or more (1).
5. **Fusion of petals to androecium:** free (0); adnate to staminal tube (1); adnate to androgynophore (2). Figs. 2J, K, Q.
6. **Number of stamens in relation to petals:** isostemonous (0); diplostemonous (1); polystemonous (2).
7. **Union of filaments:** completely free from each other (0); filaments partially connate (1); filaments completely connate (2); filaments coherent by intertwining trichomes. Figs. 2C, D, G, J, O; 3B, D, F, G, H.
8. **Shape of staminal tube:** globose (0); cyathiform (1); urceolate (2); cylindrical (3). Figs. 2F, G, H, I, L, M, Q.
9. **Anthers insersion:** at the apex of filaments (0); at the margin of staminal tube (1); on the inner side of staminal tube, all at the same height (2); on the inner side of staminal tube at different heights (3). Figs. 2C, I, L, O; 3A, F.
10. **Apical appendage of anther connective:** absent (0); present (1). Fig. 2F.
11. **Gynophore:** absent (0); present (1). Fig. 2C.
12. **Androgynophore:** absent (0); present (1). Figs. 2 J, K.
13. **Nectary (structure evidenced macromorphologically):** indistinct (0); patelliform or annular, around the base of the ovary (1); cyathiform (2); tubular (3); annular at the gynophore apex (4). Figs. 2C, D, G, M, N. 3E, F, H.
14. **Carpel number (locules):** one (0); two or three (1); four or five (2); more than five (3). Fig. 2K.
15. **Number of ovules per locule:** one (0); two (1); two and a vestigial one (2); three or more (3).
16. **Position of the ovules:** collateral (0); superposed (1). Figs. 2H, N; 3E.
17. **Stigma type:** capitate, without conspicuous lobes (0); conical (1); discoid (2); pileate (3); large stigmatic lobes (4); *receptaculum pollinis* (5); truncate (6); elongate and divergent (7). Figs. 2E, F, O, P, Q; 3A, B, C, E, G, H.
18. **Fruit type:** loculicidal capsule (0); septifragal capsule dehiscent from apex (1); septifragal capsule dehiscent from base (2); septifragal capsule dehiscent from apex and base simultaneously, with a conspicuous columella (3); septifragal capsule with a rudimentary columella dehiscent from apex and base simultaneously (4); drupe (5); nut (6); samarids (7); berry (8). Figs. 3I-P.
19. **Seed outlayer or outgrowths (arillode or sarcotesta):** absent (0); arillode present (1); sarcotesta present (2).
20. **Position of cotyledons:** superposed (0); collateral or oblique (1); completely fused (2).

Table 2. Morphological matrix of characters and their states in selected taxa of Meliaceae and Simaroubaceae.

Táxons	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Meliaceae - Cedreloideae																				
<i>Capurionianthus</i>	0	(01)	(02)	1	0	1	2	(23)	1	0	0	0	0	(12)	2	-	0	1	(02)	1
<i>Cedrela</i>	0	(01)	0	1	2	0	0	4	0	0	0	1	0	2	3	-	2	1	0	1
<i>Chukrasia</i>	0	1	2	1	0	1	2	3	1	0	1	0	1	(12)	3	-	0	1	0	1
<i>Khaya</i>	0	(01)	2	1	0	1	2	(12)	2	0	0	0	1	2	3	-	2	1	0	1
<i>Lovoa</i>	0	(01)	0	1	0	1	2	(13)	1	0	0	0	1	2	3	-	(02)	(13)	0	1
<i>Swietenia</i>	0	1	2	1	0	1	2	(12)	2	(01)	0	0	1	2	3	-	2	(23)	0	1
<i>Toona</i>	0	(01)	0	1	2	0	0	4	0	0	0	1	0	2	3	-	2	1	0	1
<i>Carapa</i>	0	(01)	2	1	0	1	2	(123)	2	0	0	0	1	(23)	3	-	2	4	2	2
<i>Xylocarpus</i>	0	1	2	1	0	1	2	(12)	2	0	0	0	1	2	3	-	2	4	2	2
Meliaceae - Melioideae																				
<i>Lepidotrichiia</i>	2	1	1	1	0	1	(12)	3	(01)	0	0	0	0	(12)	0	-	0	5	0	1
<i>Munronia</i>	3	1	0	1	1	1	2	3	1	(01)	0	0	(03)	2	1	1	0	0	2	1
<i>Pseudoclausena</i>	2	0	0	1	?	?	0	4	?	1	0	0	0	2	0	-	?	8	?	-
<i>Sandoricum</i>	3	(12)	0	1	0	1	2	3	2	0	0	0	3	2	1	0	4	5	2	1
<i>Walsura</i>	(12)	(01)	(012)	1	0	(12)	(01)	(12)	(01)	(01)	0	0	1	(12)	(01)	0	(036)	8	(01)	1
<i>Azadirachta</i>	2	1	0	1	0	1	2	3	(12)	0	0	0	1	1	1	0	4	5	2	1
<i>Melia</i>	2	1	0	1	0	1	2	3	(12)	0	0	0	1	(23)	1	1	(04)	5	0	1
<i>Aglaia</i>	1	1	0	(01)	0	(012)	2	(0123)	(12)	0	0	0	0	(012)	(01)	(01)	(01)	(068)	(12)	(01)
<i>Cabralea</i>	1	(01)	0	1	0	1	2	3	2	0	0	0	2	2	1	1	2	0	1	1
<i>Calodécaryia</i>	3	1	2	1	0	1	1	(13)	0	0	0	0	1	2	1	0	(02)	0	2	1
<i>Dysoxylum</i>	(13)	(012)	(01)	(01)	1	(12)	2	3	2	0	0	0	(123)	(123)	1	(01)	(02)	0	(12)	(01)
<i>Ekebergia</i>	1	1	0	1	0	1	(12)	(123)	1	0	0	0	1	(123)	1	1	(02)	5	0	1
<i>Guarea</i>	(13)	(12)	(01)	(01)	0	1	2	3	2	0	1	0	(04)	(123)	1	1	2	0	2	(01)
<i>Heckeldora</i>	1	1	0	1	0	1	2	3	2	0	1	0	4	0	1	0	2	(08)	2	1
<i>Lansium</i>	(13)	(01)	0	1	1	1	2	(01)	2	0	0	0	0	2	0	-	6	8	1	0
<i>Naregamia</i>	3	1	0	1	0	1	2	3	1	1	0	0	0	(12)	1	0	(25)	0	1	1
<i>Nymphia</i>	3	1	0	1	0	1	1	(13)	0	0	0	0	1	2	1	0	0	0	2	1
<i>Owenia</i>	1	0	0	1	0	1	(12)	(12)	(01)	0	0	0	1	(12)	0	-	1	5	2	1
<i>Reinwardtiodendron</i>	3	(01)	0	1	1	1	2	(01)	3	1	0	0	0	(12)	(01)	0	(03)	8	(02)	0
<i>Trichilia</i>	(123)	(012)	(01)	(01)	0	(01)	(0123)	(0123)	(01)	0	1	0	(012)	(123)	1	(01)	(01)	0	(12)	1
<i>Turraea</i>	3	(12)	(012)	(01)	0	(12)	(12)	(13)	(012)	(01)	0	0	(012)	(123)	1	(01)	(025)	0	1	1
<i>Turraeanthus</i>	1	(12)	1	1	1	1	2	3	2	0	0	0	0	2	1	1	2	0	2	0
Simaroubaceae - outgroup																				
<i>Ailanthus</i>	(01)	1	1	1	0	1	0	4	0	0	0	0	0	(12)	0	-	7	7	0	1
<i>Castela</i>	1	1	0	1	0	1	0	4	0	0	0	0	1	2	(01)	0	7	5	0	1
<i>Picrasma</i>	(01)	(01)	1	1	0	0	0	4	0	0	0	0	1	(12)	0	-	7	5	0	1
<i>Quassia</i>	3	0	2	1	0	1	0	4	0	0	1	0	(04)	2	0	-	0	5	0	1
<i>Simaba</i>	3	1	(02)	1	0	1	(013)	3	0	0	1	0	(04)	2	0	-	7	5	0	1
<i>Homalolepsis</i>	(23)	1	(02)	1	0	1	(013)	3	0	0	1	0	(04)	(23)	0	-	0	5	0	1

DISCUSSION

General aspects and probable synapomorphies: Even though only 31 out of 50 genera of Meliaceae were analysed, we believe that patterns obtained represent working hypotheses towards a better understanding of the evolutionary pathways of reproductive traits examined. Most characters studied show some degree of homoplasy, and only the isostemonous androecium (char. 6) and an androgynophore (char. 12, Figs. 2J, 6D) with petals adnate to it (char. 5, Fig. 2K) were reconstructed as unique synapomorphies helping to define the *Cedrela-Toona* clade, as well as the fused cotyledons (char. 20) and the capsule with a rudimentary columella (char. 18, Fig. 3M) emerging as synapomorphies of the *Carapa-Xylocarpus* clade. The two later genera compose a tribe apart (*Xylocarpae*) in the current classification of Meliaceae (Mabberley, 2011). However, both share some morphological apomorphies with the sister clade (*Khaya* and *Swietenia*), as a corolla with contorted aestivation (char. 3) and the anthers inserted on the inner side of the staminal tube (char. 9, Figs. 2C, 2E, 2G, 2H, 2L- N, 2P, 3A), and so the tribe could be redefined to include also these two genera, currently unplaced. (Figs.4C, 5D, 6A, 8A).

Despite the high degree of homoplasy, our reconstructions reveal that several of the characters analysed are potentially useful in helping to define clades when combined with other morphological features, and they also suggest several interesting events that may have taken place during the evolution and diversification of these genera.

Our results indicate that a putative synapomorphy of Meliaceae is the monadelphous androecium (fully connate filaments, char. 7, e.g. Figs. 2C, 2E-I, 2L-N, 2P-Q, 3A, 3C and 3F) to be discussed further below). No morphological synapomorphy was found for the Melioideae clade, while three features seem to bear strong phylogenetic signal supporting the Cedreloideae clade: a special type of fruit (the septifragal capsule - char. 18, Fig. 3O), an ovary with three to several ovules (char. 15), and the transition to monoecy (char. 1, to be further discussed in detail).

Floral characters: The evidence that the staminal tube is a synapomorphy of the Meliaceae is consistent with the statement by Pennington & Styles (1975) that the androecium is “the most important diagnostic character of the family”, as also did most traditional taxonomic treatments (e.g. Cronquist 1988; Beck, 2004). But this is an homoplastic feature, since the filaments subsequently became partially free in at least some species of eight genera (*Owenia*, *Ekebergia*, *Lepidotrichilia*, *Trichilia*, *Nymania*, *Turraea*, *Calodercarya*), or even completely free in the *Cedrela-Toona* clade and in *Pseudoclausena* and some species of *Walsura* and *Trichilia* (Fig. 5C). In fact, in these two latter genera the free filaments are dorsoventrally flattened and remain very close to each other, suggesting that they still constitute a “functional tube”. The wide variation in the degree of fusion of the filaments is expressed not only between genera, but also within a single species, as between male and female flowers of *Trichilia havanensis* Jacq. It must be pointed out that *Trichilia* is one of the largest genera of the family, with 81 Neotropical species and around 20 African species (Pennington, 2016), so several polymorphic characters would be expected (Figs. 1I, 1J, 1L, 1M). The degree of staminal fusion is constant in smaller genera as *Dysoxylum* and *Chisocheton* (the latter not studied here – Pennington & Style, 1975; Mabberley, 2011).

Our data also suggest that the ancestral state of the shape of the staminal tube was cylindrical (char. 8, Fig. 2E), which latter underwent homoplastic changes in shape (Fig. 5D). These variations in shape may be related to the particularity of the pollination process.

Another feature related to the androecium is related to the position of the anthers (char. 9, Figs. 2D–K, 3A–H), which is also highly informative at the generic level according to Pennington & Styles (1975). Here we recovered an hypothesis in which the anthers in the ancestor of Meliaceae were placed at the margin of the staminal tube and subsequent homoplastic transitions occurred along the history of the family. Anthers may be inserted at the apex of the filaments or at the margin of the staminal tube as in *Trichilia*, or on the inner side of staminal tube, as in *Guarea* and other genera; in this latter case the anthers at the same height seem to be a plesiomorphic state in relation to those on the inner side of staminal tube at different heights found in *Dysoxylum* (Fig. 6A).

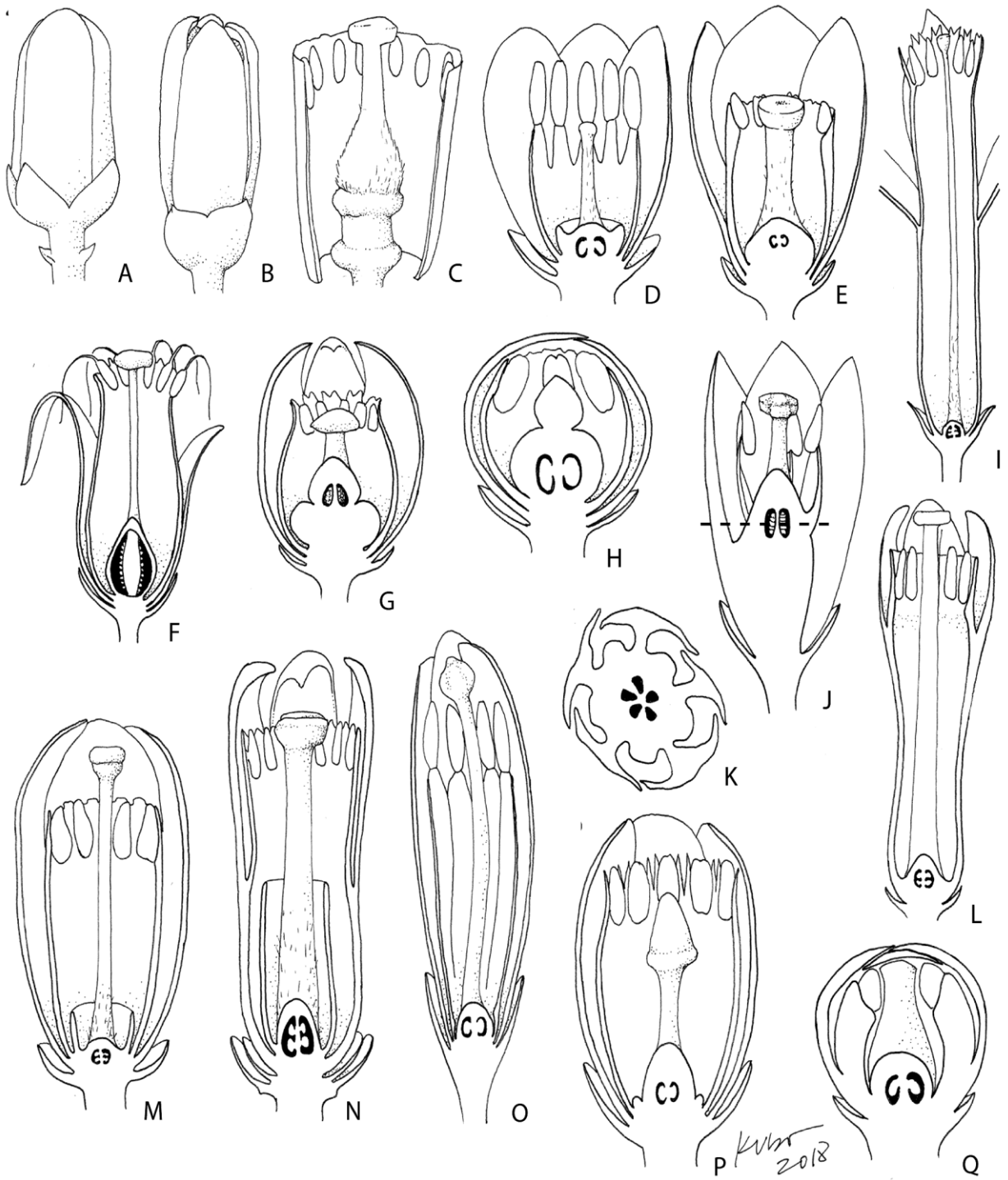
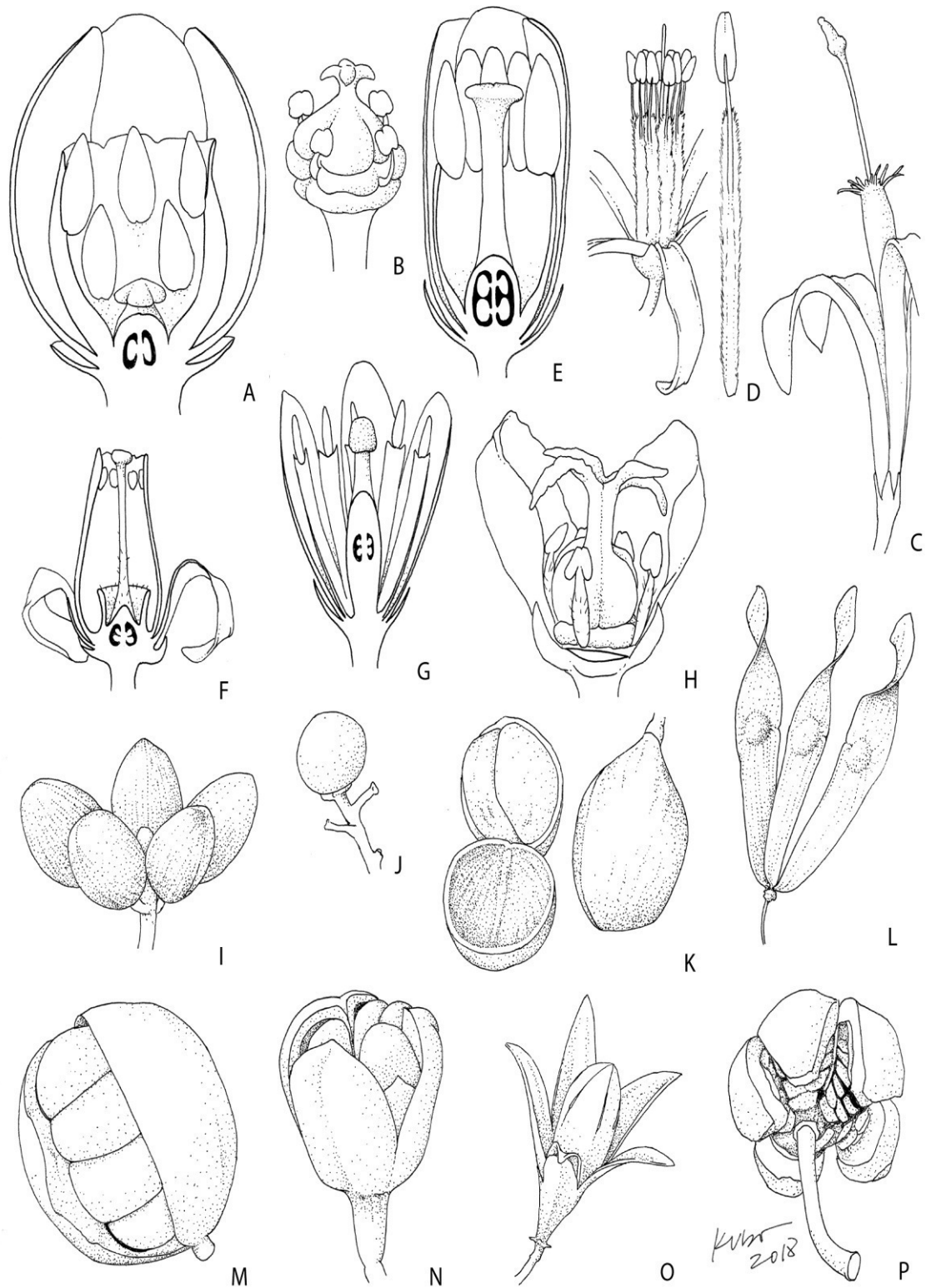


Figure 2. Diversity of floral characters of Meliaceae. A, Flower bud of *Guarea macrophylla* subsp. *tuberculata* with sepals partially connate. B, Floral bud of *Guarea kunthiana* with sepals completely connate. C, *Guarea macrophylla* subsp. *tuberculata* – gynoecium on top of a gynophore with an annular nectary at the gynophore apex; stigma discoid; androecium with filaments completely connate and anthers on the inner side of staminal tube, all at the same height. D, *Pseudobersama mossambicensis* – filaments partially connate and anthers inserted at the apex of filaments; nectary patelliform adnate to the ovary. E, *Synoum glandulosum* – stigma discoid; nectary indistinct. F, *Schmardaia microphylla* – anther connectives with apical appendage; staminal tube urceolate; stigma discoid; ovules numerous. G, *Xylocarpus granatum* – nectary annular; with filaments completely connate forming an urceolate staminal tube. H, *Aglaia elaeagnoidea* ♀ - staminal tube globose; nectary indistinct; ovules collateral. I, *Munronia unifoliolata* – staminal tube cylindrical with anthers inserted at its margin. J, *Cedrela odorata* – filaments completely free from each other; petals adnate to an androgynophore. K, *Cedrela odorata* – transverse section showing fusion of petals to androgynophore by a medial carina; five multiovulate locules. L, *Turraeanthus zenkeri* – staminal tube cylindrical with anthers inserted on its inner side, all at the same height; nectary indistinct; ovules superposed. M, *Cabrlea canjerana* subsp. *polytricha* – staminal tube cyathiform; nectary cyathiform. N, *Dysoxylum amooroides* – staminal tube tubular, partially adnate to petals; nectary tubular; ovules superposed. O, *Calodectarya crassifolia* – anthers at the apex of filaments which are only partially connate; stigma capitate, without conspicuous lobes. P, *Owenia reticulata* – staminal tube urceolate; stigma conical; ovules collateral; annular nectary. Q, *Lansium domesticum* – petals adnate to a globose staminal tube; stigma truncate; nectary indistinct. (A - C, K modified from Pirani, 1992; all remaining figures modified from Pennington & Styles, 1975) by M. T. Kubo.

Although the anther insertion is constant for most genera, in several cases it varies within a genus, for example in *Aglaia* and *Turraea* (Mabberley, 2011). The presence of the apical appendages of the anthers connective (char. 10) evolved independently several times in Meliaceae. This terminal appendage may be short or sometimes long as in *Naregamia* (Fig. 6B).

Regarding the number of stamens, our reconstruction reveals the diplostemonous androecium as plesiomorphic, since it is shared with most Simaroubaceae and also most lineages of the related families Rutaceae and Sapindaceae (Muellner-Riehl *et al.* 2016). Diplostemony was conserved throughout the evolution of most Meliaceae, with isostemony evolving three times: in *Trichilia*, *Aglaia* and in the *Cedrela-Toona* clade (Fig. 5B).

Our analyses show that the Meliaceae ancestor had partially connate sepals (char. 2), which later became free or completely connate in some groups. In some genera this character seems to be very unstable: in *Trichilia* for example, the three states are found (Fig.4B).



An imbricate corolla (char. 3) was already present in the ancestor of Meliaceae, and is maintained in most of the living genera, except for some scattered changes to the valvate or contorted types (Fig. 4C). According to Pennington & Styles (1975), contorted aestivation is frequent in the subfamily

Cedreloideae and only occasionally found in other genera. The flowers with four or more petals represent the putative plesiomorphic state, while trimerous corolla appear as a homoplastic feature of the genera *Trichilia*, *Turraea*, *Guarea*, *Aglaia* and *Dysoxylum* (Fig. 4D).

Some interesting patterns emerged in relation to the fusion of the petals with the androecium (char. 5). In several genera, for example *Turraeanthus*, *Murronia* and part of *Dysoxylum*, the petals are free from each other but adnate to the staminal tube (Fig. 5A). Even in some species of *Murronia*, the petals seem to become free as they mature (Pennington & Style, 1975). Adherence to the androecium occurred homoplastically, including the very special case in which an androgynophore developed as a synapomorphy of the *Cedrela-Toona* clade. This is a quite unusual feature within angiosperms and evolved only once in Meliaceae (Fig. 5A).

Figure 3. Diversity of floral (A-H) and fruit (I-P) characters of Meliaceae and Simaroubaceae. A, *Sphaerosacme decandra* – urceolate staminal tube with anthers inserted on its inner side, at different heights; stigma pileate. B, *Picrasma javanica* ♀ - stigma elongate and divergent; nectary annular; filaments completely free from each other (staminodes). C, *Turraea mombassana* – stigma *receptaculum pollinis*; D, *Homalolepis intermedia* – filaments coherent by intertwining trichomes (pseudotube), with a detail of an isolated stamen. E, *Astrotrichilia* sp. – stigma discoid; ovules superposed; nectary indistinct. F, *Cabrlea canjerana* subsp. *canjerana* – nectary cyathiform; filaments completely connate with anthers on the inner side of staminal tube, all at the same height; ovules superposed. G, *Trichilia hirta* – filaments completely free from each other; stigma capitate; ovules superposed. H, *Castela tweedii* - filaments completely free from each other; stigma elongate and divergent; nectary annular, around the base of the ovary. I, *Quassia amara* – fruit compound with five drupelets. J, *Walsura monophylla* – berry. K, *Aglaia korthalsii* – nut. L, *Ailanthus altissima* – fruit with three samarids. M, *Carapa procera* – septifragal capsule with a rudimentary columella, dehiscent from apex and base simultaneously. N, *Trichilia emetica* - loculicidal capsule dehiscent from apex. O, *Cedrela sinensis* - septifragal capsule dehiscent from apex, with a conspicuous central columella. P, *Swietenia mahagoni* - septifragal capsule dehiscent from apex and base simultaneously, with a conspicuous columella. (I – K, M, O) by M. Kubo; (A, E, G. modified from Pennington & Styles, 1975; B, L. modified from Clayton, 2011; C,N,P. modified from Harms (1940); D. modified from Devecchi *et al.* 2018; F. modified from Pirani, 1992; H. modified from Pirani, 1987) by M. Kubo.

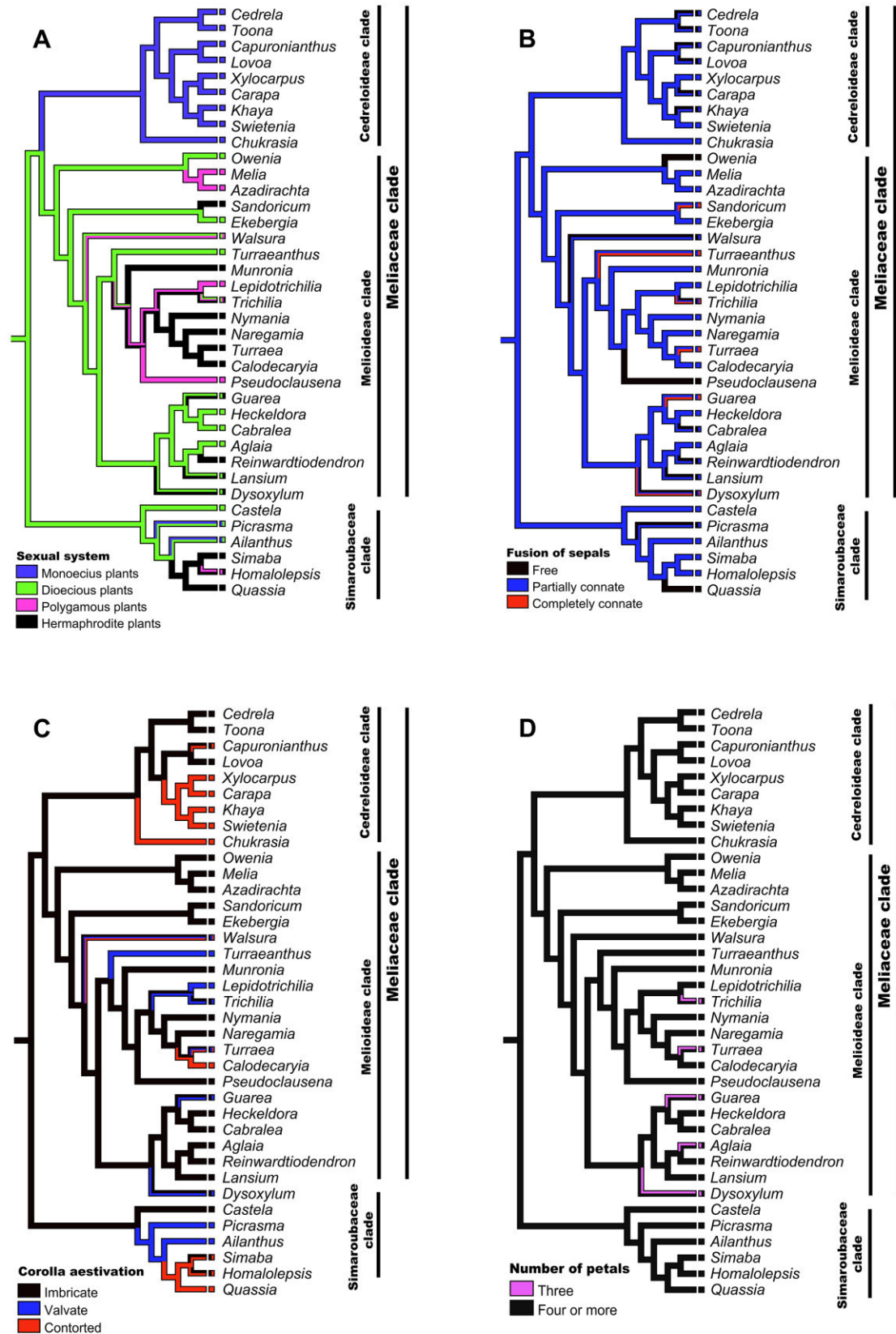


Figure 4. Ancestral character reconstruction for selected morphological characters of Meliaceae and Simaroubaceae. A, Sexual system. B, Fusion of sepals. C, Corolla aestivation. D, Number of petals.

According to Pennington & Style (1975) the *Cedrela* and *Toona* petals are adnate to androgynophore by means of a longitudinal carina (along the median vein of each petal), and this prevents petals of open flowers from spreading (Fig. 2K). The role of this configuration is discussed in the *Floral biology* section ahead.

The presence of gynophores evolved independently at least four times in Meliaceae, being present in *Chukrasia*, *Trichilia*, *Guarea*, and *Heckeldora* (Fig. 6C). The presence of an androgynophore in the *Cedrela-Toona* clade arose independently, meaning that the ancestral of this clade probably lacked a gynophore (Fig. 6D). Gynophores also occur in the sister-group Simaroubaceae as well as in several Rutaceae (e.g. El Ottra *et al.* 2013) and Sapindaceae (e.g. Beck, 2004).

In several genera of Meliaceae, e.g. *Aglaia* and *Lansium*, a disk (char. 13) is absent or indistinct (Mabberley, 2011), while in other genera it may be very conspicuous as an intrastaminal structure. Pennington & Styles (1975) state that the disk is probably nectariferous and that in many species of Melioideae it appears as an annular ring surrounding the base of the ovary. In other cases, it may be a poorly developed saucer-shaped structure (a patelliform disk), but in our reconstruction we recovered this feature more widely in the Cedreloideae clade, than in Melioideae. This latter group presents in addition to the annular or patelliform disks, other forms like the cyathiform and the long tubular disk completely surrounding the ovary height, e.g. in *Dysoxylum*, *Sandoricum*, *Cabralea* and some species of *Munronia* (Fig. 7A). The lack of anatomical studies of the disks in the family prevent us from establishing the portion holding the nectariferous tissue, nor the position of this tissue in the genera devoid of a structurally distinct disk. In the related Simaroubaceae, a recent study showed that the whole surface of the elongate gynophore of *Homalolepis* (as *Simaba*, Alves *et al.* 2017) is nectariferous. The possibility that gynophores of Meliaceae can be likewise nectariferous remains to be evaluated. This seem to be the case of the thick annular disk which is adnate to the gynophore of species of *Xylocarpus* (Fig. 2G).

Regarding the ovary (char. 14), we recovered that the ancestor of Meliaceae and Simaroubaceae had four to five loculi, but this number also is very homoplastic and can vary from 1 to 20 in living meliaceous genera (Fig. 7B).

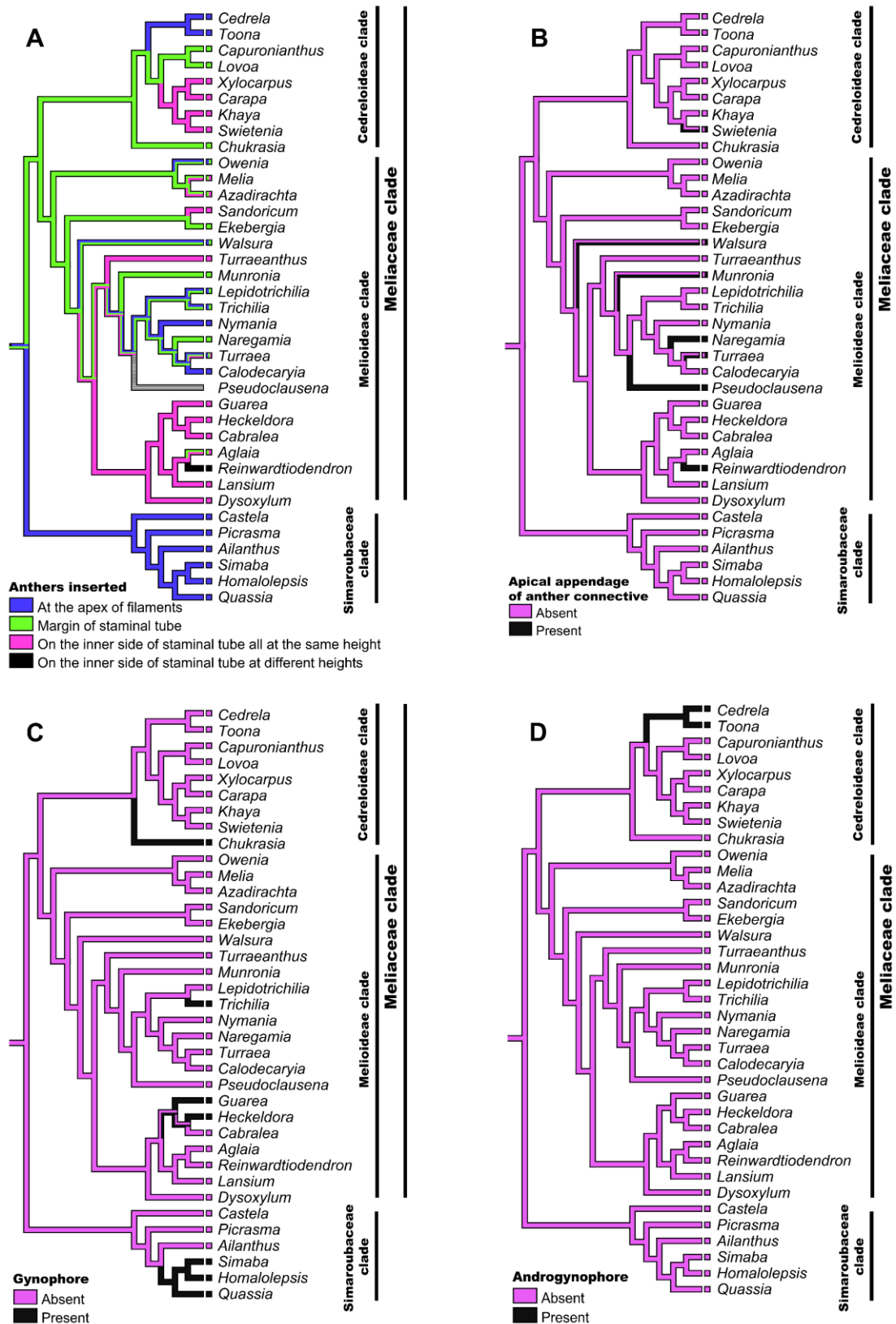


Figure 6. Ancestral character reconstruction for selected morphological characters of Meliaceae and Simaroubaceae. A, Anthers insertion. B, Apical appendage of anther connective. C, Gynophore presence. D, Androgynophore presence.

The hypothesis presented in the reconstruction confirms what was described in the Meliaceae monograph by Pennington & Style (1975). The number of ovules per loculi provides an important distinction of the subfamilies. In almost all the Melioideae each loculi can contain one or two ovules, while in the Cedreloideae each loculi is provided with three or more ovules. Two ovules with one vestigial is an autapomorphy of *Capuronianthus* (Fig. 7C). The collateral position of the ovules is widespread in the Melioideae clade, the superposed position appearing in scattered genera (Fig. 7D).

The stigma in Meliaceae shows remarkable variations in shape, and according to Pennington & Styles (1975) this structure could provide valuable characters for classification within the family. In some of the genera the stigma is capitate as in e.g. *Capuronianthus*, *Chukrasia* and *Munronia*, and this feature has evolved multiple times. In *Owenia* it is expanded into a conical structure, and in most genera of the Cedreloideae it is discoid, as well as in the clade formed by *Guarea*, *Heckeldora* and *Cabrarea*. The stigma is pileate (in the shape of a mushroom) in some species of *Walsura* and *Reinwardtiidendron*. The stigma truncate is found in *Walsura* and *Lansium*. *Melia*, *Azadirachta* and *Sandoricum* have large stigmatic lobes (Fig. 8A). In *Turraea*, the stigma reaches its greatest development, modified to form a *receptaculum pollinis* of various shapes (Fig. 8A), with a marked role in secondary pollen presentation (White & Styles, 1963; Yeo 1993; see discussion below).

Floral biology: Several of the remarkable variations in floral designs of the Meliaceae discussed above suggest a strong association to specializations in pollination biology. Among the genera or even among congeneric species treated herein, structures differ either in the spatial arrangement of female and male sexual organs, or in their position in relation to other floral structures, resulting in various forms of attraction of visitors, protection against nectar robbers, and also in the location where pollen is deposited on visiting pollinators.

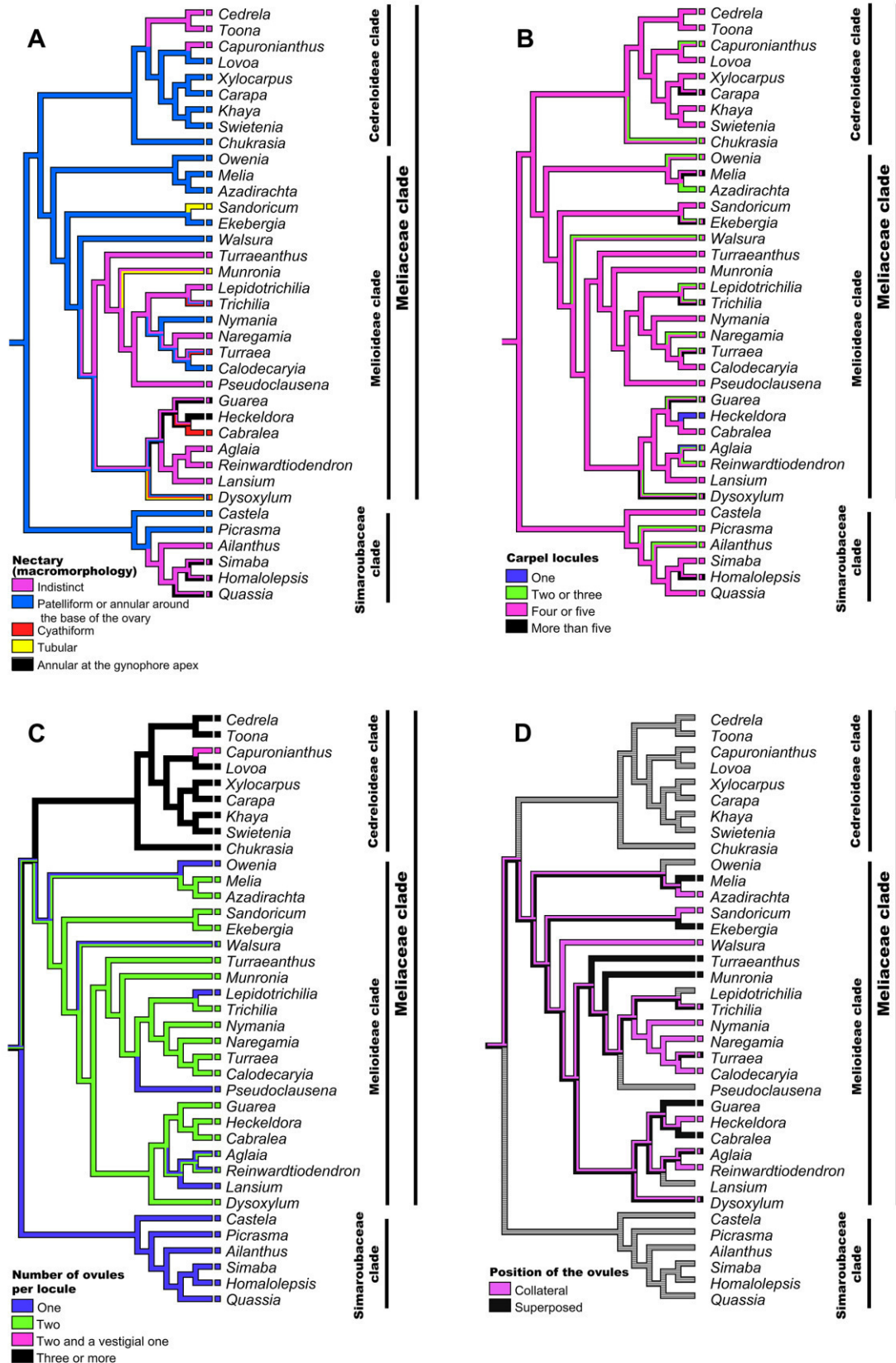


Figure 7. Ancestral character reconstruction for selected morphological characters of Meliaceae and Simaroubaceae. A, Nectary. B, Carpel number (locules). C, Number of ovules per locule. D, Position of the ovules.

Either the monadelphous androecium or the structure resulting from the coherence of enlarged or appendiculate filaments surrounding the gynoecium of most Meliaceae, and of some of the Simaroubaceae here studied, are likely to act by restricting access to nectar deposited at the bottom of the staminal tube or “pseudotube” (see also Alves *et al.*, 2017; Devecchi *et al.*, 2018). Our reconstruction implies that coherent appendaged stamens evolved independently in a small clade of Simaroubaceae, while in the Melioideae clade ancestors with staminal tube gave rise to lineages with partially free stamens at least four times (Fig. 5C). Anyway, we may supposed that pollination of most of these flowers is accomplished by animals with long mouth parts, which are able to access the concealed nectar at the base of the floral tubes. It is interesting to note that in some genera of the Cedreloideae clade, which lack a staminal tube, the adnation of the petal carina to the androgynophore prevents petals of open flowers from spreading resulting in their “tubular-like” flowers (Pennington & Styles 1975, Fig. 2K). As nectar is accumulated in the longitudinal furrows surrounding the androgynophore of the Cedreloideae flowers, this resource is apparently available only to animals with long mouth parts. In fact, there is evidence that *Cedrela* flowers are pollinated by moths (Morellato, 1991), and this has been demonstrated also for some taxa of the Melioideae clade as *Cabralea* and *Guarea* (Morellato, 1991; Carmo, 2005; Franceschinelli *et al.*, 2015). Although most species of *Turraea* are sphingophilous (Yeo, 1993), there is a report of an observation of honey-birds visiting flowers of a species with long gradually widened staminal tube (White & Styles, 1963). Finally, it is important to highlight the parallel evolution of “tubular flowers” within the Meliaceae and Simaroubaceae, although they may be constructed in several different ways as discussed above.

Also, several of the enlarged stigmas of the Meliaceae seem to block the opening of the staminal tube and may even have a role in presentation of pollen (e.g. Pennington & Styles, 1975). Yeo (1993) refers that *Turraea* is the only genus of the family with secondary pollen presentation, with a stigma remarkably modified as a *receptaculum pollinis* of various shapes. White & Styles (1963) observed in living plants of *Turraea* that the pollen is poured over the head of the style before the flower opens (detailed data in Yeo, 1993).

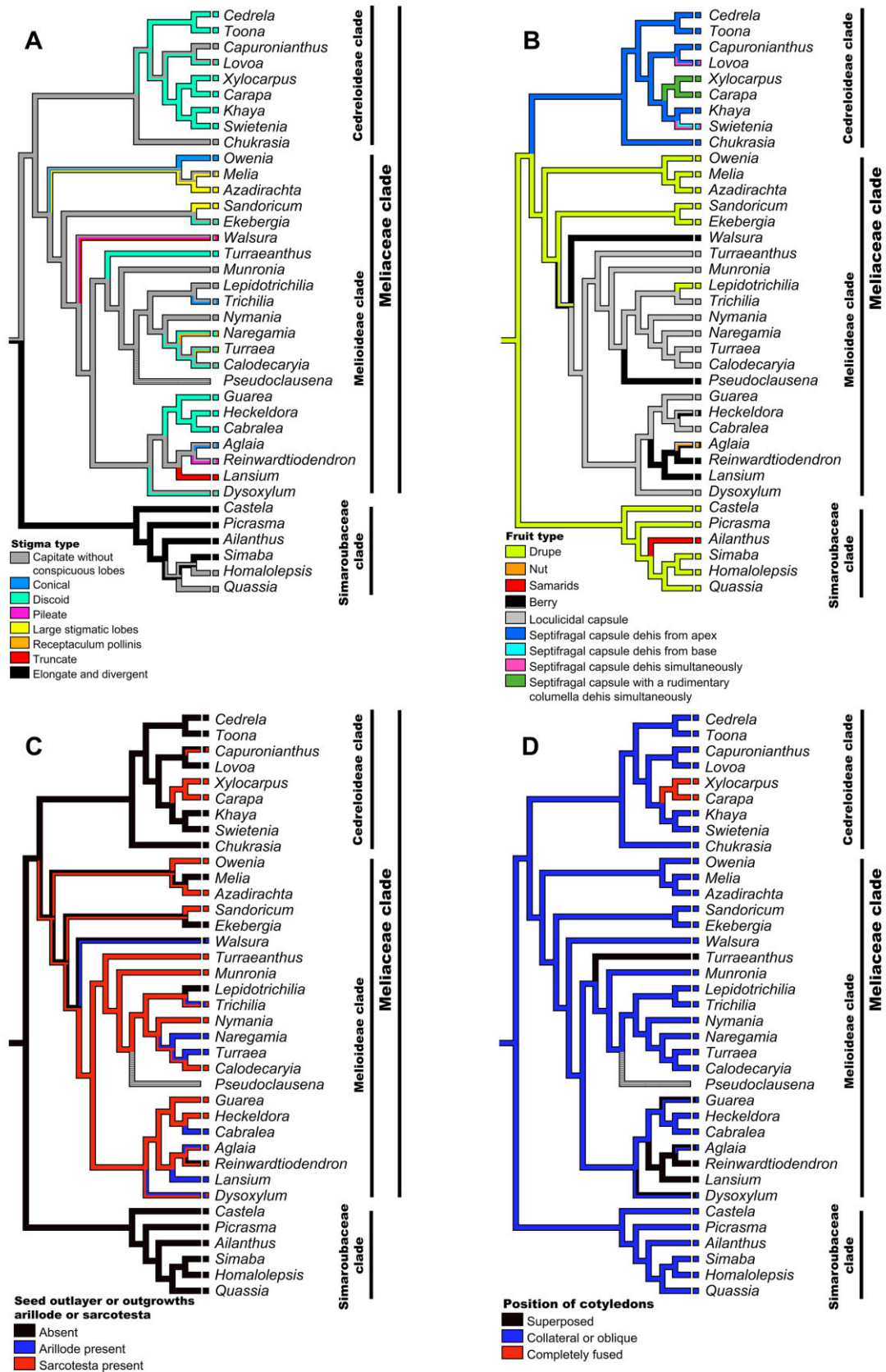


Figure 8. Ancestral character reconstruction for selected morphological characters of Meliaceae and Simaroubaceae. A, Stigma type. B, Fruit type. C, Seed outer layer or outgrowths (arillode or sarcotesta). D, Position of cotyledons.

Dichogamy is likely to occur in such flowers, but this remains to be evaluated. More pollination biology studies are needed to reveal the effective role of the observed structural variations in the floral biology of these plants.

Sexual systems: As Bawa & Beach (1981) stated, “the evolution of a particular sexual system must be influenced by the dynamics of the pollination system”. Thus, the role of pollinators in the evolution of Meliaceae requires further examination of several types of interactions between flowers and pollinators.

The analyses regarding the sexual systems indicate that the ancestor of Meliaceae and Simaroubaceae was possibly a plant that had unisexual flowers in dioecious individuals (Fig. 4A). The distinction between flowers of different sexualities is very subtle in most Meliaceae, in which a pistillode is barely distinguishable from the functional gynoecium, and the staminodes are only slightly smaller than the stamens, while Simaroubaceae usually show a more conspicuous floral dimorphism. Previous authors as Gouvêa *et al.* (2008) pointed out the possibility that functionally either male or female flowers were plesiomorphic traits within Meliaceae. In the ancestor of the Cedreloideae clade there was a unique transition to monoecy. Apparently none of the available works on evolution of sexual systems in flowering plants discuss the rise of monoecious plants from dioecious ancestors (e.g. Bawa & Beach, 1981; Sakai & Weller, 1999; Ainsworth, 2000; Barrett, 2002; Barrett & Hough, 2013). Nonetheless, since all flowers of dioecious Meliaceae usually are provided with both sexual organs, along with the fact that some species are in fact subdioecious and others even polygamous, it seems that these plants probably still maintain the fundamental genetic material which could allow variations in the sexual expression of phenotypes. Therefore, the transition that is likely to have taken place in the ancestor of the Cedreloideae was a reversal to male and female flowers being produced in a single individual. However, even assuming that plants in Cedreloideae are mostly monoecious, an important issue must be pointed out here. Styles (1972) demonstrated that the sex-distribution is not random within the inflorescence of monoecious species of *Cedrela*, *Swietenia* and *Toona*, in

which the central flower of each lateral cymule is female, whereas the lateral flowers are male. Such an arrangement allows a temporal sequence of flowers that become anthetic within the inflorescence or within a plant. For example, Pennington & Muellner (2010) suggest that flowering in *Cedrela* follows the pattern described for species of *Cupania*, Sapindaceae (Bawa 1977) and *Dictyoloma*, Rutaceae (Pirani 1989): each flowering event in a plant involves two phases of anthetic male flowers separated by an intermediate female phase. Hence, though structurally monoecious, these plants in fact behave as xenogamous, due to the temporal separation of male and female phases leading to almost complete outcrossing.

The conservation of parts of the opposite sex in an unisexual flower (staminodes in a female flower; pistillode in a male flower) is observed in several other angiosperm families as well, although usually not quite remarkable as within Meliaceae provided with subtle distinct morphotypes. This feature has been interpreted sometimes as a gradual step of an evolutionary loss still underway during transition from a hermaphrodite ancestor. On the other hand, some authors explain the phenomenon as a result of intraspecific mimicry: it has been suggested that the staminodes would make female and male flowers look alike to pollinators; without anthers the pollinators' visitation rate to female flowers would be very low (e.g. Bawa 1977). According to Dafni (1984) a quite common intraspecific mimicry is observed in plants as papaya (*Carica*, Caricaceae), in which nectarless female flowers attract pollinators by mimicking the nectar-producing male flowers; pollinators keep visiting the female flowers "by mistake", because they anticipate the reward they usually get from male flowers. Nevertheless, Schaefer & Ruxton (2010) critically reviewed the hypotheses of mimicry in plants, by contrasting it with the perceptual exploitation by animals, and they conclude that mimicry remains "contentious in plants". Anyway, these are interesting subjects demanding further studies in Meliaceae, since fundamental aspects involved (as nectar on both male and female flowers or not) are still poorly known in most Meliaceae groups. Secondary sexual characters should also be further investigated in the family, regarding features as inflorescence size, number of flowers produced, sex ratio across populations, among others.

Fruit and seed characters: The most widespread type of fruit found in Meliaceae is the capsule (char.18). As we previously discussed, a septifragal capsule appears as a synapomorphy of the Cedreloideae clade (Fig. 8B), but at this point we emphasize the variation in the dehiscence, which can begin from the base, or from the apex or from the base and apex simultaneously. According to Pennington & Styles (1975), the Cedreloideae septifragal capsules are generally woody and contain a central columella, where the seeds are adhered (Figs. 1E, 8B). In *Xylocarpus* and *Carapa* this columella is rudimentary and vanishes (Mabberley, 2011). The loculicidal capsules appeared once in the Melioideae clade, but they do not occur in all genera of the group. These capsules lack a columella and can be woody, coriaceous, membranous or even fleshy, and they always dehisce from the apex downwards. It is also interesting to point out that even though capsules are usually fruits of the “dry” type, in the Melioideae clade capsules may be fleshy, as seen in all species of *Cabralea* and several ones of *Guarea* (Figs. 1 C, G, H). Pennington & Styles (1975) stressed that further field observations are required to determine the mode and time of dehiscence of several species.

Indehiscent fruits are also widespread in Melioideae. The berries evolved at least four times and occur in genera as *Lansium* and *Aglaia*. Although the distinction between berries and drupes in the family is not accentuated, genera with drupaceous always show some differentiation of the endocarp, as observed in some species of *Lepidotrichilia* and *Sandoricum* (Pennington & Styles, 1975). The nut fruit is only found in a few species of *Aglaia* (Pannell 1992, Fig. 8B).

Regarding the seed coat (char. 19), facing the lack of anatomical and ontogenetic studies, Pannell (1992) proposed to denominate as “aril sensu lato” all fleshy outer seed coats in Meliaceae. Here we follow the definitions by Pennington & Styles (1975), according to whom the sarcotesta is applied to the fleshy part that appears as a localized or general swelling of the seed coat, while arillode the fleshy outgrowth that forms partially or completely free flap of tissue involving some or all of the seed. However, in some cases the distinction is not clear between the two states, and both also are found within a single genus (e.g. the polymorphic *Trichilia*). Gama *et al.* (in prep., see Chapter 1)

demonstrated that seeds of *Trichilia clausenii* are provided with a true aril, developed from a protrusion in the funiculus. Our reconstructions show that an arillode probably evolved at least seven times in the history of the family, while the sarcotesta evolved at least three times. In *Munronia*, the sarcotesta covers the surface of the seed and disintegrates before maturity, leaving the mature seed with a large, hollow and membranous structure, similar to what occurs in *Nymania* (Fig. 8C). In *Ruagea*, the sarcotesta is attached to the chalazal portion of the seed. In species where the arillode or the sarcotesta act as visual attractions for birds or mammals, they are red or orange, contrasting with the seed coat and with the pericarp color (Figs. 1G, 1H, 1I, 1J); in contrast, in the species with indiscriminate fruits, they are always white or colorless (Pennington & Styles, 1975 – Fig. 1K).

Embryos with collateral, oblique cotyledons are the most common in the Meliaceae, being recovered as a plesiomorphic state of character. The completely fused cotyledons are an autapomorphy of the *Xylocarpus* – *Carapa* clade. The superposed cotyledons seem to have appeared at least twice in the family (Fig. 8D).

The evolutionary hypotheses generated here in have to be taken with caution. However, several if not all of them emerge either as patterns to be tested or aspects demanding detailed structural investigation. They also indicate possible evolutionary pathways that the reproductive structures passed through as lineages were diversifying, certainly related to specialization on floral biology and dispersal strategies.

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FINAL CONSIDERATIONS

In this work we performed the analysis on the structure, development and sexual-functional aspects of the flowers of *Trichilia clausenii* C.DC. and *Guarea macrophylla* subsp. *tuberculata* (Vell.) T.D.Penn (Meliaceae, Melioideae), in order to contribute to a better understanding of the floral and evolutionary diversity of the group. We also analyzed some morphological reproductive characters upon the current phylogenetic relationships of Meliaceae, aiming to find probable synapomorphies for some clades of the family, and to evaluate hypotheses about floral evolution and sexual systems in the group.

The comparison between the structures of the two species studied herein, and in relation to other representatives of the family, showed interesting variations in structure and development. We found some novelties, such as the existence in *Trichilia clausenii* of at least two functional floral morphotypes. Pollen viability tests, stigma receptivity, fruit formation, associated to morphoanatomical data, reveal the viability and integrity of the androecium and gynoecium in both floral morphotypes. However, in the female morphotype the anther is sterile due to the presence of the secretion of the tapetum, which forms a mass that adheres to the pollen and prevents its release and eventually causes its sterility. In addition, our pollination tests allow to characterize this species as gynodioecious. The two floral morphotypes found in *T. clausenii* do not differ in relation to their chemical structure, basic structural development and vascularization; they differ only by the external morphology and the concealed pollen grains in the female morphotype. Although sharing several morphological, anatomical and histochemical features, the studied species of *Guarea* and *Trichilia* differ in some characteristics of both the gynoecium and the androecium. Even though there are descriptions in the literature of sexual dimorphism in *Guarea*, we found no dimorphism in the population analyzed herein, since all flowers had fully developed ovules and fully developed pollen grains. Additionally, all individuals presented abundant fruit set during the study, but further detailed pollen viability and reproductive biology studies are still needed for this species.

The evolutionary hypotheses raised on the analysis upon the molecular phylogeny must be taken with caution, but they suggest some macroevolutionary

patterns that should be tested and encourage detailed structural research focused on some particular floral aspects. They also indicate possible evolutionary pathways that the reproductive structures may have undergone related to the specialization in floral biology and strategies of dispersion. In this way, we were able to increase the knowledge about the flowers of Meliaceae, mainly in relation to the structure and development of the gynoecium and androecium of two selected species of *Guarea* and *Trichilia*. Our data contribute to the improvement of the systematics of the family and to the discussions about evolution and diversification of the floral structure and sexuality within the family and in the Sapindales order.

