

Yasmin Vidal Hirao

**Estudos morfológicos e ontogenéticos
com inflorescências e flores de
Lepidagathis Willd. (Acanthaceae)**

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with inflorescences and flowers of
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São Paulo

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Prof. Dr. Diego Demarco
(orientador)

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em uma das minhas primeiras lembranças botânicas,
me ensinou a amar a Natureza.

**Quando eu flor
Quando tu flores
E ela flor
Nós flores seremos
E o mundo florescerá**

Sandra Braconnot

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RESUMO

A filogenia de Acanthaceae, sobretudo baseada em dados moleculares, confirma a monofilia da família, no entanto, ainda não se conhece uma sinapomorfia morfológica que a caracterize. Apesar de estar bem representada em nossa flora, a identificação de suas espécies é dificultada por problemas taxonômicos e pela falta de estudos morfológicos. A família apresenta muitas inconstâncias morfológicas dentro dos gêneros, por exemplo no padrão de inflorescências. *Lepidagathis* Willd. serve como exemplo, apresentando três tipos de inflorescências e morfologias florais divergentes que tem dificultado sua sistemática. Assim, explorando a morfologia, anatomia, vascularização e desenvolvimento das inflorescências e flores, foi possível encontrar homologias entre as espécies estudadas a especular sobre sua evolução. Descobriu-se que os padrões das inflorescências são formas enriquecidas ou escassas da mesma arquitetura. Em algumas espécies, existem mais ou menos meristemas reprodutivos nas axilas das brácteas e, portanto, mais ou menos possibilidades de desenvolvimento de flores e inflorescências parciais. A filogenia disponível para o grupo sugere que há uma probabilidade igual de ganho ou perda dos meristemas reprodutivos nas inflorescências. O desenvolvimento dos verticilos florais nas espécies estudadas é igual, apesar das diferenças na morfologia externa. No entanto, a vascularização revelou aspectos importantes sobre a evolução floral no gênero, mostrando que a anatomia pode reter características ancestrais que relacionam as espécies. Portanto, a disposição e o volume de flores por inflorescência e o tamanho e arranjo das flores tem mais chances de estarem relacionadas com a síndrome de polinização de cada espécie. Os resultados corroboram a circunscrição atual de *Lepidagathis* e encorajam mais investigações com as espécies de Acanthaceae que possam levar a descobertas importantes sobre homologias e ajudar nos estudos filogenéticos com a família.

Palavras-chave: anatomia, Barlerieae, desenvolvimento, evolução, Lamiales, vascularização.

ABSTRACT

The phylogeny of Acanthaceae, chiefly based on molecular data, confirms the monophyly of the family, however, a morphological synapomorphy to characterize it is still unknown. Apart from being well represented in our flora, the identification of its species is quite difficult due to taxonomic problems and lack of morphological studies. The family presents many morphological instabilities within genera, for example with the pattern of inflorescences. *Lepidagathis* Willd. serves as an example, presenting three types of inflorescence and divergent floral morphologies that have challenged its systematics. Therefore, exploring the morphology, anatomy, vascularization and development of the inflorescence and flowers, it was possible to find homologies between the studied species and speculate around its evolution. The patterns of the inflorescence were discovered to be enriched or depleted forms of the same architecture. On some species, there were more or less reproductive meristems on the axil of bracts, and therefore, more or less possibilities of developing flowers or partial inflorescences. The available phylogeny for the group suggests an equal probability of gain or loss of such reproductive meristems on the inflorescences. The development of the floral whorls on the studied species was the same, despite their different external morphology. Nevertheless, the vascularization of the flowers revealed important aspects of the floral evolution of the genus, showing that the anatomy may retain ancestral characteristics that relate the species. Thus, the display and volume of flowers of each inflorescence, and the size and arrangement of the flowers are more likely related to the pollination syndromes of each species. The results corroborate the current circumscription of *Lepidagathis* and encourages further investigations with Acanthaceae species that may lead to interesting discoveries on homologies and assist the phylogenetic studies with the family.

Key words: anatomy, Barlerieae, development, evolution, Lamiales vascularization.

INTRODUCTION

Acanthaceae Juss.

Acanthaceae has approximately 200 genera and over 3500 recognized species distributed throughout the tropics and sub-tropics, rarely reaching the temperate zones (Olmstead, 2012). Around 40 genera and 449 species are estimated to occur with a broad distribution through Brazil, mainly on the Semideciduous Seasonal Forests inland (Profice *et al.*, 2015). However, their natural habitats are in a process of degradation, and, despite the family's abundance in the Brazilian flora, a great number of non-catalogued New World species are endangered (Wasshausen, 1975; Giuliatti *et al.*, 2005, McDade *et al.*, 2008; Wanderley *et al.*, 2009).

The Acanthaceae are herbs, plants with climbing habits, shrubs, often growing extensively tall, and even small trees. The phyllotaxis is mainly decussate, with some species presenting a congest whorled phyllotaxis, and the leaves are simple with entire or, less frequently, toothed margins. The flowers are pentamerous, gamosepalous and gamopetalous, usually with a bilabiate corolla showing different degrees of zygomorphism. The androecium has four functional stamens, or two functional stamens and two staminodes, or, less frequently, a fifth staminode or only two functional stamens. The gynoecium is syncarpic, bicarpellary, superior, the style is terminal, and the ovary is bilocular, each locule with 2-10 ovules attached to the central septum. There is usually an annular nectary around the base of the ovary. The fruit is a loculicidal capsule, or, less often, a berry. Another common feature of these plants is the spike or thyse inflorescence, made by decussate conspicuous bracts and a pair of bracteoles before each flower (Braz *et al.* 2002, Wasshausen & Wood 2004, Souza &

Lorenzi 2012). Other frequent morphological aspects are the presence of cystoliths on vegetative organs (Solereider 1908, Patil & Patil 2011) and the presence of a retinacula (lignified funicule) sustaining the seeds on the explosive capsules (Tieghem 1908, Wortley *et al.* 2005). Both are key features that help identify subfamilies and tribes (Scotland & Vollesen 2000, McDade *et al.* 2008). Since the relations amongst the families of the Lamiales are not yet clearly defined, other families related to Acanthaceae also share these characteristics (Stevens, 2001 onwards; Schäferhoff *et al.* 2010; Refulio-Rodriguez & Olmstead, 2014). Therefore, the family lacks a morphological synapomorphy to characterize it (McDade *et al.* 2012).

Driven by the need for new approaches to resolve the family's and its taxa delimitation, some researchers have been concentrating their analysis on phylogenetic studies based on molecular data. It has helped define and support infra-familial relations (Schwarzbach & McDade 2002, McDade *et al.* 2005, McDade *et al.* 2012) and the monophyly of the family (Scotland *et al.* 1995, McDade & Moody, 1999, Scotland & Vollesen 2000, McDade *et al.* 2008).

The association of the molecular data and studies of the external morphology, like type of corolla aestivation and pollen morphology, have resulted in the recognition of three subfamilies in Acanthaceae (Scotland & Vollesen 2000). Nelsonioideae is the subfamily with more symplesiomorphies that reappear in later groups (Wenk & Daniel 2009). Thunbergioideae has species with climbing habits, flowers with reduced calyx and only two prophylls preceding them, amongst other characteristics which are autapomorphic of this clade (Borg *et al.* 2008, Borg & Schönenberger 2011). Acanthoideae, referred to as Acanthaceae *s.s.*, has as synapomorphy the presence of the retinacula on the explosive capsule (Scotland & Vollesen 2000, McDade *et al.* 2008).

Morphology, anatomy and ontogenetic studies

In Acanthaceae the external morphology of the vegetative organs is highly variable, for instance the size of leaves, which suffer changes according to environmental pressures and chromosome variations (Kameyama, 1995). It is usual that, within species belonging to the same genus, the inflorescences suffer small changes relative to the size of internodes and bracts, creating congest or sparse architectures. Such variations in morphology were also observed among specimens of a single population (Ramsey & Schemske 2002).

Acanthaceae presents a great diversity of inflorescence types, even within a single genus, such as *Lepidagathis* Willd. (Kameyama 2008). Spikes are a common inflorescence structure in the family, and are made of monochasia, usually sessile or sub-sessile, preceded by two small bracteoles and subtended by a conspicuous and colored bract, arranged around an indeterminate main axis (Wasshausen & Wood 2004, Souza & Lorenzi 2012). Eventually, on the axil of the bracteoles, there is the formation of floral buds, evidencing the determinate nature of this unit of the inflorescence (Sell 1969, Moylan *et al.* 2004). This kind of increment on the structure creates a thyrses, as in *L. alopecuroidea*, where the flowers are organized in decussate dichasia around an indeterminate axis.

The basic spike pattern often goes through reduction of flower production, with the extreme form becoming the solitary flower, like in *Thunbergia* Retz. and *Mendoncia* Vell. ex Vand., whose inflorescences are basically a single flower preceded by two prophylls (Schönenberger & Endress 1998). The extreme increase in flower production may also happen, developing complex and, many times, congest inflorescences with repeated units along the axis. The reduction of internodes and great amount of flowers

grant the inflorescence a glomerulate aspect that is difficult to interpret, leading to vague or confuse descriptions, as for *Dicliptera* Juss. (Sell 1969).

When the flowers appear in opposite pairs, 90° across at each node on the axis of the inflorescence, this is considered a decussate spike, like that of *L. diffusa*. However, one of the bracts of the decussate pair might become sterile, characterizing a secundiflorous spike, such as in *L. floribunda*. As in *Lepidagathis*, other non-related genera of Acanthaceae have secundiflorous spikes where the sterile bract suffers a displacement in relation to the flower-bearing, or fertile, bract. Thus, the pairs turn alternate along the inflorescence axis, with the sterile bract of one pair becoming close to the fertile bract of the next pair (Kameyama 2008, Indriunas & Kameyama 2012).

The leaves of Acanthaceae are generally hypostomatic with diacitic stomata and subsidiary cells transverse to the pores. Non glandular and glandular trichomes are always present, and the head of the glandular trichomes is composed by a single or multicellular disk with vertical walls (Ahmad 1978, Patil & Patil 2011, Larcher & Boeger 2006). The xylem rays are narrow, the perforation plates of the vessel elements are simple, and the fibers might be septate and have simple pitting (Solereder, 1908).

The stem wood anatomy of the climbing species of Thunbergioideae is well studied because of the cambial variants. On these plants there are successive cambia, internal phloem, and new vascular bundles appearing at the boarder of the pith (Carlquist 2007, Angyalossy *et al.* 2012). Bicolateral bundles were observed in stems and leaves of species not related to the Thunbergioideae, like the African lineages of the Barlerieae tribe (Solereder 1908, Patil & Patil 2012).

The presence of cystoliths on the epidermis is an important characteristic of Acanthaceae *s.s.*, and was well characterized by Larcher & Boeger (2006) and Patil & Patil (2011, 2012). There are many studies with species used in popular medicine

(Bhogaonkar & Lande 2012) and ornamental species (Zuffellato-Ribas *et al.* 2005). Other studies focus on the tropical species that live under high temperatures, investigating the xeromorphic characteristics (Akhani *et al.* 2008; O'Neill 2010; Muhaidat *et al.* 2012).

Few developmental studies have been published with Acanthaceae, mostly focusing on the corolla ontogeny to resolve taxonomic issues, or its shape, correlating it with the clades evolution and pollination syndromes. Some studies have revealed important aspects to the taxonomy of groups, like the work of McDade & Turner (1997), who studied a group of species of *Aphelandra* R. Br. that present dark spots at the base of the bracts, classified as nectaries. Through anatomical investigations, the authors verified that these nectaries vary in number and size of glands, and thus serve as taxonomic identification characters.

The corolla of mature flowers of Acanthaceae have a constant morphology, apart from a few taxa that are characterized by peculiar floral features. An analysis of the aestivation of the corolla can reveal different patterns that help with classification and delimitation of taxa (Scotland & Endress 1994). Ontogenetic studies evidence evolutionary steps that are different or camouflaged on mature structures. It is the case of the corolla of *Avicennia* L., which is tetramerous at anthesis phase. However, at the beginning of development, five distinct primordia are found on the floral meristem, the upper two, later, connating and growing together (Nadia *et al.* 2013).

Scotland & Vollensen (2000), assisted by molecular biology, conducted one of the first general revisions of the classic phylogeny of Acanthaceae, since the Works of Lindau (1895 *apud* Scotland & Vollesen 2000) and Bremekamp (1965), which were based on corolla aestivation and pollen morphology. The general conclusion is that the

lack of profound morphological studies to elucidate the nature of certain structures makes it difficult to position certain groups, despite the molecular techniques.

The curious inclusion of *Avicennia* in the family, attested by molecular studies, could have been less intriguing if the morphology of this mangrove genus and its sister clade, the Thunbergioideae, were better understood. The construction of the clade was tested by Schwartzbach & McDade (2002), based on three genetic sequences of DNA from the nucleus and chloroplast, and the analysis showed the clade to be well supported. However, the authors point to morphological evidences that would position *Avicennia* closer to the Acanthoideae subfamily. Anatomically, *Avicennia* is recognized by the presence of successive cambia on the stem (Zamski, 1979; Carlquist, 2007), besides other characteristics resulting from its convergent evolution with other woody species from the mangrove. Nevertheless, cambial variants are also present on lianas from the Thunbergioideae subfamily (Angyalossy *et al.* 2012). In this case, ecological and ontogenetic studies can acknowledge which are homologous characteristics, supporting the molecular analysis and requesting new studies with different groups and structures. Following this lead, Borg & Schönenberger (2011) analyzed the floral development in *Avicennia* and the Thunbergioideae. They found many synapomorphies regarding the ovules, and similarities between the androecium and the corolla aestivation that endure the clade. The authors call attention to the fact that other morphological aspects could establish the relations between the taxa, like the inflorescence structure, however the evolution of these traits is not known for the family, and, thus, can guide to misleading conclusions.

An opposite case, the segregation of *Thomandersia* Baill from Acanthaceae, was supported by studies with the fruits development. Wortley *et al.* (2005) verified that the retinacula from the *Thomandersia* fruits are homoplastic to those characteristic of the

Acanthoideae subfamily, presenting an example of parallel evolution within the Lamiales. Therefore, the genus was elevated to the rank of family, as Thomandersiaceae Sreem. Other particular structures have interested new researches, like on the Ruellieae tribe where the connated basal portion of the stamens' filaments create a "filament curtain", partitioning the corolla tube (Manketlow 2000, Moylan *et al.* 2004, Tripp *et al.* 2013). Thus, on account of such discoveries, the knowledge of the morphology and anatomy of Acanthaceae must continue to grow. Analysis that investigate the true nature of structures on this family collaborates with other areas of study, not only for the family, as for the Lamiales.

***Lepidagathis* Willd.**

Lepidagathis has approximately 100 species with pantropical distribution (Durán & Ramírez 2011). In Brazil there are 16 known species, mainly on semideciduous and tropical rain forests on the middle west and southwest of the country (Silva & Nogueira 2012, Profice *et al.* 2015). This genus is located in the Barlerieae lineage, characterized by the quincuncial aestivation of the corolla, inside a clade within the Acanthoideae subfamily that has as sinapomorphy the presence of cystoliths on vegetative organs (Fig. 1) (Scotland & Vollesen 2000; McDade *et al.* 2008).

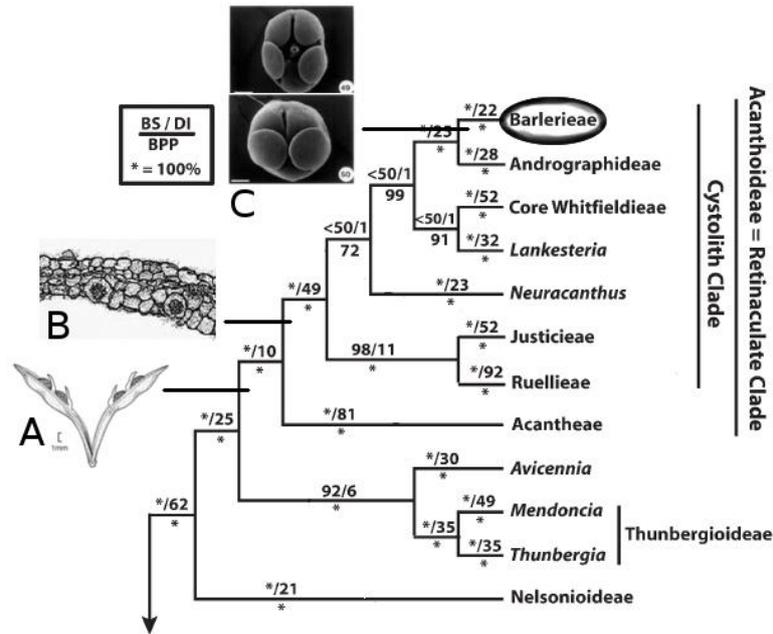


Fig. 1. Simplified phylogenetic tree of Acanthaceae (adapted from McDade *et al.* 2008). (A) Capsule fruit of *Stenostephanus lyman-smithii* Wassh. (Wasshausen & Wood 2004). (B) Cystoliths on the leaf epidermis of *Hemigraphis alternata* (Burm. f.) T. Anderson (Moylan *et al.* 2004). (C) Developing corolla of *Crabbea velutina* S. Moore (Scotland & Endress 1994).

Lepidagathis has four didynamous stamens, the anterior pair bithecous and the posterior pair with monothealous or bithecous anthers, sometimes reduced to staminodes or completely missing. The fruit is a capsule with oblong outline, thin walls and the seeds are recovered by hygroscopic trichomes that expand when hydrated, secreting a mucilaginous substance (Schnepf & Deichgräber 1983, Wasshausen & Wood 2004, Kameyama 2008). In some species, the calyx appears to be tetramerous, with unequal sepals. However, a closer examination reveals that the anterior segment is constituted by the two anterior sepals that are connate with various degrees of fusion amongst the species (Benoist 1911).

The anterior sepals' fusion was used to separate two genera: *Lepidagathis*, with a pentamerous calyx where the anterior sepals were connate only at the base, and species

mainly paleotropical; and *Lophostachys* Pohl, in which the anterior sepals could have more than $\frac{1}{3}$ of its margins connate, so the calyx appeared to be tetramerous, and its species were restricted to the neotropics, with the diversity center in Brazil. Later, based on the different architectures of the inflorescences, about 10 neotropical species were attributed to a new genus *Teliostachya* Nees, with thyrses made by dichasia displayed around an indeterminate axis in opposition to the simple spikes of the other species (Kameyama 2008). Divergences and misinterpretations of the pollen morphology, number of stamens and geographical distribution were also taken in account to separate the three genera, but an analysis of these characters showed them to be too variable. Therefore, Kameyama (2008) put *Lophostachys* in synonymy with *Lepidagathis*. Some authors have been treating *Teliostachya* under *Lepidagathis* as well, but further studies with the development of its inflorescences may reveal and corroborate this inclusion (Wasshausen & Wood 2004; Kameyama 2008).

AIMS

- Analyze the development of the three inflorescence architectures, the fusion of the anterior sepals and the androecium constitution;
- Hypothesize about the patterns of evolution of such traits in the genus, in account of reduction or enrichment of structures.

To achieve the proposed aims, the dissertation was divided into two chapters written in a manuscript format.

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CHAPTER 1

Comparative flower anatomy, ontogeny and vascularization in *Lepidagathis* Willd.

(Acanthaceae): evidences of flower evolution

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ABSTRACT

- *Premise of the study:* Molecular studies have helped to understand the relations within Acanthaceae, although its basic morphology has not yet been properly investigated. As an example, *Lepidagathis* is characterized by different inflorescences, calyx and androecium morphologies. To contribute with the phylogenetic comprehension of the family and its taxa, the present study examined the floral anatomy, ontogeny and vascularization of three species of *Lepidagathis*.
- *Methods:* The selected species were *L. alopecuroidea*, *L. diffusa* and *L. floribunda*. Flowers in different stages of development were analyzed under light microscopy, particularly regarding the vasculature divergences. In addition, meristematic phases were observed on scanning electron microscopy for developmental examinations.
- *Key results:* The vascularization is different for each sepal, resulting on the zygomorphic calyx, while the connation of the anterior sepals is most likely linked with the flowers' size. The staminodes of *L. diffusa* are vascularized, and a fifth trace on the tetramerous androecium of *L. floribunda* was observed vascularizing part of the nectary. Anatomy and ontogeny revealed the carpels are fused by their margins, with the intrusive growth of a false septum constituted of placental tissues dividing the locule.
- *Conclusions:* The vasculature of the calyx and androecium, as well as the development of the sepals and the carpels with its septum constitution demonstrated that the studied species, despite their different external morphologies, share common features in the

internal morphology. This highlights the need to find proper homologies for phylogenetic analysis and, therefore, new morphological studies would be of great value to this family.

Key words: development; false septum; intrusive placenta; sympetaly; staminode; trace.

INTRODUCTION

Acanthaceae Juss. is a family of pantropical distribution, with approximately 200 genera and over 3500 species (Olmstead, 2012). In Brazil, 40 genera and 449 species are recognized (254 of which are endemic), and can be found in all vegetation types of the country, including the mangrove forests (Profice et al., 2015). The species are mainly herbs, shrubs and lianas of great importance constituting the understory vegetation, especially, in the Atlantic Forest (Profice, 2013). The family's phylogenetic analysis, mostly based on molecular data, strongly support its monophyly, although a morphologic synapomorphy characterizing the whole clade remains uncertain (McDade et al., 2008; Tripp and McDade, 2014). Furthermore, the identification of its species becomes intricate due to taxonomic divergences and the lack of studies with micro and macro morphological characters (Wasshausen, 1975; Kiel and McDade, 2014).

Within the Barlerieae tribe, *Lepidagathis* Willd. figures as one of the largest genera, with approximately 100 species of pantropical occurrence (Kameyama, 2008). Placed on an understudied branch of the phylogenetic tree of Acanthaceae, Scotland and Vollesen (2000) proposed the quincuncial aestivation of the corolla as the synapomorphy of this tribe. On phylogenetic analysis of all lineages of Acanthaceae (chiefly based on DNA sequences) by McDade et al. (2008), the monophyly of the Barlerieae is corroborated, but the circumscription of *Lepidagathis*, as known by then, is questioned.

On its prior descriptions, *Lepidagathis* consisted of dry, tropical, Old World species with congest glomerulate inflorescences and calyces of five segments connate only at the base; *Lophostachys* Pohl, a close neotropical genus, was characterized by presenting secundiflorous spikes, and calyces with four segments (Kameyama, 2008). Nees von Esenbeck was the first author to consider these the same genera, but soon after treated them separately (1847), also describing a third genus, *Telyostachya* Nees, characterized by having fascicled inflorescences and five, almost entirely free, segments. The inflorescence and floral morphology of these genera complex only came to be better understood after Benoist's studies (1911). He suggested that the divergent morphological characteristics (calyx composed of four or five segments and type of inflorescence) were merely a misinterpretation. To this author, the number of segments composing the calyx resulted of a longer or shorter conation of the anterior sepals, and the different inflorescence architectures were a simpler or enriched form of the same pattern. Therefore, following Benoist's ideas, Kameyama (2008) combined *Lophostachys* species under *Lepidagathis*, also including those of *Telyostachya* on a broad new circumscription for the genus. Nevertheless, she calls attention for the need of further studies to support or refute the definite inclusion of *Telyostachya* in *Lepidagathis*.

Despite its implications in external morphology, anatomical features are rarely explored under an evolutionary aspect in Acanthaceae (Bhatt et al., 2010). The few hitherto knowledge of anatomy for the family gathers around the vegetative parts: the leaves (mainly emphasizing the presence of cystoliths, which characterizes some clades), and the stem (showing cambial variants on climbing species and on *Avicennia*) (Solereeder, 1908; Zamski, 1979; Patil and Patil, 2011, 2012). Only recently the origin and nature of reproductive structures have been investigated, like the retinacula of *Thomandersia* (a genus elevated to the rank of family), and the structure of the flowers of Thunbergioideae and *Avicennia* (which are part of Acanthaceae

s.l. and show different floral morphologies from those of Acanthaceae s.s.) (Wortley et al., 2005; Borg and Schönenberger, 2011). This new information provided by an anatomical approach emphasizes the importance of ontogenetic studies to understand the evolution, development, and relations amongst the family.

On account of such facts, we studied the anatomical and ontogenetic floral characteristics of some *Lepidagathis*, as to provide morphological information on their possible phylogenetic relations and evidences of floral evolution, thus, setting the basis for new studies with the group.

MATERIALS AND METHODS

The species chosen for this study were: *L. alopecuroidea* (Vahl) R.Br. ex Griseb., a small herb, which presents congest thyrses, and has the anterior sepals shortly connate at the base; *Lepidagathis diffusa* (Nees) Lindau, also a small herb with decussate spikes, and anterior sepals connate to about a third of its length; and *L. floribunda* (Pohl) Kameyama, a shrub that has flowers arranged in secundiflorous spikes, with small green bracts and glaring pink calices, with the anterior sepals connate for about half its length. Specimens of *L. diffusa* were collected in Parque Estadual da Serra do Mar, in Caraguatatuba, SP, Brazil (*Y. V. Hirao et al.* 2, Parque Estadual da Serra do Mar, Caraguatatuba, SP, Brazil); both other species were obtained from Harri Lorenzi's collection of live plants at the Instituto Plantarum, in Nova Odessa, SP, Brazil (*L. diffusa* and *L. floribunda* respectively: *H. Lorenzi 3343*, Reserva Ducke, Manaus, AM, Brazil; and *A. Campos-Rocha 16*, cultivated, Instituto Plantarum, Nova Odessa, SP, Brazil). The vouchers are deposited at SPF herbarium at Universidade de São Paulo.

Inflorescence's apical meristems and flowers in various stages were collected and fixed either in formaldehyde-acetic acid-ethylic alcohol 50% (FAA) for 24 hours (Johansen, 1940) or in buffered neutral formalin (BNF) for 48 hours (Lillie, 1965). After fixation, the material was dehydrated and stored in ethanol 70%.

For the light microscopy analyses, the fixed inflorescences were dissected, removing bracts and bracteoles to isolate flowers in meristematic stages, and the flowers were classified in different stages of development from the meristematic phase with whorls primordia formation to the flower in post-anthesis. Then, these were dehydrated in butyl alcohol series, and embedded in paraffin. The paraffin blocks were sectioned sequentially in transverse and longitudinal planes with a rotary microtome (10-20 μm thick), and stained with astra blue 1% and safranin 1% (Gerlach, 1984). The slides were permanently mounted with synthetic resin, and photomicrographs were taken with a Leica DMLB light microscope.

Scanning electron microscopy (SEM) analyses were made with flowers in pre-anthesis and inflorescence apices fixed in FAA. The inflorescence apices were dissected, removing bracts and bracteoles, in order to expose the meristematic flowers. The material was dehydrated in an ethanol series and critical point dried, mounted on stubs, and coated with gold. The results were observed and images were taken with a Zeiss DSM-940 scanning electron microscope.

RESULTS

Morphology and anatomy—The three studied flowers of *Lepidagathis* are pentamerous and zygomorphic. Their calyces are gamosepalous, fused only at the base, with unequal sepals; and the corollas gamopetalous with a bilabiate tube, the upper lip shallowly bilobate and the lower lip trilobate. The androecium on these flowers is composed of 2-4 functional

stamens, which are epipetalous, connate with the corolla tube, didynamous and antesealous. The fifth stamen, which would be positioned in the middle of the upper corolla lip, is always missing from the external morphology. All species have a nectary disk around the base of the gynoecium, which is composed of two carpels. The ovary has two locules with two stacked ovules each (Fig. 1A–C). The calyces of *L. alopecuroidea* and *L. diffusa* are green and have almost the same size of the bracts and bracteoles, which are also green colored and conspicuous (Fig. 2A). Their corollas are short and white, sometimes with purple spots acting as a nectar guide on the lower lip of *L. alopecuroidea* (Fig. 2B). Distinctively, the flowers of *L. floribunda* are long and the calyx and the corolla are brightly pink colored, so the calyx is more conspicuous than the green bracts and bracteoles (Fig 2C).

Calyx—The anterior sepals may have longer or shorter lengths of connation on this genus. On *L. alopecuroidea* and *L. diffusa* the anterior sepals are shortly fused at the base, just slightly above the brief calyx tube (Fig. 3A). *L. floribunda* has the longer fusion of the studied species, with about $\frac{3}{4}$ of the anterior sepals connate (Fig. 3B). The sepals of *L. alopecuroidea* have an epidermis of cubic to flattened cells on both surfaces. The mesophyll is composed of a chlorenchyma of isodiametric cells filled with raphides. The vascular bundles are positioned closer to the adaxial epidermis (Fig. 3C). *L. diffusa* has a similar anatomy, although the mesophyll has one or two layers of much smaller cells next to the adaxial side, and the occasional presence of a sclerenchyma on the vascular bundles. The anatomy of the sepals of *L. floribunda* differs from the other species due to the presence of a distinctive sclerenchyma forming a sclereids band between the vascular bundles across the mesophyll. This tissue appears on the free portion of the calyx, and expands as a band to interlink the vascular bundles in each sepal (Fig. 3D). Also on *L. alopecuroidea* and *L. floribunda*, from the middle

to the apex of the sepals, it was observed that the mesophyll cells are brachiform, creating pronounced intercellular spaces (Fig. 3E).

Corolla—The corolla of *L. alopecuroidea* has a wider lower lip than that of *L. diffusa*, but the corolla tube of both species are short, with the free labiate portion spreading just after the apex of the sepals (Fig. 2A). The corolla of *L. floribunda*, contrarily, has narrow lips and a slender tube that extends to about twice the length of the sepals (Fig. 2C). The corolla tubes of *L. alopecuroidea* and *L. diffusa* have a similar anatomy (the cells of *L. diffusa* being smaller): an epidermis of slightly elongated cells; a mesophyll of three to five layers of isodiametric ground parenchyma cells with large intercellular spaces; and vascular bundles positioned close to the inner surface of the tube (Fig. 3F). The corolla of *L. floribunda* has from five to ten layers of parenchyma on the mesophyll and the vascular bundles are distributed on the middle of the tissue. The three species have an indumentum composed of non-glandular and short stalked glandular hairs with multicellular secretory heads. On *L. alopecuroidea*, there are sparse non-glandular and glandular hairs on the inside of the tube, starting at the base of the style height until the separation of the corolla lips. On the outside of the lips there are only glandular hairs and on the outside of the lobes there are both types of hairs. *L. diffusa* has non-glandular and less frequent glandular hairs on both sides of the tube (Fig. 3F) and lips, with a denser indumentum on the outside of the lobes. On the inside of the corolla lips, the epidermis is papillate and the contents of the vacuoles are densely stained on anatomical slides (Fig. 3G), which could be evidence of an osmophore. Apparently the flowers do not exhale an odor, but it would be necessary to perform histochemical tests to verify the presence of volatile oils. The corolla of *L. floribunda* has a dense indumentum. There are long and retrorse non-glandular hairs on the inside of the tube, starting after the nectary height, until the middle of the corolla (Fig. 3H). On *L. alopecuroidea* there is a structure similar to a

channel denominated “rugula”, formed by two ridges of parenchyma, on the adaxial side of the upper corolla lip. This structure encloses the style during the flower anthesis (Fig. 3I). On *L. diffusa* (Fig. 3J) and *L. floribunda* (Fig. 3K) it is the lower lip of the corolla that folds itself to form a channel where the style passes through.

Androecium—The morphology of the androecium is highly variable on this genus. On *L. alopecuroidea*, there are four functional stamens inserted on the same height on the corolla tube (Fig. 1A). The posterior stamens are shorter than the anterior ones, the anthers are bithecal and tetrasporangiate, and the thecae divergent (Fig. 4A). On *L. diffusa*, only the two anterior stamens have functional anthers (Fig. 1B). Sometimes, the posterior stamens appear as two inconspicuous staminodes protruding on a lower position, or they do not project and are completely missing from the external morphology of the flower. The functional stamens have bithecal tetrasporangiate anthers, with a wide connective between the divergent thecae (Fig. 4B). On *L. floribunda* the four stamens are functional, the posterior anthers are monotheical bisporangiate and the stamens shorter than the anterior ones, which have bithecal tetrasporangiate anthers with parallel thecae (Fig. 1C, 4C). The stamens of the studied species have cylindrical filaments, with an epidermis and a parenchyma of isodiametric cells. The anthers walls have a single layered epidermis of round to flattened cells and one layer of endothecium cells with lignified secondary wall thickening that installs only on mature anthers of flowers in pre-anthesis (Fig. 4D). On younger anther walls, there is a single middle layer (Fig. 4E) that collapses as soon as the secretory tapetum begins to degenerate and detach from the inner wall of the microsporangia. On *L. floribunda* the epidermal cells of the anthers are bigger than those of the endothecium, and there are pollen sac placentoids projecting from the septum that divides the sporangia (Fig. 4F). The mature pollen grains of the studied species are tricellular and dispersed in monads (Fig. 4G).

Nectary—The nectary disks on these species are cupulate. The epidermal cells range from round to cubic, the cells of the nectariferous parenchyma are isodiametric, and the vascularization is provided by phloem bundles that stop near the top of the nectary. The secretion of the nectar occurs by modified stomata near the apex of the nectary (Fig. 5A). The nectary of *L. alopecuroidea* extends higher than on the other species, to about half the ovary's extent, and the nectary of *L. floribunda* has a denser vascularization and a greater concentration of stomata near its apex.

Gynoecium—The gynoecium, unlike the other features of these flowers, has a constant morphology throughout the studied species. In the three species, it is bicarpelar and syncarpic, with a bilocular superior ovary, one style, and a bilobate stigma with a secretory palisade epidermis. The carpels are fused by the margins, so the ovary is divided into two locules by a false septum made of an intrusive placenta (Fig. 5B). On transverse section, at the middle of the locule height, the carpels are composed of flattened cells on the outer and inner epidermis, one or two layers of isodiametric parenchyma cells on the mesophyll, and two layers of longitudinally elongated cells with a reduced lumen next to the inner epidermis (Fig. 5C). The mesophyll thickens at the connate margins, where the individual ventral bundles are located (Fig. 5B). At this site, the merged placenta projects into the locule, constituted by isodiametric cells and longitudinally elongated cells (transmitting tissue), which happen close to the carpels' margin and at the middle of the placental septum (Fig. 5D). The funiculus are made of a high amount of parenchyma, having a crescent shape and holding the ovules from underneath. One end of the crescent carries the vasculature into the chalaza, and the other is placed under the micropile, where the cells show a secretory activity (Fig. 5D-E). This indicates a funicular obturator, part of the transmitting tissue, which is connected to the

longitudinally elongated cells of the placenta. The styles of the studied *Lepidagathis* have an epidermis and a parenchyma of isodiametric cells. The center of the cylinder is continuing with the transmitting tissue of the placenta, and runs along the entire style, ending, together with the dorsal vascular bundles, just below the secretory stigma (Fig. 5F).

The ovules of the studied species are tenuinucellate and unitegmic. The integument has many layers of parenchyma, filled with raphides, forming a straight micropyle that faces the base of the funiculus below the ovule (Fig. 5G). The ovule is partially inverted, exhibiting an embryo sac conspicuously bended in the middle along both upper and lower sides and a vascularization curved too, characterizing the ovule as ana-amphitropous (Fig. 5H).

Ontogenesis—The studied flowers development begin with the emergence of the sepals primordia on the flower meristem, which is preceded by two bracteoles, in the axil of a bract. The development of the calyx whorl is the most different step in the ontogeny of these species. On *L. alopecuroidea*, five independent primordia appear simultaneously on the periphery of the meristem, but the posterior and anterior sepals' primordia expand much faster than the lateral ones (Fig. 6A-C). As soon as the lateral sepals' primordia begin to expand (Fig. 6D), the calyx segments are joined at their base (Fig. 6E), developing the brief fusion of the anterior sepals and the short calyx tube. On *L. diffusa*, the posterior and anterior sepals' primordia emerge on the floral meristem and expand individually before the lateral primordia appear (Fig. 6H-I). Only then the calyx short tube is formed. On *L. floribunda*, the posterior sepal primordium emerges first and shows a conspicuous growth before the other primordia appear on the meristem (Fig. 6K). The anterior sepals' primordia than emerge apart from each other (Fig. 6L), but soon begin their fused expansion (Fig. 6M). Lastly, the lateral sepals' primordia, which are positioned internally in relation to the others, emerge and expand completing the calyx whorl morphology (Fig. 6N).

The ontogeny of the other flower whorls are similar on all studied species. The corolla begins development with five separate petals primordia arising simultaneously on the borders of the flower meristem, soon followed by the emergence of the stamens' primordia (Fig. 6E). These are still free from the corolla tube, which is beginning to form as the petals' primordia expand. The stamens' primordia expand laterally (Fig. 6F-G), forming the anthers, and then begin to extend to form the filaments, which grow fused with the expanding corolla tube (Fig. 6F,O). The remaining meristem on the center of the flower bud will differentiate into the marginally fused carpels. The meristem gains an annular-oval shape and begin to grow upwards into a tube format, which than closes at the top creating the ovary (Fig. 6F-G). The placenta protrudes from both fused carpel margins into the ovary cavity as the funiculi and ovules are being formed. With the expansion, the placentas meet and merge at the middle of the ovary creating the false septum and a bilocular ovary (Fig. 5B). On developing flowers it is possible to observe that the apical portion of the ovary, where there is no ovule or placenta, is still unilocular. The style and stigma are formed later by elongation of the upper portion of the ovary. The nectaries are the last structures to develop in the flowers, appearing around the base of the gynoecium at the same time as the anther wall layers are formed.

Vascularization—The pedicel of the studied flowers of *Lepidagathis* have an amphiphloic stele with an internal phloem next to the pith (Fig. 7A).

The vascularization of the calyx of the studied species has significant variations amongst them, correspondingly to their external morphology. Some constant characteristics are the collateral arrangement of the traces, the initial divergence of 10 traces and their distribution around the central stele of the receptacle as follows: one small in an anterior position (given out to the anterior segment of the calyx), somewhat flanked by other two traces; in an opposite position to the small trace, there is a solitary and bigger one (which goes

to the posterior sepal); and six opposite traces more or less grouped on lateral positions (Fig. 7B).

On *L. alopecuroidea*, when the 10 traces enter the calyx whorl, the marginal bundles from the lateral groups bifurcate, as the lateral sepals soon begin to separate from the short tube. The small bundle in the middle of the fused anterior segment divides a little way above, when this segment divides (Fig. 7C). On *L. diffusa* every two of the ten traces, which diverged from the receptacle, divide before reaching the connate base of the calyx, including the small trace on the anterior position (Fig. 8A). Thus, the number of traces arriving at the calyx (15 traces) is bigger than the number of gaps left on the stele (10 gaps). At the base of the short calyx tube, the marginal bundles of the lateral sepals divide once again, and the outermost new bundle of each side migrate to the margin of the adjacent sepal, as the lateral sepals separate from the tube (Fig. 8B, C). The calyx vasculature of *L. floribunda* is similar to that of *L. diffusa*, as it also has more traces entering the whorl than gaps left on the receptacle stele, but the traces have a different pattern of branching. At first, the marginal bundles of the lateral groups immediately bifurcate as the ten traces are given out to the calyx. The number of traces that are directly given out from the receptacle can be misleading, due to the great amount of phloem on the stele that conceals the divergence events of the traces. Then, still on the receptacle, the bundle in-between the two anterior segments divide, and, thus, there are 15 independent traces arriving at the calyx. After the separation of the calyx tube from the receptacle, the marginal bundles of the lateral sepals may or may not bifurcate again. If so, the outermost new bundle always migrates to the margin of the adjacent sepal before its separation from the tube, in the same way as in *L. diffusa*.

At the height in which the corolla traces diverge, on *L. alopecuroidea* and *L. diffusa*, five traces are given out to the corolla, alternated with the sepals and are immediately followed by four traces in-between, which will be driven into the epipetalous stamens (Fig.

7C, 8B). The posterior staminodes of *L. diffusa* are vascularized, although externally they appear simply as a small projection (Fig. 8D). On the area where the fifth stamen should be located, opposite to the posterior sepal, there is no vascularization. Unlike the other species, on *L. floribunda* there is a fifth trace diverging together with the androecium vasculature. It can be driven into the nectary, fading into small phloem bundles (Fig. 8E), or act as part of the upper lip vascularization. No staminode was observed on this species. Each stamen, therefore, has one vascular bundle per filament, but it bifurcates at the connective, thus vascularizing each of the anther's thecae individually (Fig. 7I).

The phloem that vascularizes the nectaries of these species comes from both the corolla and the androecium traces (Fig. 7D, 8C, E). The remaining vascular tissue on the receptacle dissect in six traces: two groups of three collateral traces related to each carpel (Fig. 7D). Two bigger bundles derived from the marginal bundles in the lower portion of the ovary proceed to a central position into the intrusive placentas (Fig. 7E, F). The funiculus vasculature is provided by the placental bundles, which end as it enters the last pair of funiculi (Fig. 7G). The marginal bundles also reduce as they pass the top of the ovary, and only the middle dorsal bundles are carried on into the style, fading just below the stigma (Fig. 7H). On *L. floribunda*, the vascular events follow quickly after each other, despite the longer length of its flowers.

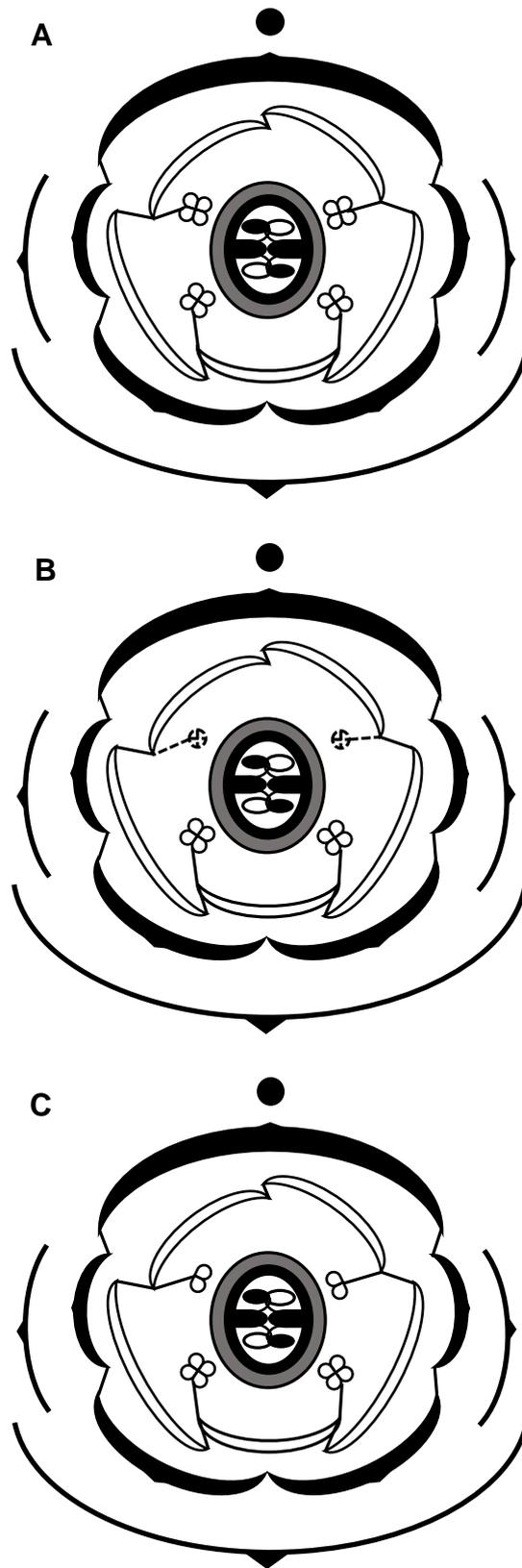


Fig. 1. Floral diagrams with bracts and bracteoles. Note the inner position of the lateral sepals, the quincuncial aestivation of the corolla, and the different number of thecae per anther in each species. Gray circle represents the nectary. (A) *Lepidagathis alopecuroidea*. (B) *L. diffusa*. Dashed lines indicate the possible presence of staminodes. (C) *L. floribunda*.

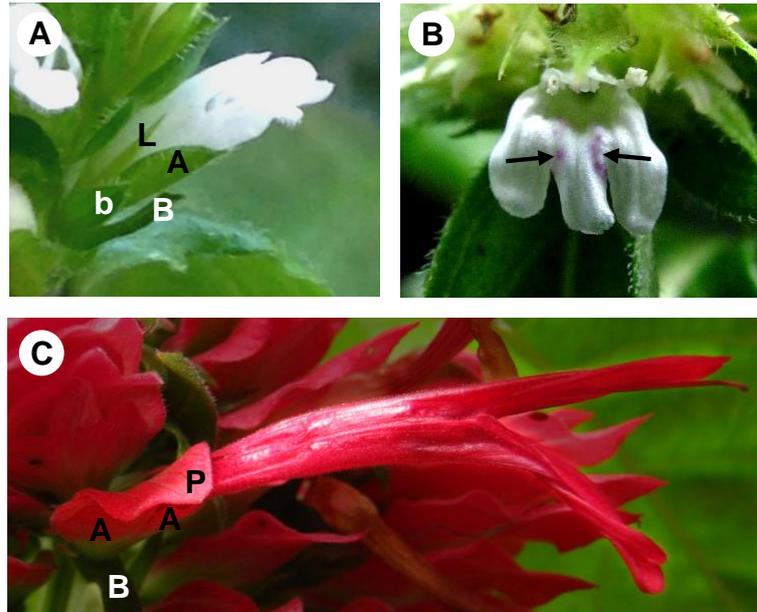


Fig. 2. External morphology. (A) Lateral view of *L. diffusa*. (B) Front view of *L. alopecuroidea*. Arrows indicate the nectar guide on the lower lip of the corolla. (C) Lateral view of *L. floribunda*. Abbreviations: B, bract; b, bracteole; A, anterior sepal; L, lateral sepal; P, posterior sepal.

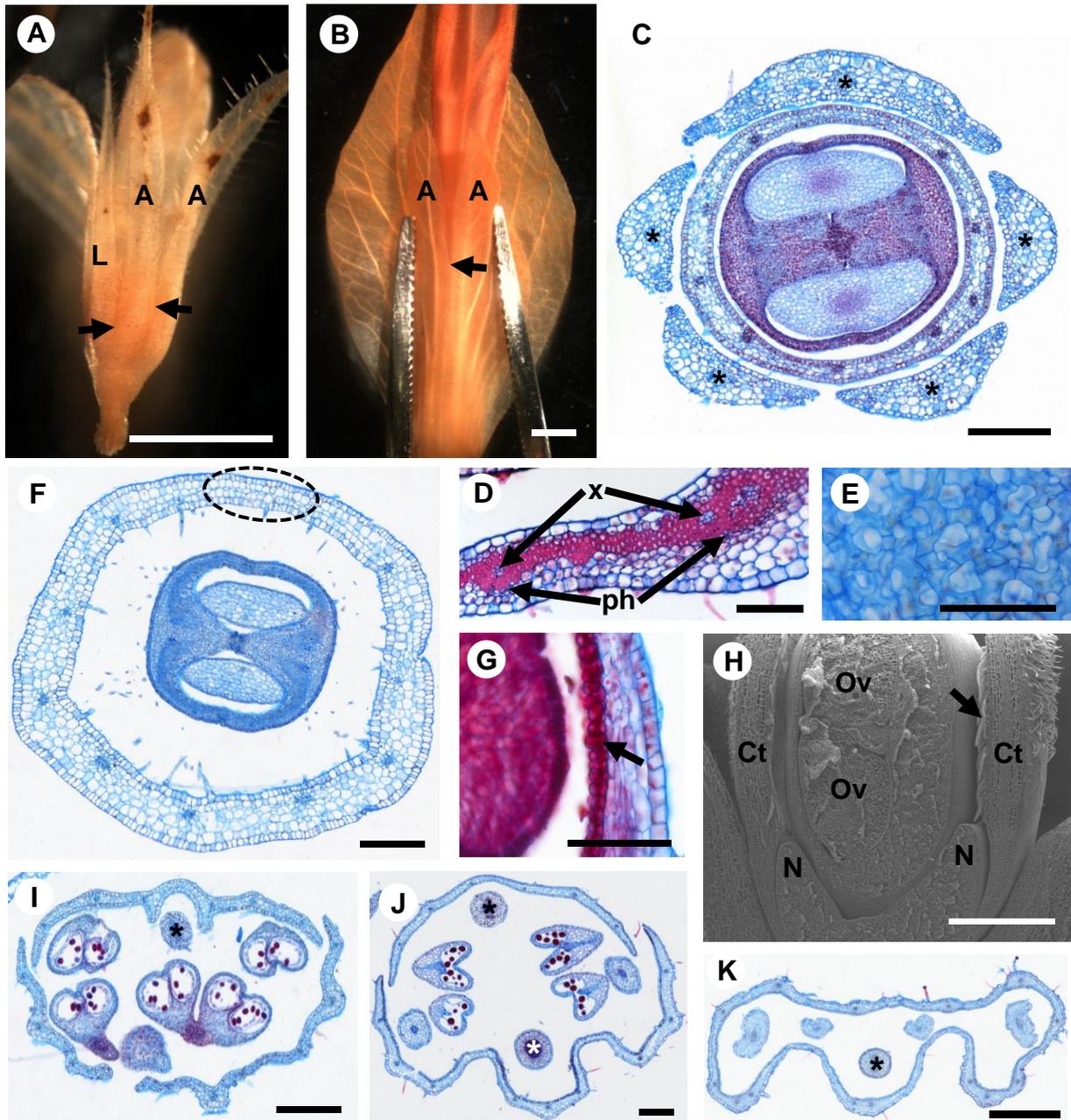


Fig. 3. Calyx and corolla morphology and anatomy. (A, B) Stereomicroscopy images of fixed flower buds. Arrows indicate the beginning of the free portion of the calyx. (A) *L. alopecuroidea*. (B) *L. floribunda*. (C) Transverse section (TS) of flower of *L. alopecuroidea*. Asterisks indicate the middle bundle of each sepal. (D, E) *L. floribunda*. (D) TS of a sepal. Note the sclerenchyma band of cells with lignified walls interlinking the vascular bundles. (E) Paradermal section of a sepal showing brachiform cells and large intercellular spaces on the mesophyll. (F, G) *L. diffusa*. (F) TS of the corolla tube. Note the absence of vasculature on the dashed area, between the upper lobes' vascular bundles. (G) Longitudinal section (LS) of a corolla lobe. Arrow indicates the adaxial papillose epidermis with dense contents on the cells. (H) SEM image of a *L. floribunda* flower sectioned longitudinally. Arrow indicates retrorse trichomes on the inner side of the corolla tube. (I–K) TS of corollas. The upper lip is positioned at the top of the images. Asterisks indicate the style. (I) *L. alopecuroidea*. Note the rugula channel formed by ridges of parenchyma on the upper lip. (J) *L. diffusa*. (K) *L. floribunda*. Abbreviations: ph, phloem; x, xylem; Ct, corolla tube; N, nectary; Ov, ovule. Scale bars: A, B = 2 mm; C, F, I, J = 200 μ m; D, E, G = 100 μ m; H, K = 500 μ m.

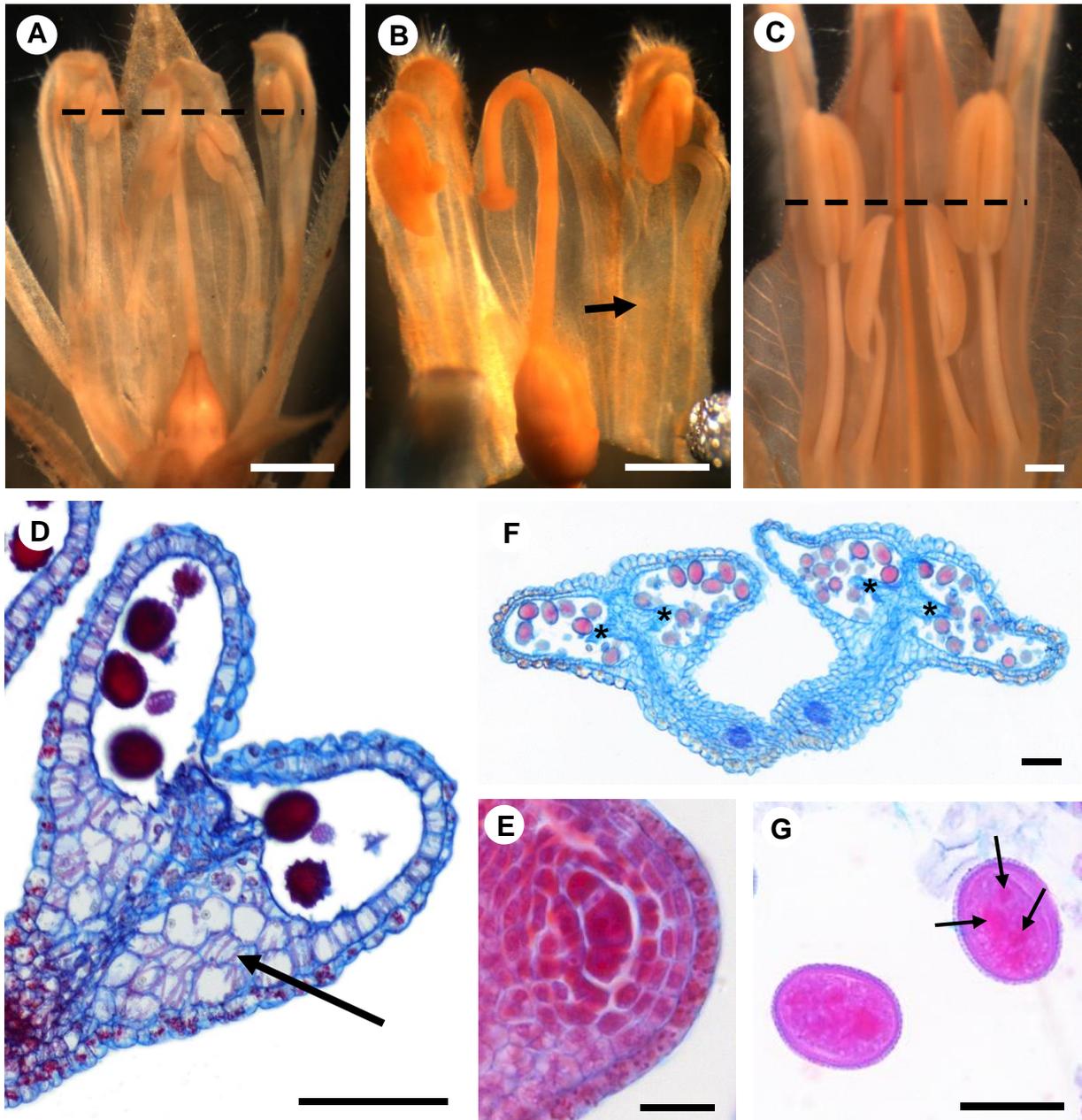


Fig. 4. Androecium morphology and anatomy. (A–C) Stereomicroscopy images of fixed flower buds in pre-anthesis phase. Buds were spread open longitudinally through the lower lip's middle lobe. Dashed lines indicate the posterior stamens height. (A) *L. alopecuroidea*. (B) *L. diffusa*. Arrow points the end of a staminode vasculature which is adnate to the corolla tube. (C) *L. floribunda*. (D, E) TS of anthers of *L. diffusa*. (D) Mature anther. Note the arrow indicating the fibrous thickening also on the connective cells. (E) Young anther going through sporogenesis phase. Note the four layered anther wall. (F) TS of anthers of *L. floribunda*. Asterisks indicate the protruding pollen sac placentoids. Note the vascular bundle of each thecae connective. (G) Tricellular pollen grains of *L. alopecuroidea*. Scale bars: A–C = 1 mm; D, F = 100 μm; E, G = 20 μm.

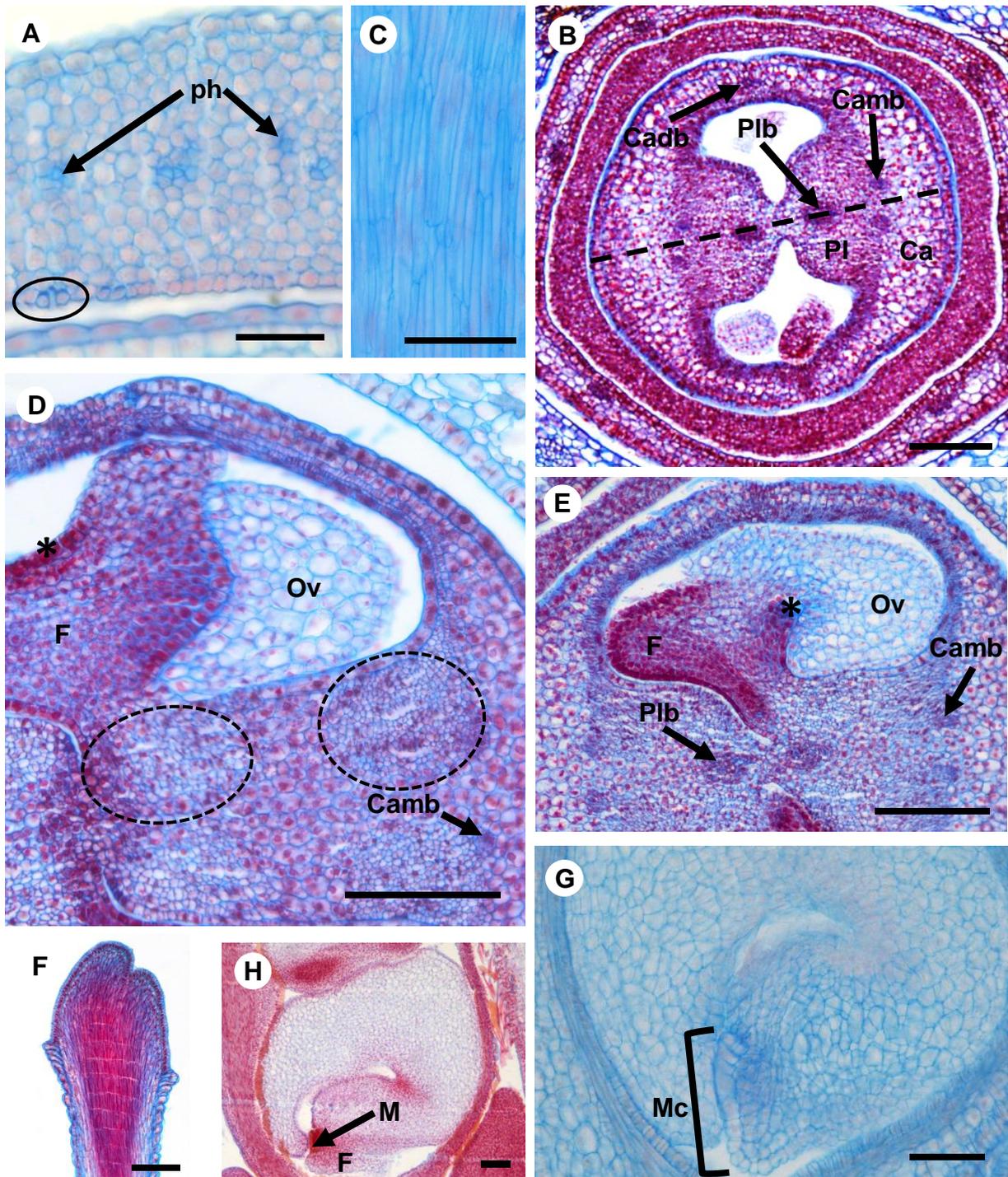


Fig. 5. Nectary, gynoecium and ovules morphology and anatomy. (A) TS of the nectary of *L. floribunda*. Note a circled stomata. (B–E) *L. alopecuroidea*. (B) TS at the base of the ovary height. Dashed line indicates the fusion of the carpels. (C) LS of the longitudinally elongated cells of the placenta. (D, E) TS at the funiculus height. Dashed circles indicate the masses of transmitting tissue in the placenta. Note the strong staining of the epidermal funicular obturator (asterisk) under the micropile. (F) LS of stigma of *L. floribunda* showing the secretory epidermis and transmitting tissue below. (G, H) LS of ovules. (G) *L. alopecuroidea*. Note the micropilar channel formed by the thick integument. (H) *L. floribunda*. Note the curved embryo sac. Abbreviations: Ca, carpel; Cadb, carpel dorsal bundle; Camb, carpel marginal bundle; F, funiculus; M, micropile; Mc, micropilar channel; PI, placenta; Plb, placental bundle. Scale bars: A, C, G = 50 μ m; B, D, E, F, H = 100 μ m.

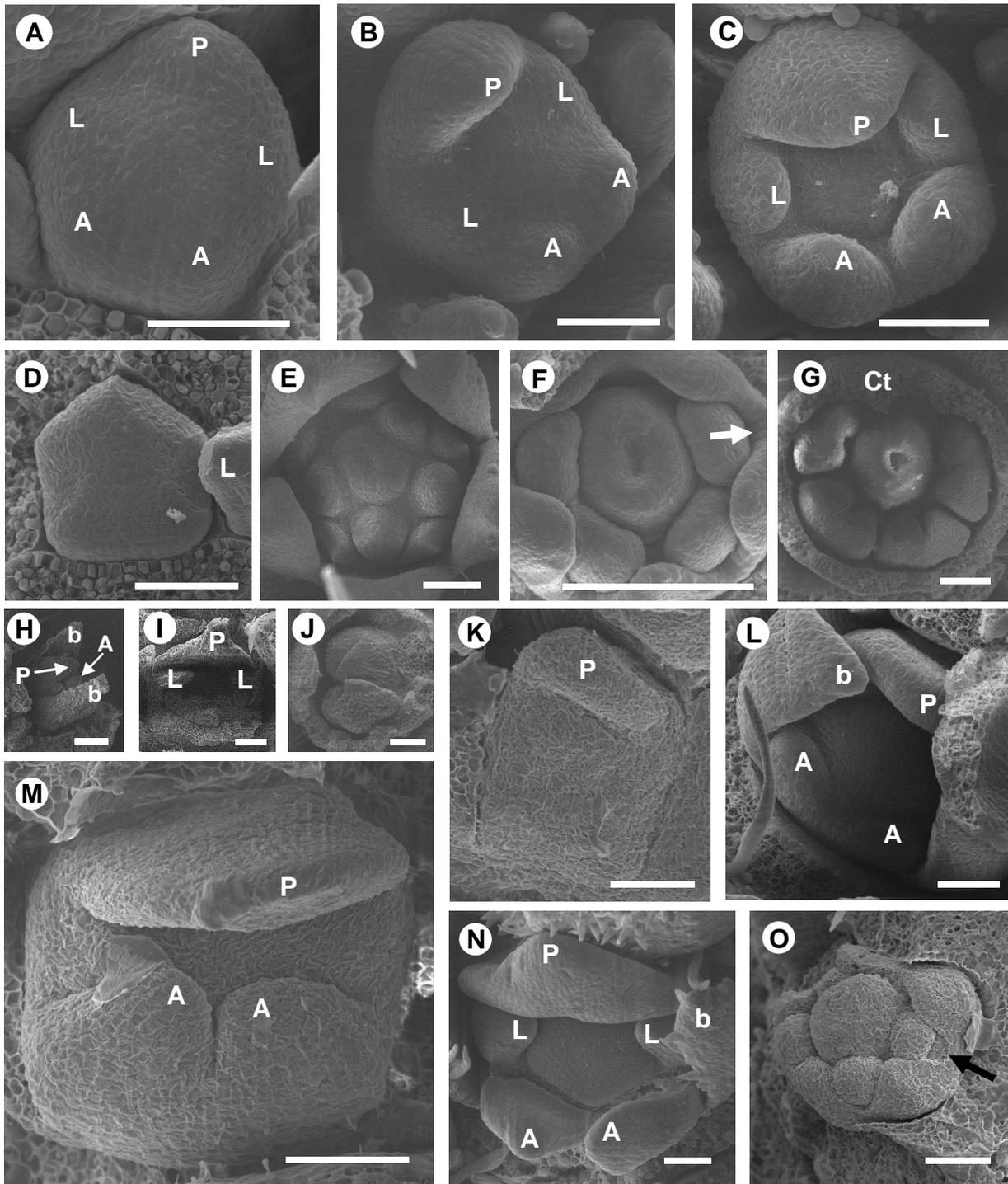
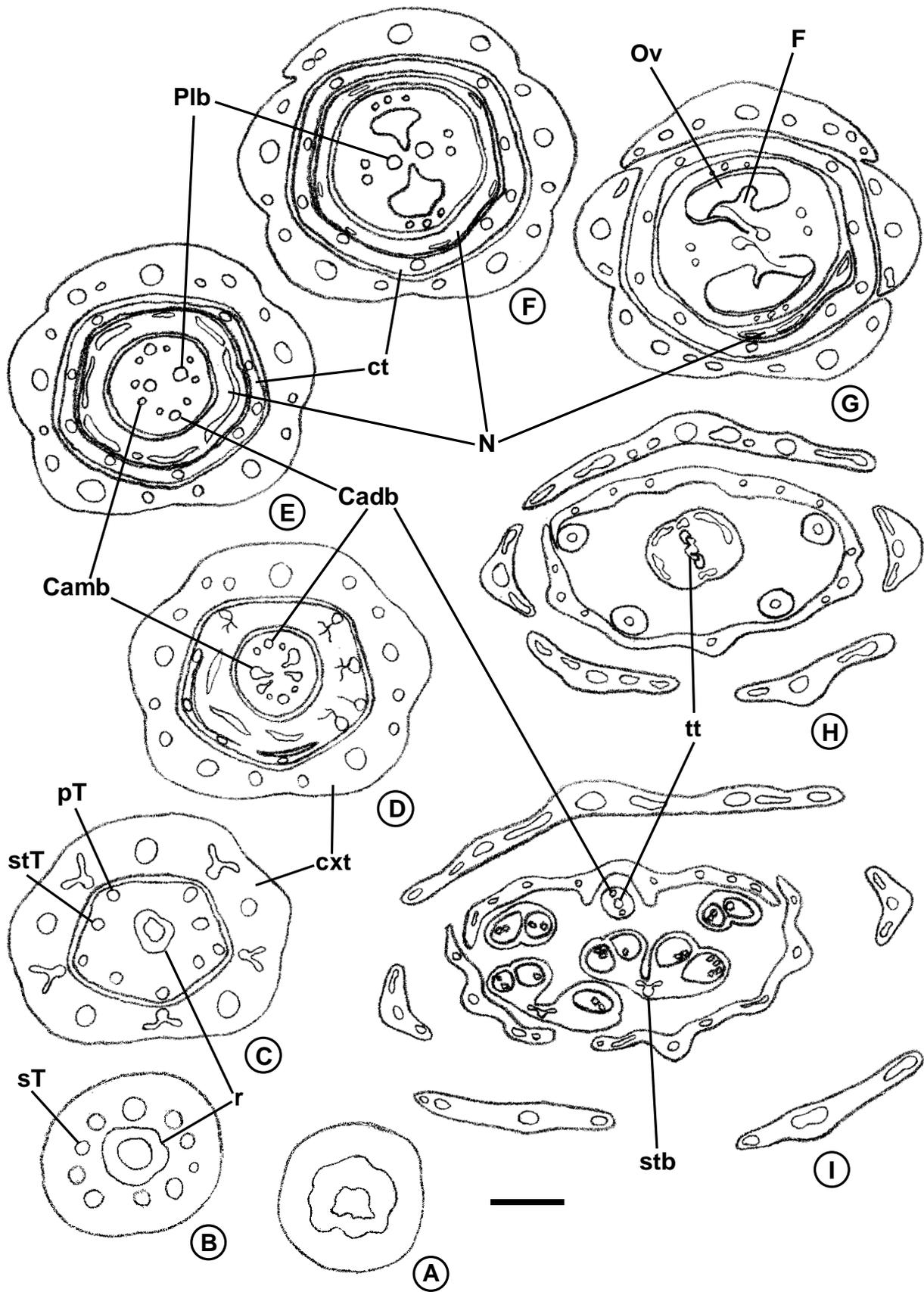


Fig. 6. Flower development. (A–C) Calyx development of *L. alopecuroidea*. Note the primordia are still separate. (D–G) Corolla, androecium and gynoecium development of *L. alopecuroidea*. (D) Calyx has been partially removed. (E) Emergence of the stamen's primordia. (F) Arrow indicates the beginning of the corolla tube. (G) Corolla tube (Ct) has been removed. Note the open tubular expansion of the carpels. (H–J) Flower development of *L. diffusa*. (H) Lateral view. Note the absence of the lateral sepals' primordia. (I) Anterior sepals have been removed. (J) Corolla tube and one stamen have been removed. (K–N) Calyx development of *L. floribunda*. Note the late development of the lateral sepals. (O) Corolla, androecium and gynoecium development of *L. floribunda*. Arrow indicates the fusion of the corolla primordia. Abbreviations: A = anterior sepal; b = bracteole; L = lateral sepal; P = posterior sepal. Scale bars = 50 μ m.

Fig. 7. Vasculature *L. alopecuroidea* from serial transverse sections, upwards from the pedicel. Posterior sepal at the top of the illustrations. (A) Pedicel. (B) Receptacle at divergence of the calyx traces. (C) Receptacle at divergence of the corolla + stamens traces. (D–F) Base of ovary. (G) Middle of ovary. (H) Top of ovary. (I) Middle of anthers. Abbreviations: cxt, calyx tube; pT, petal trace; r, receptacle; sT, sepal trace; stb, stamen bundle; stT, stamen trace; tt, transmitting tissue. Scale bar = 200 μm .



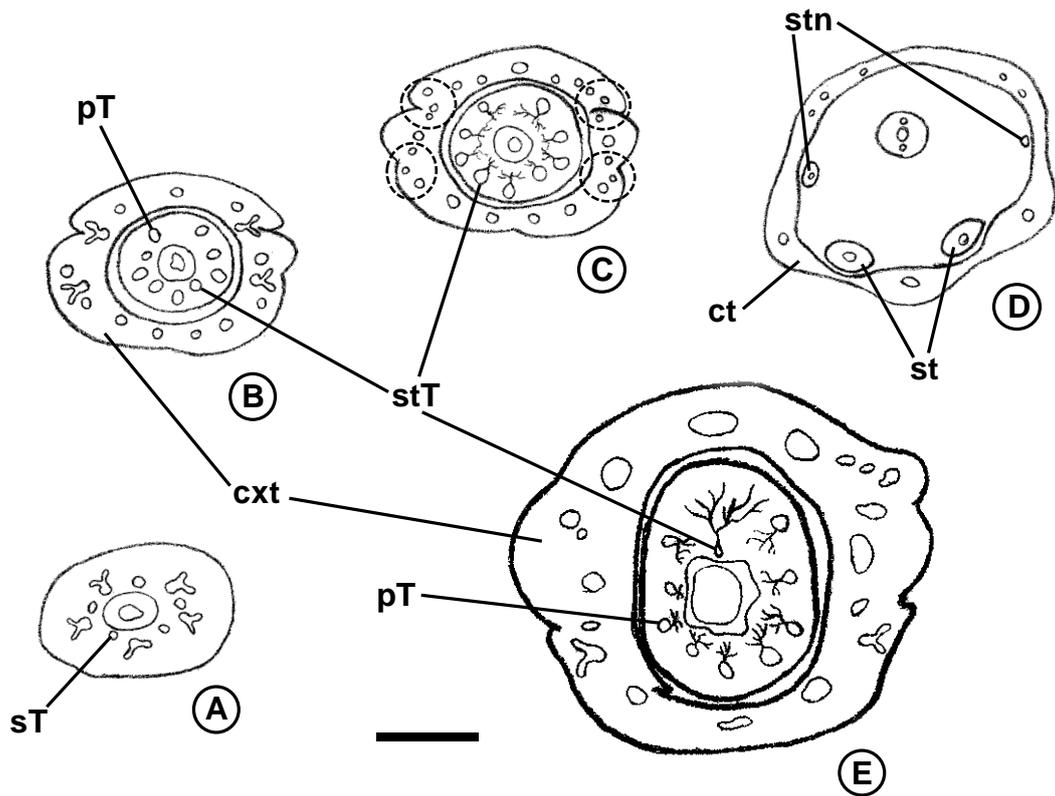


Fig. 8. Vasculature *L. diffusa* and *L. floribunda* from serial transverse sections, upwards from the pedicel. Posterior sepal at the top of the illustrations. (A–D) *L. diffusa*. (A) Receptacle at divergence of calyx traces. (B) Receptacle at divergence of corolla + stamens traces. (C) Receptacle at corolla + stamens traces' divergence to nectary. Dashed circles indicate the bundles resulting from the divergence of the lateral sepals' marginal bundles. (D) Corolla tube at stamens and staminodes' separation. (E) *L. floribunda*. Receptacle at corolla + stamens traces' divergence to nectary. Abbreviations: st, stamen; stn, staminode. Scale bar = 500 μm .

DISCUSSION

Whereas taxonomic works with *Lepidagathis* have placed the three studied species in different genera in the past, the morphological investigations of the present study sustain the recent circumscription proposed by Kameyama (2008). The species share common floral characteristics regarding the histological composition, development of the whorls and vascularization, which emphasize their affinity. The analysis revealed important details on the stamen reduction and the nature of the zygomorphic calyx and the ovary, which contribute to understand the evolution of the group.

Calyx—The anatomy and development of the calyx is similar on the three species. The development of the calyx is initiated by the individual and rapid expansion of the posterior and both anterior sepals, which indicates that the fusion of the five calyx segments is post-genital. In addition, despite the different number of traces vascularizing the sepals on each species, the number of gaps left on the stele is the same. The major differences concern the size of each flower and the color of the sepals. On *L. floribunda*, the largest flower studied, the fusion of the anterior sepals is longer than on the other species, and the volume of tissue constituting the calyx is also bigger.

The explanation to the morphological variations of the calyx in *Lepidagathis*, and the differences between its sepals can be achieved from different points of view. The study of vascularization and ontogeny of these three species revealed changes in the floral development programs that probably led to the current floral morphologies.

The different sizes of the calyx segments may be interpreted as a result of the vascular traces arrangement, divergence and relocation, because the vasculature is less variable than the external parts and it may reveal the former boundaries, relative positions, numbers, and

categories of organs, or their parts, which may now be obscured by reduction, connation, and adnation (Schmid, 1972). On the studied calices, after the bifurcation of half of the original traces, four of them, now associated with the margins of the lateral sepals, bifurcate a second time, as observed on *L. diffusa* and *L. floribunda*. The originated bundles, however, migrate towards the adjacent margins of the other sepals, instead of remaining as part of the lateral sepals' vascularization. Hence, the posterior and anterior sepals are enlarged by being partially vascularized by the lateral sepals. The correlation between the shape of an organ and its vasculature is well-known in many families and large organs will usually have many and highly branched bundles, whereas small organs will have few (Schmid, 1972).

The morphology of the calyx can also be analyzed from a functional evolutionary perspective. Organs that lost their original function may become labile in number and shape because the functional constraints on shape are lacking. Sepals are basically the protective organs in floral buds but, in many groups, the protective function has been evolutionarily transferred from the sepals to another floral or extrafloral organ. In these cases, the sepals have commonly become small and sometimes irregular in shape and number. In Acanthaceae, the basic sepal number is five, however, in Thunbergioideae, the mechanical protective function for the flowers has been transferred to two large prophylls and the sepals are much reduced in size and sometimes completely lost (Endress, 2008).

Corolla—The development of the corolla is the same on the studied species, and the anatomical differences, as observed in the calyx whorl, result from the different size of the flowers. The five petals' primordia emerge simultaneously, although separate, on the floral meristem. Therefore, these species show a post-genital, or late, sympetaly, consistent with the description of the family (Erbar and Leins, 1996; Leins and Erbar, 1997). Each of the corolla

lobes is vascularized by the bundles derived from a single trace, but the corolla tube vasculature comprises the epipetalous stamens bundles, which are fully integrated to its morphology.

The absence of the posterior fifth stamen, especially of its primordia, may have influenced the morphology of the upper lip of the corolla, that has longer fusion between its lobes (Borg and Schönenberger, 2011). The monosymmetry of flowers can be achieved by an elaborate synorganization through the flower's development, or purely by the curvature or simplicity of structures, and it has been associated with the presence of a lower corolla lip and bee pollination (Endress, 2012). Both *L. alopecuroidea* and *L. diffusa* show the morphology of bee-pollinated flowers, with short tubes, wide lower lips and the possible presence of an osmophore, while *L. floribunda* is clearly pollinated by humming-birds, with the long and brightly pink corolla tube (Fenster et al., 2004; Matias and Consolaro, 2015; Silva and Nogueira, 2012). On *L. alopecuroidea*, the rugula creates a channel that guides the style and holds it in an elevated position facilitating the pollination process (Manktelow, 2000; Moylan et al., 2004). On *L. diffusa* and *L. floribunda*, the style also appears to be enclosed by the folding of the lower lip of the corolla, but this position would not assist the pollination purposes, and so these features may not be correlated.

Androecium—The stamens begin their development free from the petals, but soon as the anthers differentiate at the distal portion of the primordia, the filaments grow adnate to the expanding corolla tube. On *L. diffusa*, the two short staminodes are vascularized, therefore the apex of the corolla no longer has their vasculature. On the contrary, the fifth stamen that was observed on *L. floribunda*, when not completely given to the nectary, is continuous with the upper lip vasculature.

The reduction of stamens within a whorl is mainly caused by two factors: zygomorphy and reduction of the number of carpels, both of which are quite common in Lamiales (Borg and

Schönenberger, 2011; Ronse Decraene and Smets, 1995) and evident in *Lepidagathis*. The number of stamens varies within Acanthaceae with records of androecium composed of: (1) four fertile stamens and one posterior staminode; (2) four fertile stamens and one posterior stamen absent (as observed in *L. alopecuroidea* and *L. floribunda*); (3) two lateral anterior stamens and two lateral posterior staminodes (as observed in *L. diffusa*); (4) two lateral anterior stamens (Ronse Decraene and Smets, 1995; Wasshausen & Wood 2004).

Reductions of the androecium linked with zygomorphy are a progressive phenomena and a continued stamen loss occurs in many Lamiaceae and Scrophulariaceae through reduction, usually, of the posterior pair of stamens (Cronquist, 1981; Ronse Decraene and Smets, 1995; Singh and Jain, 1975), as occurred in *L. diffusa*, and by reduction of one theca of each anther (Cantino, 1992), as observed in *L. floribunda*. The influence of the zygomorphy in the suppression of stamens is supported by the presence of radially symmetric flowers with an entire whorl of stamens occurring independently in several lines of Lamiales (Walker-Larsen and Harder, 2000). In addition, peloric mutants of usually zygomorphic species, as *Antirrhinum*, also lose stamen suppression (Coen, 1996; Luo et al., 1996).

In the present species, the ontogeny of the monosymmetric calyx may act as an influence on the development of the androecium. According to the inhibitory field theory (Hofmeister's rule), new organs originate on the floral meristem in the space first available between the already existing organ primordia (Leins and Erbar, 1997). During floral ontogeny, it was observed that on *Lepidagathis* the posterior primordium is the first to grow, and this wide sepal occupies a large space on the meristem. The fifth antesepalous stamen would occupy the position opposite to this sepal, so the unequal origin and size of the sepals may explain the absence in the formation of a stamen in that position.

The second main factor that influences the reduction of stamens is the reduction of the number of carpels in the gynoecium. Apparently, reduction to three or two carpels often induces

a reshaping of the floral symmetry and a loss of some of the stamens; the remaining stamens usually alternate with the carpels and the floral symmetry becomes restored in that way (Ronse Decraene and Smets, 1994, 1995).

The pollen sac placentoids that were observed in the anthers of *L. floribunda* are a common feature amongst the Lamiales and were also observed in immature anthers of other genera of Acanthaceae, possibly being associated with the pollen grains development (Borg and Schönenberger, 2011; Moylan et al., 2004)

Nectary—In *Lepidagathis* the nectary disc around the ovary base, according to the vasculature analysis, is originated from the base of the corolla tube plus androecium, as it is vascularized by both of these whorls. In general, this nectary is interpreted as a gynoecial structure in the members of the order (Kumari, 1986; Nicolson et al., 2007). Other nectaries formed by the androecium have been cited for the family, like the disc in *Thunbergia* that is much more intrastaminal-receptacular than gynoecial (Schönenberger, 1999), and for the order, like the nectary formed by fusion of the basal part of the filaments and the corolla tube in Plantaginaceae, although this is considered an unusual type for the family (Sérsic and Cocucci, 1999). These records and the results observed on *Lepidagathis* demonstrate the importance of further studies to determine the real origin of the floral nectaries in the group.

Gynoecium—The gynoecium of Acanthaceae is described as formed by two congenitally fused carpels, as of most Lamiales (Schwartzbah and McDade, 2002). However, there are different records for the way in which the carpels are connate and for the composition of the septal tissue that creates the ovary locules.

Witztum and Schulgasser (1995) studied the mechanisms of seed expulsion in *Ruellia*, describing the capsule's septum as the connate tissue of two carpels joined along their ventral

surfaces. They divide it into three layers: a resistance layer, closer to the rupture point, and an active layer, connected to the ovary walls, both composed of small diameter fibers, in addition to an intermediate layer, made of parenchymatous cells. Moylan et al. (2004), working with *Strobilanthis*, a genus placed in the same tribe as *Ruellia*, describe it as the fusion of the abaxial surface of the carpels, and also characterizes the septum into three layers: an inner and outer layer composed of vascular tissue, and an intermediate one made of parenchyma. However, these studies are not clear about the ontogeny of the gynoecium.

As observed on the studied species of *Lepidagathis*, the two carpels' primordia arise as a ring, being the result of the growth of the carpels congenitally connate margins. The bilocular division of the ovarian cavity is due to the intrusive growth of the parietal placentas, which form a false septum. This is interpreted from the placental vascular bundles that are derived from the marginal traces of the carpels. The placental bundles enter the funiculi to vascularize the ovules, evolutionarily relocated in a central/axile position.

This interpretation changes the traditional view of the gynoecium formation in the group and has important evolutionary implications. The presence of an intrusive placenta is widespread in some groups and seems to be the plesiomorphic state for Asterales, helping to sustain clades with moderate support in phylogenetic analysis (Lundberg and Bremer, 2003). Unlikely, it is only described for Acanthaceae in few dated papers (Tieghem, 1908; Singh and Jain, 1975). Also, description of ovaries completely or partially subdivided by false septa is not a rarity, occurring in different groups. The best known example occurs in Brassicaceae where the partition wall is derived from the contact of the placentas at a very early stage of ontogeny (Weberling, 1989). On Lamiaceae, the septum that divides the ovary is a combination of parietal placental tissue and a partial septum that arises from the floral apical meristem, in addition to a false septum originated from the lateral walls of the ovary that invaginate into the locules (Sharma and Singh, 19982). New ontogenetic studies are necessary to evaluate the gynoecium

origin in other Acanthaceae and Lamiales to propose a new hypothesis to the evolution of the syncarpic gynoecium in the order.

Although molecular phylogenetic studies within Acanthaceae are solving many of the relations previously cloudy in the family (McDade et al., 2008; Tripp and McDade, 2014), morphological characteristics of the flowers are still underutilized and poorly investigated, regardless of its wide spectrum of states (Moylan et al., 2004). The present work demonstrates the importance of the morphological and ontogenetic studies in understanding floral development and evolutionary changes in developmental programs that result in patterns of zygomorphy, reduction/suppression of stamens, and ovary formation. It was possible to identify that the studied flowers of *Lepidagathis* corroborate the current circumscription of the genus, opening new questions about the floral evolution of the group and the order.

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**Comparative study of inflorescence architectures in *Lepidagathis* Willd.
(Acanthaceae) through the differential meristematic activity**

Yasmin V. Hirao & Diego Demarco

ABSTRACT

- *Premise of the study:* *Lepidagathis* is a genus positioned in an understudied clade of Acanthaceae. Due to its taxonomic history, it comprises different patterns of inflorescences. To aid in the comprehension of the taxa relation, this study explored the ontogeny of such inflorescence architectures.
- *Methods:* The studied species and their respective inflorescence patterns were *L. alopecuroidea* (thyrses), *L. diffusa* (decussate spike), and *L. floribunda* (secundiflorous spike). Apexes of young and mature inflorescences were processed and analyzed under light microscopy and scanning electron microscopy.
- *Key results:* *L. diffusa* has simple decussate spikes with bracts that hold cyme units (a flower preceded by two bracteoles). The cymes' bracteoles do not hold reproductive meristems. On *L. floribunda*, only the fertile bracts of the spikes hold cyme units. No evidence of axillary buds was found on the sterile bracts or bracteoles. On *L. alopecuroidea* however, all bracts and bracteoles subtend buds that replicate the branching pattern, creating lateral cymose inflorescences, or dichasia. These dichasia are arranged around a main racemose axis, characterizing this inflorescence as a thyrses.
- *Conclusions:* The different patterns of inflorescence arise from the presence or absence and differential activity of axillary meristems, although this does not imply in a linear enrichment or impoverishment of the structure. Specific patterns are more likely associated with the pollination syndrome of each species, since the clade in which this

genus is placed also do not follow a linear evolutionary pathway for its inflorescence architectures.

Key words: Acanthaceae; development; inflorescence; meristem; ontogeny.

INTRODUCTION

Inflorescences are important structures to the flowering plants and to the environment, because of their role in how flowers are displayed and presented to pollinators, directly intervening with the plants reproductive success (Wyatt, 1982; Cohen and Shmida, 1993). Due to their often complex and plastic structure, studies with inflorescences were scarce in the past, resuming to classification reviews and the evolutionary affinities between solitary and grouped flowers (Parkin, 1914; Rickett, 1944, 1955; Weberling, 1989).

However, nowadays, this situation has changed and many botanical fields, such as taxonomy, systematics, ecology, and agronomy, acknowledge the importance of inflorescences, addressing the classical questions – evolution, terminology, and function – and seeking for answers through different approaches in developmental studies (Tucker and Grimes, 1999; Kirchoff and Claßen-Bockhoff, 2013). Investigating and understanding the ontogeny of inflorescences, in order to comprehend their different architectures, are the basis for current researches that deal with statistical techniques and the advances in genetic approaches (Prenner et al., 2009; Bull-Hereño and Claßen-Bockhoff, 2013).

The different patterns of mature inflorescences are a result of its apical meristem activity, which produces and arranges the primordia on the inflorescence apex under two principles: space and time (Prusinkiewicz et al., 2007). These attributes undergo a genetic control that works inhibiting or promoting the primordia differentiation and position at the inflorescence apex (Claßen-Bockhoff and Bull-Hereño, 2013). Such molecular characters suffer fewer equivocations of interpretation and, therefore, are broadly used in phylogenetic

studies to understand the relationships between monophyletic groups (Stevens, 2001 onwards). However, most of the DNA sequences used do not have visual phenotypes, so that many families, like Acanthaceae, are known to be monophyletic, but lack a morphologic synapomorphy (McDade et al., 2008).

Acanthaceae has species with significantly different inflorescences concerning its branching and arrangement of flowers (Wasshausen and Wood, 2004). Additionally, other morphologic variations that do not follow a pattern occur within genera, especially in widespread species (Bennett et al., 2008), as well as smaller changes between specimens of the same population, like in the size of bracts and internodes (Ramsey and Schemske, 2002). A good example of such a variation in the family is *Lepidagathis* Willd., which is a genus of pantropical distribution that has different types of indeterminate inflorescences (Kameyama, 2008).

During her taxonomic review, Kameyama (2008) reduced the neotropical species belonging to *Lophostachys* Pohl to synonymy in *Lepidagathis*. The former were distinguished by having a special kind of inflorescence named secundiflorous spike, in opposition to the congest glomerulate inflorescences of *Lepidagathis*, and a different number of segments in the calyx, which proved to be a misinterpretation of morphology, as previously pointed by Benoist (1911). The author also recommended a closely related group of species with racemes of compound cymes (thyrses), known as *Telyostachya* Nees, to be treated under *Lepidagathis*, due to floral morphological affinities. Hence, the genus, as currently recognized, presents three distinct patterns of inflorescences within its species.

The secundiflorous spike is characterized by the absence of flowers in the axil of two adjacent rows of bracts, known as sterile bracts, and is frequent in Acanthaceae (Indriunas and Kameyama 2012). Unlikely, there are no studies about the nature of these bracts and the activity of its axillary buds to clarify the absence of flowers on part of the inflorescence.

Developmental studies are a way of analyzing the affinities between different character states, such as inflorescence branching patterns, and verifying their homologies (Naghiloo et al., 2013). Likewise, studying the internal anatomy of inflorescences can provide new insights to their external arrangement. Plant anatomy is not always associated with its external morphology, e.g. there can be more traces of stamens present at the receptacle than androecium parts on a flower (Puri, 1951).

In that sense, the three different inflorescence architectures present in *Lepidagathis* were examined from early meristematic stages to mature structures, using light microscopy and SEM. The sequence of emergence and disposition of primordia on the inflorescences apex were described and compared, and the results used to discuss probable relationships within the genus, supporting future taxonomic and phylogenetic studies.

MATERIALS AND METHODS

The studied species were: *Lepidagathis alopecuroidea* (Vahl) R.Br. ex Griseb., a pantropical herb of moist disturbed habitats that has indeterminate congest thyrses; *L. diffusa* (Nees) Lindau, a small herb, which grows in the Brazilian Atlantic Forest, and whose inflorescences are simple decussate spikes; and *L. floribunda* (Pohl) Kameyama, a shrub which grows in the semi deciduous forests of Cerrado, and has secundiflorous spikes. The specimens of *L. diffusa* were collected in Parque Estadual da Serra do Mar, in Caraguatatuba, SP, Brazil. *L. alopecuroidea* and *L. floribunda* specimens were obtained from Harri Lorenzi's collection of live plants at the Instituto Plantarum, in Nova Odessa, SP, Brazil. The vouchers specimens were deposited at the SPF herbarium, respectively: *Y. V. Hirao et al.* 2, Parque Estadual da Serra do Mar, Caraguatatuba, SP, Brazil; *H. Lorenzi* 3343, Reserva Ducke,

Manaus, AM, Brazil; *A. Campos-Rocha 16*, cultivated, Instituto Plantarum, Nova Odessa, SP, Brazil.

Inflorescences in different stages of development were collected (mainly meristematic stages, before the elongation of branches, and developed inflorescence apices), and fixed either in formaldehyde-acetic acid-ethylic alcohol 50% (FAA) for 24 hours (Johansen, 1940) or in buffered neutral formalin (BNF) for 48 hours (Lillie, 1965). After fixation, the material was dehydrated and stored in ethanol 70%.

For the light microscopy analyses, the fixed inflorescences were dissected: some of the older bracts and bracteoles were removed to isolate the inflorescence apex and the axillary buds. Next, these were dehydrated in butyl alcohol series, and then embedded in paraffin. Serial transverse and longitudinal sections were made with a rotary microtome (10-20 μm thick) and stained with astra blue 1% and safranine 1%-ethanol 50% (Gerlach, 1984). The slides were permanently mounted with synthetic resin, and photomicrographs were taken using a Leica DMLB light microscope.

For the scanning electron microscopy (SEM) analyses, the inflorescences apices fixed in FAA were dissected in order to expose the meristematic apex and the axillary buds. The samples were dehydrated in an ethanol series and critical point dried, then mounted, and coated with gold. Results were observed and images were taken with a Zeiss DSM-940 Scanning Electron Microscope.

RESULTS

The thyrses of *L. alopecuroidea* are terminal and subterminal compound inflorescences. They consist in approximately 20 decussate pairs of bracts displayed along a simple racemose main axis, with each bract subtending a cymose partial inflorescence on its axil (Fig. 1A, B).

The inflorescences of *L. diffusa* are simple terminal decussate spikes. The main indeterminate axis produces approximately 10 nodes of opposite bracts, each of which, in turn, subtend a pair of bracteoles and a flower (Fig. 1C, D). *L. floribunda* has inflorescences similar to those of *L. diffusa*, which are terminal and lateral secundiflorous spikes. The indeterminate growth of the main axis can produce approximately 30 pairs of bracts on this species, however, not all of them hold bracteoles and flowers on their axils (Fig. 1E, F). Each of the pair's bract has a different outcome on the development of this inflorescence.

The partial inflorescences of *L. alopecuroidea* are dichasial cymes that start as a reproductive meristem, which produces two bracteoles preceding a terminal flower. Each bracteole subtends another reproductive meristem whose activity produces a new pair of bracteoles and a flower, and so on (Fig. 2A). The flowers of the same orders in the dichasia develop in a temporary unequal manner, so they can notably be on different stages (Fig. 2B). Additionally, flowers from higher orders, especially on the most distal dichasia, do not fully develop before the senescence of the inflorescence, perishing in a juvenile phase. The cymose branching is more pronounced on older dichasia, at the proximal region of the inflorescence, which can have fourth, sometimes fifth, order branches on longer inflorescences, assigning an almost conical outline to the thyrses. At the same time, the sessile bracts, bracteoles, and flowers, allied with the not much elongated internodes, cause this inflorescence to appear very congest and agglomerated, making it difficult to understand morphologically (Fig. 2C).

The initial activity of the reproductive meristems of *L. diffusa* (Fig. 2D) and *L. floribunda* (Fig. 2E) is similar to the branching of the dichasial cymes of *L. alopecuroidea*: they produce a pair of bracteoles, before completely differentiating into a flower. These bracteoles, however, do not hold axillary buds, and cannot continue a cymose branching as in *L. alopecuroidea*. This set of bracteoles and a flower do not branch further, so the spikes of *L. diffusa* have a cylindrical outline (Fig. 2F). Although the same happens on the secundiflorous

spikes of *L. floribunda*, their inflorescences are not alike (Fig. 2G). There are two main morphological differences between the spikes of *L. diffusa* and *L. floribunda* that can be promptly noticed: the presence of flowers only on one side of the inflorescence, and the angle formed by the pairs of flowers. Following the ontogenetic sequence, the steps and features that differentiate these inflorescences were identified.

The first event on all the studied inflorescences ontogeny is the emergence of the bracts primordia on the inflorescence apex, and, after a quick expansion of the bracts, the emergence of a reproductive meristem on their axil (Fig. 3A–C). This meristematic tissue will be completely consumed as it originates two bracteoles and then transitions to a floral meristem, which originates a terminal flower (Fig. 3D–F). On *L. alopecuroidea*, the bracteoles also hold a reproductive meristem after their elongation (Fig. 3G), and these reproductive meristems behave likewise, continuing the cymose branching of the dichasia. Thus, each of the terminal flower bracteoles' become the subtending bract of the next order's terminal flower, and so forth. On *L. diffusa*, the bracteoles do not develop reproductive meristems (Fig. 3H), and on *L. floribunda*, apart from the bracteoles not holding reproductive buds (Fig. 3I), there are two rows of bracts on the main axis that also have no axillary buds on their axils (the absence of meristematic cells was also confirmed by longitudinal sequences on anatomical slides).

The secundiflorous spikes of *L. floribunda* than create two sides: an anterior side with two rows of fertile bracts (Fig. 4A, B), and a posterior side with two rows of sterile bracts (Fig. 4, C). It was designated a fertile bract the ones that hold in their axil an axillary bud. Unlikely, the sterile bracts do not evolve an axillary bud at any phase of its development (Fig. 4D).

Scanning electron microscopy images revealed that the pairs of bracts primordia on *L. alopecuroidea* and *L. diffusa* emerge in a decussate manner on the apex of the inflorescences, i.e. in an angle of 90° across from each other, as can also be observed on transverse sequences

on anatomical slides, following the vascular traces that connect the bracts to the axis stele (Fig. 5A–F). In *L. floribunda* the sterile bract emerges ahead of the fertile bract, and has a rapid growth before the next node is set on the apex (Fig. 5G). The pair begins decussate, but the expansion of the sterile bract is conspicuously asymmetric and more emphasized on the posterior side of the spike (Fig. 5H). Therefore, already on the second node from the apex downwards, it can be noted that the sterile bract is curving itself to the anterior side of the spike, embracing the next node's fertile bract, and displacing the pair of bracts to a more acute angle (Fig. 5I). The distinctive growth of these bracts, associated with an oval shape of the inflorescence axis, makes the nodes, not decussate, but “X” shaped in transverse section (Fig. 5J). The compilation of this features characterize the unique outline of the secundiflorous spike of this species.

An arched shape can also be observed on the axis of the inflorescences of *L. floribunda*. At the beginning of the spike development, the fast expansion of the sterile bracts on the posterior side of the inflorescence, bend it forward (Fig. 5K). As the reproductive meristems on the fertile bracts mature, the volume on the anterior side immediately forces the inflorescence to curve backwards (Fig. 5L).

On all species studied, it was observed a phylloscopic accessory bud, i.e. arising on the axil between the inflorescence peduncle and the leaf that subtend it. They develop into a pair of leaf primordia, but remain dormant in this stage until the senescence of the inflorescence, or stop at early stages of bract primordia differentiation (Fig. 6A, 7A–D). On few occasions, it was observed that the accessory bud could mature into a fully developed accessory inflorescence, in case the main inflorescence suffered with an injury.

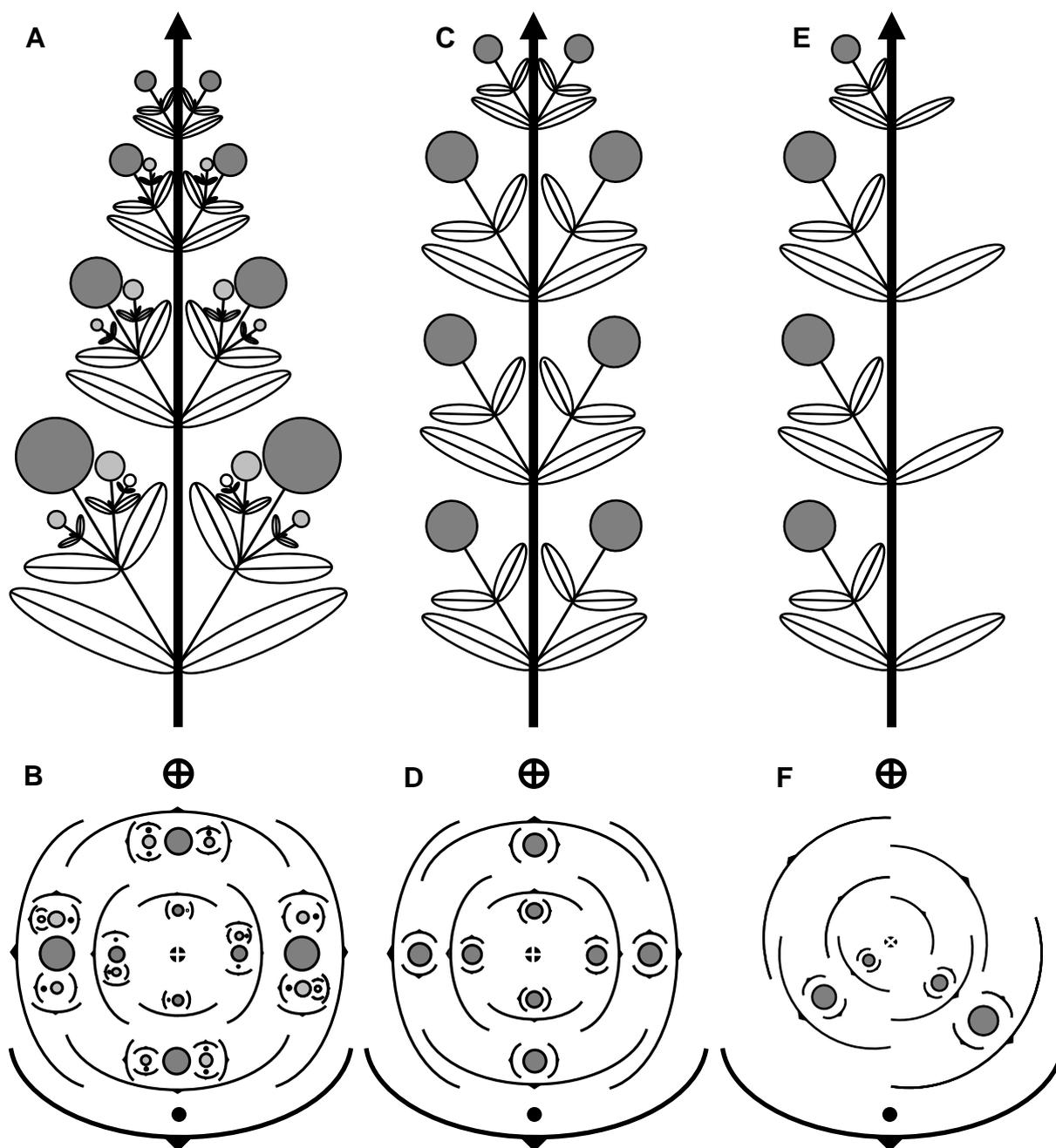


Fig. 1. Inflorescences schemes and diagrams. Peduncles and pedicels are elongated for clarity, all flowers, bracteoles, bracts, and partial inflorescences are sessile. Black dots represent meristems. (A, B) *Lepidagathis alopecuroidea*. (A) Scheme of the entire inflorescence. Note the acropetal enrichment of branching of the partial inflorescences, which follow the thyrsoid development. (B) Thyrsoid diagram. Note the decussate arrangement of the bracts on the main axis, as well as inside the partial inflorescences. (C, D) *L. diffusa*. (C) Scheme of the entire inflorescence. (D) Decussate spike diagram. Note the decussate symmetry of the spike and the absence of axillary meristems on the bracteoles. (E, F) *L. floribunda*. (E) Scheme of the entire inflorescence. Note the complete absence of flowers and meristems on the axil of one bract of the pair (sterile bract). (F) Secundiflorous spike diagram. Note the displacement of the nodes around the main axis. Note also the conformation of the inflorescence, with two rows of sterile bracts on the posterior side, next to the inflorescence peduncle, and two rows of fertile bracts on the anterior side, next to the inflorescence subtending bract.

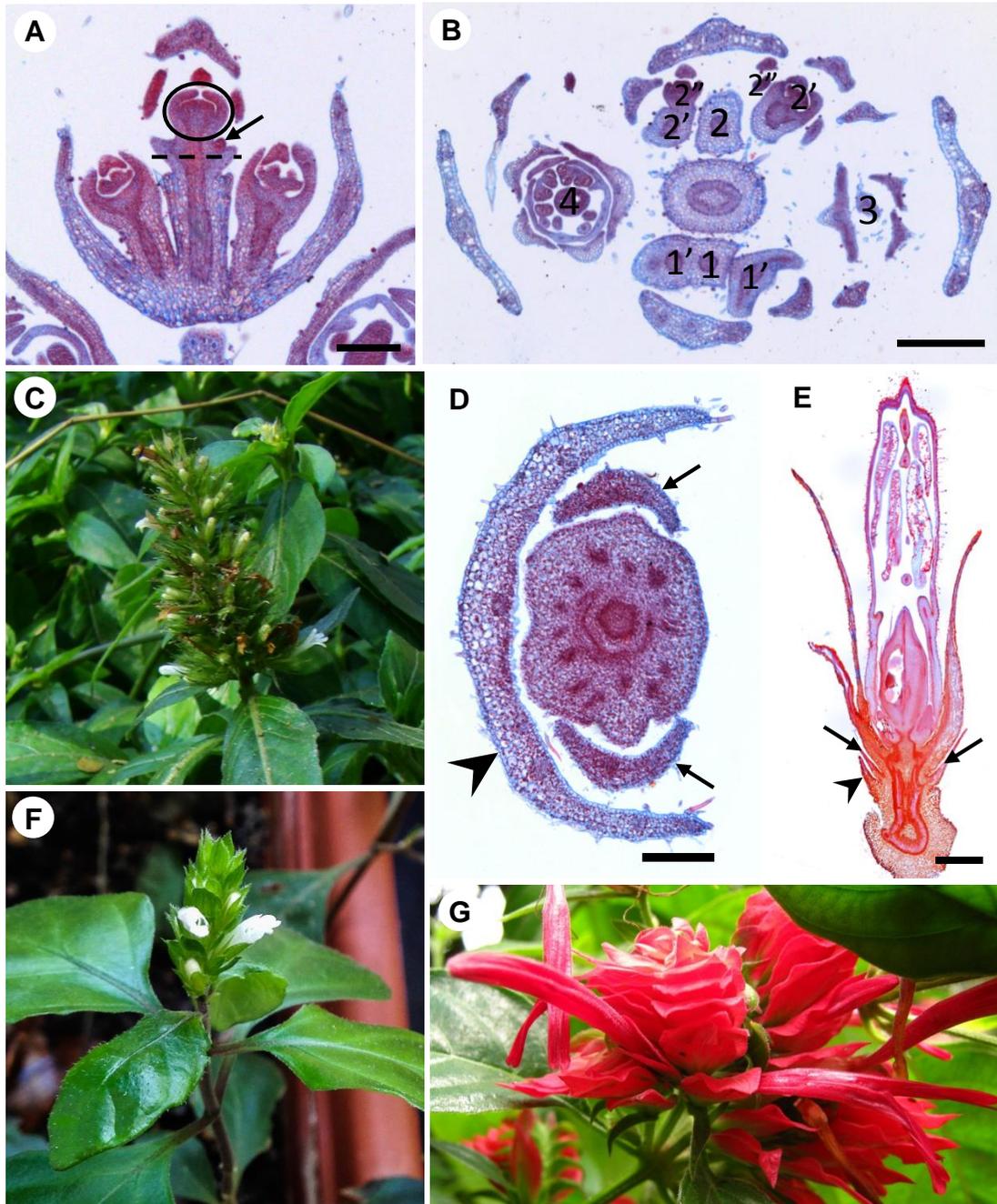


Fig. 2. Composition and architecture of the inflorescences. (A–C) *L. alopecuroidea*. (A) Longitudinal section (LS) of a dichasial cyme. Circle comprises the terminal flower, dashed line indicates bracteoles insertion, arrow points to a reproductive meristem (RM) on the axil of a bracteole. (B) Transverse section (TS) of the thyrses. Dichasia are numbered from youngest to oldest. Within the dichasia, orders are numbered from lower to higher. Note how each bracteole associates itself with the next orders flower. (C) Photo of a thyrses. Note the congest appearance of the inflorescence and its conical outline caused by the development of higher orders on the proximal dichasia. (D, E) Anatomy of cymose units. Arrows indicate bracteoles, arrow heads indicate bracts. (D) TS of *L. diffusa*. (E) LS of *L. floribunda*. (F) Photo of a *L. diffusa* decussate spike. Note the cylindrical outline of the inflorescence. (G) Photo of a *L. floribunda* secundiflorous spike. Note the tubular flowers enclosed by pink leafy calyx segments. The bracts and bracteoles are green and less conspicuous. Scale bars = 200 μm , except E = 1 mm.

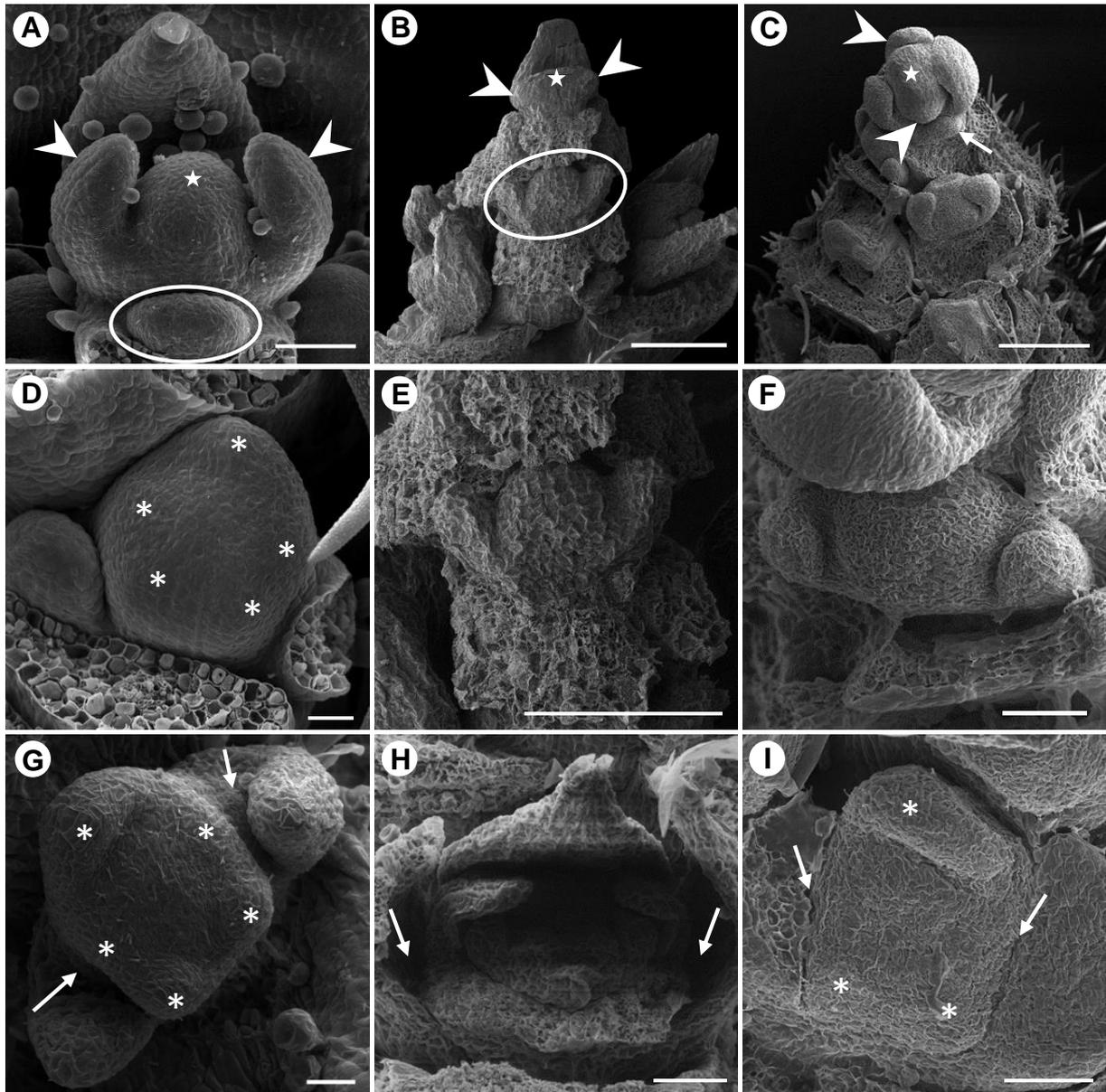


Fig. 3. Development of the inflorescences. Note that many of the inflorescence bracts have been dissected. (A–C) Inflorescences apex. Stars indicate inflorescence’s apical meristems, arrow heads indicate newest pairs of bracts primordia, circles comprise RMs. (A) *L. alopecuroidea*. RM on the axil of a dissected bract. (B) *L. diffusa*. RM with two bracteoles primordia. (C) *L. floribunda*. RM (arrow) on the axil of a dissected bract. (D–I) RM development. Arrows indicate bracteoles axils, asterisks indicate calyx segments primordia. (D) *L. alopecuroidea*. One of the bracteoles primordia has been removed. Note that the RM is already completely differentiated into a floral meristem (FM), with emerging calyx primordia. (E) *L. diffusa*. Detail of RM. (F) *L. floribunda*. Transition of a RM to an FM: expansion of the meristem surface indicates the beginning of calyx segments emergence. (G) *L. alopecuroidea*. Note the RMs on the axil of the bracteoles primordia. (H) *L. diffusa*. Flower with expanding calyx (the anterior segments have been removed) Note the absence of RMs. (I) *L. floribunda*. Note the absence of RMs. Scale bars: A, F, H, I = 50 μ m; B, E = 100 μ m; C = 200 μ m; D, G, = 20 μ m.

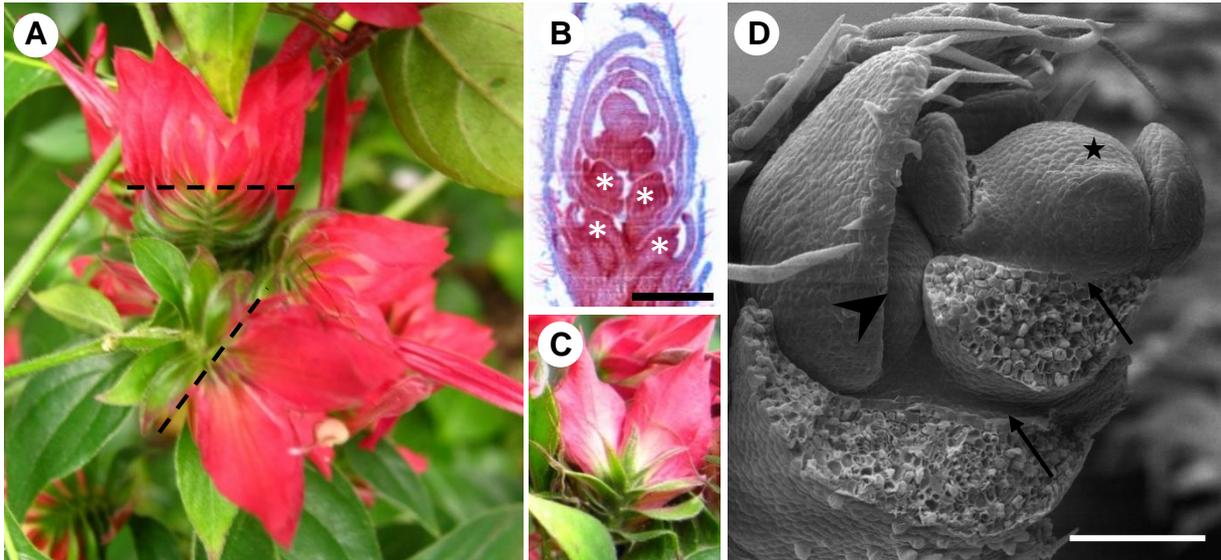


Fig. 4. Structure of the secundiflorous spike of *L. floribunda*. (A) Apical view photo of some inflorescences. Dashed line separates the anterior side (distinguished by the presence of two rows of pink calices), from the posterior side (consisting of two rows of sterile green bracts). (B) LS cutting through FMs (asterisks), present on the anterior side of the inflorescence apex. (C) Back view photo of an inflorescence. Note the two rows of green sterile bracts. (D) Back view of an inflorescence apex. Star indicates inflorescence apical meristem, arrows indicate sterile bracts axil. Note the presence of a RM (arrow head) on a fertile bract axil on the anterior side of the inflorescence. SBs have been dissected. Scale bars: B = 500 μm ; D = 100 μm .

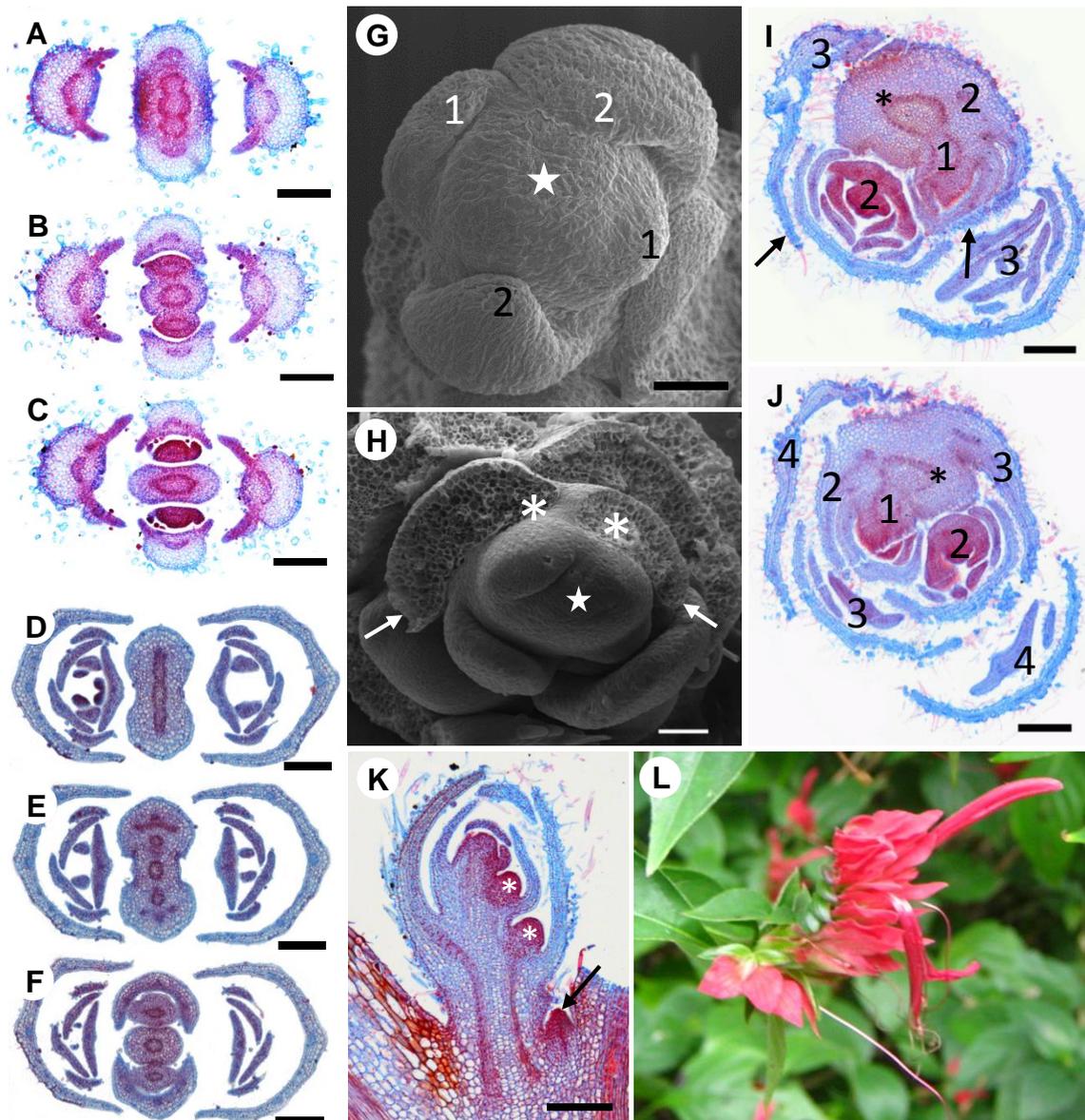


Fig. 5. Bracts development and arrangement. (A–F) TS in acropetal sequence of inflorescences. Note the decussate arrangement of the nodes around the inflorescences main axis. (A–C) *L. alopecuroidea*. (D–F) *L. diffusa*. (G–J) Fertile bracts (FB) and sterile bracts (SB) in *L. floribunda*. (G, H) Apical view of secundiflorous spikes apex. Star indicates inflorescence apical meristem. (G) Note the pronounced growth of the SBs (white numbers) over the FBs (black numbers) of the same nodes. (H) Note how the margins of the SBs have a free portion (arrow), next to the FBs rows, and another (asterisk) adnate to the inflorescence axis. SBs have been removed. (I, J) TS of inflorescence apex. Nodes are numbered from newest to oldest, asterisks indicate the connection between the stele and the last SB trace (not shown). (I) Note the free margin of the SBs (arrow) embracing the upper node's FB. (J) Note how the SBs appear to be oblique, in view of the FBs, demonstrating its asymmetrical growth. (K, L) Secundiflorous spike curvature in *L. floribunda*. (K) LS of inflorescence in meristematic stage. Asterisks indicate RM on the axil of FB. Note the apex curved towards the anterior side of the inflorescence, and an accessory bud (arrow) between the inflorescence peduncle and its subtending leaf. (L) Photo of mature inflorescence. Note that now the spike is bended backwards, exposing the tubular flowers from the calyx involucre. Scale bars = 200 μm , except G, H = 50 μm .

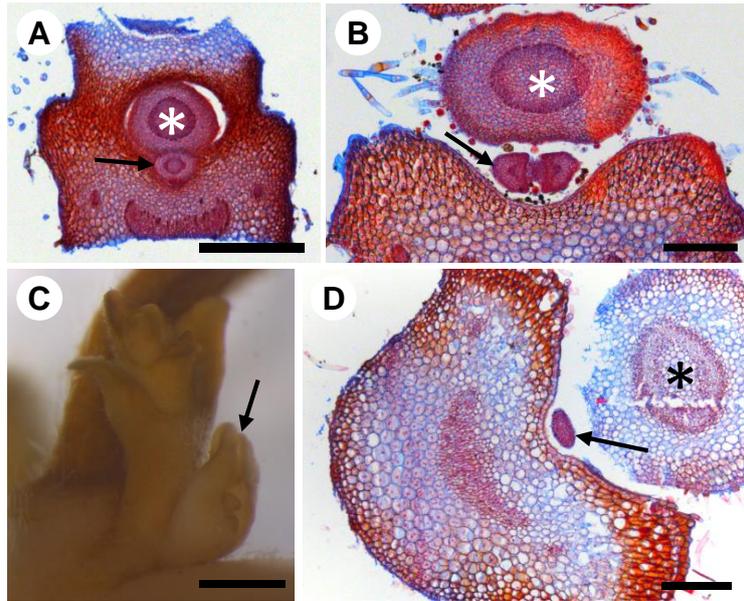


Fig. 6. Accessory buds (arrows). Note their phylloscopic position. (A, B) *L. alopecuroidea*. TS acropetal sequence at the base of the thyrses peduncle (asterisk). Note the two bract primordia enclosing the meristem on B. (C) *L. diffusa*. Stereomicroscope image of meristematic spike with accessory meristem. Note how the bracts in the accessory meristem are closed around its apex. (D) *L. floribunda*. Note the position of the accessory bud between the secundiflorous spike peduncle (asterisk) and the inflorescence subtending leaf. Scale bars: A = 500 μm ; B, D = 200 μm ; C = 1 mm.

DISCUSSION

The great majority of Acanthaceae species have flowers preceded by two bracteoles disposed in inflorescences that have been referred to as spikes or racemes (Nees, 1847a, 1847b; Wasshausen and Wood, 2004), or yet as cymes that have been reduced to spikes (Wasshausen and Smith, 1969). The flowers and bracteoles subtended by each bract are a product of the same reproductive meristem, i.e. they share a common origin, and are the smallest flowering unit of these inflorescences. On the three studied species of *Lepidagathis*, the reproductive meristems on the axil of bracts have the same developing activity, being responsible for the production of two bracteoles and then transitioning into a flower meristem. Considering *L. alopecuroidea*, the reproductive meristems, apart from producing a pair of bracteoles and then a terminal flower, also contribute to the appearance of new reproductive meristems on the bracteoles axils. On both other studied species, there are less reproductive meristems, and hence, a reduced possibility of branching for the inflorescences. However, the inflorescence branching pattern of *L. alopecuroidea* indicates that a more enriched branching is possible for the flowering units of the other species as well.

In the revision of *Barleria* (a genus from the *Barleria* clade that is sister to the *Lepidagathis* clade, forming the Barlerieae tribe), Balkwill and Balkwill (1997) discuss the mainly cymose pattern of its inflorescences. There are many species that present solitary flowers as a reduction, although the authors point out that the inflorescence in this genus may be otherwise interpreted, due to various definitions given by different authors. Sister to the Barlerieae tribe is the Andrographidieae tribe, characterized by genera with flowers mostly displayed in racemose inflorescences and panicles; and sister to [Andrographidieae + Barlerieae] is the Whitfieldieae tribe, with mostly thyrsoid inflorescences. Therefore, based on the latest phylogenetic tree presented to the family by Tripp and McDade (2014), it can be

proposed that the plesiomorphic state of the inflorescence for the Barlerieae tribe was probably a raceme, an indeterminate main axis with a variable number of second order axes with no further branching (Fig. 7). From this state, the inflorescences' architecture took divergent paths. In the *Barleria* clade, there was a transition of the apical meristem from its indeterminate growth to a floral meristem (cymose pattern), and a subsequent reduction of axillary buds development and growth of the inflorescence (solitary flowers). On the *Lepidagathis* clade there were two opposite events, an increase (*L. alopecuroidea*) and a reduction (*L. floribunda*) of axillary buds development, but the indeterminate inflorescence apex was maintained.

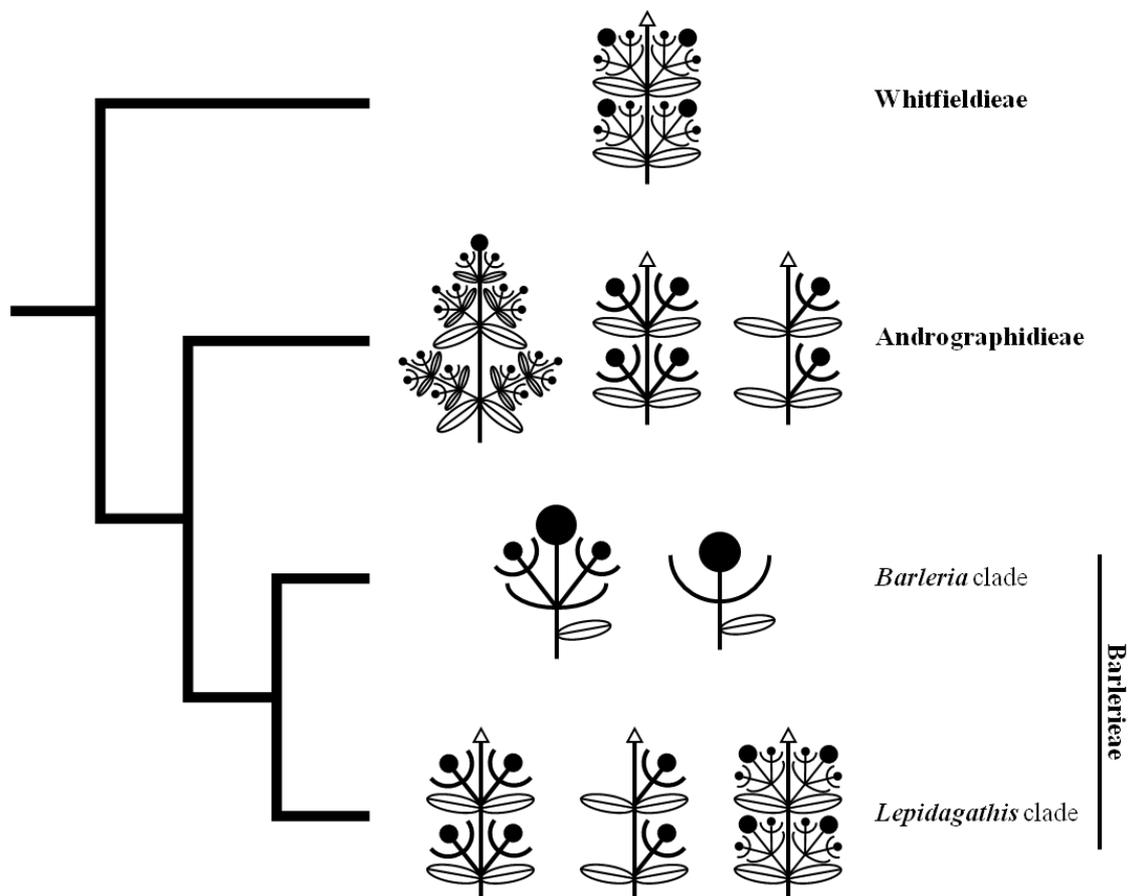


Fig. 7. Simplified cladogram, based on Tripp and McDade (2014), showing the Whitfieldieae, Andrographidieae and Barlerieae tribes and their respective inflorescences (naming in order of appearance on the cladogram: thyrse, panicle, raceme/spike, secundiflorous spike, dichasia, and solitary flower).

Following the molecular inference of Glover et al. (2015), losing or gaining a feature is equally likely in an evolutionary scenario, because at the molecular level both changes have the same probability of taking place. According to Weber (2013), in the Lamiales there has been a passage from determinate to indeterminate inflorescences, meaning the loss of the terminal flower on the main axis. Nonetheless, within the indeterminate inflorescences, there has been a gain in branching growth, as the secondary axes, which were formerly solitary flowers, may become cymes, by the activity of reproductive meristems, giving rise to thyrses.

Axillary meristems are, overall, an alternative in the growth pattern for the plant: the site in which they appear and their activity determinate the plant form and the inflorescences architecture (Kerstetter and Hake, 1997; Schmitz and Theres, 2005). The presence of axillary buds is usually associated with a subtending leaf (Snow and Snow, 1942), but the opposing argument, the presence of leaves without axillary buds, seems to be valid only for modified leaves, usually associated with the reproductive phase of the plant, e.g. bracts, sepals and petals (Endress, 2010). A recurrent example of the activity of the axillary meristems on flowering plants is the model angiosperm *Arabidopsis thaliana* (L.) Heynh. A study by Long and Barton (2000) showed that on this species, the axillary buds on leaves from the vegetative portion of the plant become visible late after the leaf primordia initiation. On the contrary, the axillary buds from the leaves on the reproductive portion, i.e. the bracts of the inflorescence, develop without an apparent subtending leaf primordia. They demonstrated by in situ hybridizations that the subtending bract is actually present, but is repressed in early developmental stages.

The establishment of primordia on the inflorescence apex is induced by the interaction of hormones, genetic control, and many other factors, which also have been reported to delay or suppress the development of flower buds by action of the shoot apical meristem dominance (Coen and Nugent, 1994; Kwiatkowska, 2008; Schmitz and Theres, 2005; Tooke et al., 2005). Snow and Snow (1942) has already noticed the delay in the appearance of a conspicuous

axillary meristem on species of Lamiaceae, where they refer to buds that form on the axils of old leaves from meristems that were already present. Leyser (2003) rank the axillary meristem's ontogeny into two categories: the first of meristematic cells that become detached from the apical meristem and remain undifferentiated from the tissue around, until they are released from this repressed state and develop into an axillary bud; and the second category of apical meristem cells that lose their meristematic characteristics, differentiating into the tissue they are submerged into, at the axil of a subtending leaf, and thus requiring some kind of signaling to regain the meristematic activity and develop into a functional axillary meristem.

Considering these knowledge, some inferences can be made on the matter of the axillary meristems of the secundiflorous spikes of *L. floribunda*. The absence of a distinguishable bulge, or even an internal region of more stained cells on anatomical sections (characteristic of meristematic cells, which have dense protoplasts), may imply that the axillary meristems on the axil of the sterile bracts of the inflorescence have a development of the second kind, losing their meristematic characteristics. Therefore, these cells become indistinct from the rest of the tissue, although, through the action of a signal, might be able to develop back into a functional reproductive meristem.

The presence of accessory buds accompanying the main inflorescence meristem was constantly observed on the studied species of *Lepidagathis*. These buds often do not develop into more than a pair of leaf primordia. Sell (1969a) studied particular condensed and ramified inflorescences of many Acanthaceae species, and the presence of accessory structures, especially the accessory flowers and partial inflorescences which constituted the complexity of such flowering shoots. Other families of Lamiales are also known for the common occurrence of accessory buds in the inflorescences (Weber, 2013), as well as on many families amongst the Myrtales (Briggs and Johnson, 1979; Sell and Cremers, 1987; Weberling, 1988), and even on the sister family to all Angiosperms, Amborellaceae

(Posluszny and Tomlinson, 2003). These structures are reported to increase the complexity of the inflorescence architecture and the number of displayed flowers by developing into new flowers or branches; and they begin their growth after the associated inflorescence has started its own development (Cavalcanti and Rua, 2008; Naghiloo et al., 2013).

On the investigated species of *Lepidagathis*, the presence of accessory buds is regarded as a separate aspect from the number of flowers produced per inflorescence, or from the number of inflorescences produced during the reproductive period of the plant. This is evidenced by *L. floribunda*, which is the studied species that has the less amount of active reproductive meristems (denoting the lack of flowers subtended by the sterile bracts). However, it presents accessory buds and the greater number of mature inflorescences taking place concomitantly. Thus, the development of accessory flowers and partial or full inflorescences cannot be correlated with any special type of inflorescence, as happens in the formerly mentioned families.

Sell (1969b) also investigated the frequent anisophylly and anisoclady on Acanthaceae species, emphasizing his discussion on the flowering shoots as well. Together with the distribution of active reproductive meristems on the axil of bracts, the anisophylly of the sterile bracts is responsible for the assembly of the secundiflorous spikes of *L. floribunda*. The lack of a prominence on the axil of the bract allows it to grow more adpressed to the inflorescence axis. The anisophylly of the sterile bract lamina is accentuated by the fused growth of one of its margins with the inflorescence axis, making it seem as there has been a displacement of the bract. It has been stated in the “Hofmeister’s rule” that the organization of primordia on the shoot apical meristem is given by its spatial constraints, and this is physiologically understood due to the inhibitory field created by the previous produced primordia (Leins and Erbar, 1997). Since the mature structure of the secundiflorous spikes do not follow the initial positioning of the primordia on the inflorescence apex, which is clearly

decussate, this spatial developmental assumption may explain the peculiar conformation of the inflorescence.

It may also be inferred that such diversity of inflorescence architectures has an ecological importance for the reproductive success of the plants. Studies show that the main pollination in Acanthaceae species occur by hummingbirds, and secondly by bees (Matias and Consolaro, 2015). The flowers of *L. floribunda* have long pink colored tube corollas, most probably pollinated by hummingbirds. The curvature and predominance of flowers on the anterior side of the inflorescence serve as by better display of the flowers for the pollinators. As for *L. alopecuroidea*, the flowers have small white tubes, with nectar guides, more probably pollinated by insects, such as bees. On this species, there is an increased number of viable concomitant flowers displayed on the thyrses, which is associated with bees behavior of visiting many flowers repeatedly.

An overview of the results concludes that the many forms of inflorescences present on *Lepidagathis* derive from the presence and activity of reproductive meristems on the axil of bracts. This diversity may be correlated with the pollination syndrome of each species, more than with a sequence of evolution, given the fact that this treat also does not follow a linear pattern within the clade. More studies with the group will help to comprehend the phylogenetic relations between the Barlerieae clade, as well as its patterns of dispersal, and ecological values.

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CONCLUSIONS

Considering the diversity of inflorescence architectures and floral morphologies and the obscure taxonomy of the genus, *Lepidagathis* proved to be a prominent model for morphological and developmental studies to understand its evolution. The investigations of this dissertation provided base information, with which it is now possible to hypothesize about evolutionary trends. On the inflorescences, it was proposed that a reduction of reproductive meristems on the inflorescences, creating patterns like the secundiflorous spike, or an increment of reproductive meristems on the axil of bracteoles, that creates the thyrses pattern of inflorescence, have the same probability of having occurred on the evolution of the group. The constant tetramery of the androecium was proved by the presence of vascularization, even on flowers with only two stamens, and the probable extinction of the vascular traces, as a fifth trace was only found on one species. The study of the floral anatomy revealed an intriguing characteristic of the ovaries of *Lepidagathis*, and may be extended to Acanthaceae s.s. The recognition of a marginal fusion of the carpels is important considering phylogenetic homologies and proves that anatomical investigations have a critical value to botanical studies.

Therefore, studying the ontogeny of an organ, a solitary flower or a whole inflorescence, is important, for only then the activity of the meristems that will lead to its final morphology are able to be accompanied. By analyzing the origin and sequence of emergence of the primordia and its anatomy, it is possible to look for homologies and reflect about the fusions and reductions on the plant body. These factors are essential to comprehend the phylogenetic relations between the taxa and contribute with the knowledge of our flora.