Linking the Evolution of Development of Stem Vascular System in Nyctaginaceae and Its Correlation to Habit and Species Diversification

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Research

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Abstract

Background: The presence of alternative patterns of secondary growth in stems of Nyctaginaceae has been known for a long time. Still, the interpretation of types of cambial variants are controversial. The knowledge on stem anatomical diversity in Nyctaginaceae, which is diverse also in habits, offers the unique opportunity not only to investigate the evolution of complex developments, but also to address how these anatomies shifted within habits and how the acquisition of novel cambial variants and habit transitions impacted the diversification of the family.

Methods: We integrated developmental data with a phylogenetic framework to investigate the diversity and evolution of stem anatomy in Nyctaginaceae using phylogenetic comparative methods, reconstructing ancestral states, and examining whether anatomical shifts correspond to species diversification rate shifts in the family.

Results: Two types of cambial variants, interxylary phloem and successive cambia, were recorded in Nyctaginaceae, which result from four different ontogenies. These ontogenetic trajectories depart from two distinct primary vascular structures (regular or polycyclic eustele) yet, they contain shared developmental stages which generate stem morphologies with deconstructed boundaries of morphological categories (continuum morphology). Unlike our *a priori* hypotheses, interxylary phloem is reconstructed as the ancestral character for the family, with three ontogenies characterized as successive cambia evolving in few taxa. Cambial variants are not contingent in habits, and their transitions are independent from species diversification.

Conclusions: Our findings suggests that multiple developmental mechanisms, such as heterochrony and heterotopy generate the transitions between interxylary phloem and successive cambia. Intermediate between these two extremes are present in Nyctaginaceae, suggesting a continuum morphology across the family as a generator of anatomical diversity.

Background

In the context of evolutionary developmental biology and phylogenetic research, morphological and anatomical comparative studies have played a major role in our challenge to unravel the complexity and diversity of both living and fossil organisms [1, 2]. Thus, one of the fundamental pillars of this discipline remains to investigate how modifications in developmental programmes of organisms contributes to the diversity of phenotypes encountered in nature [1, 3–5]. All organisms have an ontogeny hence, the evolution of diversity is in large part achieved by modifications in developmental processes across these ontogenies (e.g., heterochrony, heterotopy, homeosis, heterometry) [6–8]. In stem development, previous studies have demonstrated different developmental programmes interacting in the evolution of various anatomical architectures, which evidenced also anatomical shifts that likely triggered their diverse forms, coped with possible hydraulic and biomechanical functions critical to plant survival [9–12]. Therefore, by studying the alterations in developmental trajectories in stem vasculature formation, we are indirectly

studying possible modes of how evolutionary mechanisms have acted in the evolution of some of nature's most complex stem architectures.

Regular secondary growth – a single bifacial cambium producing wood and bark – is believed to have first appeared in the ancestor of progymnosperms, gymnosperms, and angiosperms [13–15]. Within this large, diverse lineage, known as the lignophytes, alternatives to this regular growth are not uncommon [16–19]. Several modifications on the regular growth include a single cambium with differential activity and/or multiple cambia [18, 20–22]. These alternative patterns of secondary growth produce diverse and complex stems architectures, also known as cambial variants [20, 21]. Many types of cambial variants are found in lineages containing lianas, although they also occur in plants with distinct growth forms, such as trees, shrubs and herbs [22, 23]. While the development of certain cambial variants has been investigated in recent years, the evolutionary history underlying the formation of these complex patterns is still being amassed. The few previous studies integrating stem anatomical data with a phylogenetic framework have reviewed how disparate macromorphologies evolved from regular anatomies [11, 12], showing that their evolution involved different developmental mechanisms, especially heterochrony. Nevertheless, much has yet to be investigated to understand the total realm of changes in developmental trajectories that can contribute to the major complexity and diversity of the vascular system of plants in phylogenetically distant lineages.

Nyctaginaceae is a family of c. 400 species of broad distribution across the Americas, Africa and Indo-Pacific, which grows in a wide range of habits from arid deserts to tropical rain forests [24, 25]. The family includes herbs, shrubs, scandent delicate plants, bulky lianas, large trees and suffrutescent species [24, 25]. Regardless of growth forms, the stem vascular anatomy of most Nyctaginaceae is remarkable for their polycyclic eustele [26] and cambial variants that appear in stems under secondary growth [27–29]. Most other plant families only have cambial variants in certain clades or their lianescent taxa (Malpighiaceae [30]; Sapindaceae [12, 31]), although they are also found in self-supporting plants derived from lianescent ancestors (Bignoniaceae [11]; Convolvulaceae [32]). According to Gianoli [33, 34], the evolution of climbing of climbing habit substantially increased the species richness of clades compared to their non-climbing sister groups. However, since many clades containing climbing plants (mostly lianas) are also characterized for showing cambial variants, we asked whether it is the shifts in habit or the appearance of cambial variants that are associated with an increased species richness within these lineages. Given that Nyctaginaceae have a wide diversity of habits and because all lineages have cambial variants, the family is perfect as a first approach to test if the transition to the lianescent habit promotes species diversification or not.

Distinct types of cambial variants have been reported for Nyctaginaceae and more recently new approaches to their types have been proposed, demonstrating that taxa initially described as having successive cambia have interxylary phloem instead [35]. As for their origin, both the pericycle [36, 37, 38] and a meristem derived from the cortex (i.e., the master cambium [39]) have been suggested as the place where the cambial variants arise [29, 40, 41]. Either way, the understanding of anatomical and developmental diversity of the vascular system in Nyctaginaceae has been limited given the absence of

ontogenetic studies in a broader taxonomic scale. In the family, few genera and species have been investigated in previous studies, which include generally only the ornamental charismatic taxa, such as *Bougainvillea* and *Mirabilis* [28, 29, 40, 41]. Given the diversity of vascular anatomies, in this study we aim to investigate the developmental processes causing the evolution of disparate vascular architectures, which is widespread in the family and likely independent of habits and habitats.

Here, we performed a comparative stem developmental analyses in the context of a well-supported phylogenetic hypothesis to understand how developmental processes evolved over time and shaped the diversity of stem architectures in Nyctaginaceae. Among the remarkable findings of this evo-devo study we highlight: i) the anatomical changes underlying the evolution of four ontogenetic trajectories in stem development; ii) the anatomical, developmental and evolutionary lability of vascular meristems, especially the vascular cambium; and iii) the significance of developmental mechanisms for evolutionary diversity of stem anatomical architectures. In addition, we evaluated whether species diversification rates have changed in Nyctaginaceae to explore the potential impact of both the multiple transitions in vascular anatomies and the lianescent habit in the diversification of the family.

Materials And Methods

Taxon sampling and anatomical analysis

This study represents the broadest taxonomic sampling for stem anatomical studies in Nyctaginaceae to date. Stem samples of 55 species (~75 specimens) from 25 genera were collected, representing all major clades within the family, based on the most recent phylogenies for the group [24,25,42,43]. Specimens were obtained mostly from field collections in different countries in both North and South America (Additional file 1: Table S1). Additional samples were obtained from dried stems from either herbarium vouchers or wood collections (Additional file 1: Table S1).

Samples from living plants were harvested at different heights of the stem to ensure that different developmental stages would not be missed. For herbs, complete stems were collected; for shrubs, lianas and scandent-shrubs, samples were obtained at the base of the plant and at least three different heights towards the shoot apex. For trees, we collected trunk samples at breast height (1.30m) and at different heights of selected branches. See Additional file 1: Table S1 for information on stem diameter for each specimen.

For the ontogenetic analyses, 27 species belonging to 22 genera of Nyctaginaceae were selected to account for all the variation both in terms of their phylogenetic distribution and anatomical patterns (Fig. 1; Additional file 1: Table S1). For these species, sections were taken from different internodes beginning at the shoot apex until reaching the fully developed stem. For the remaining species, analyses of adult stems (the most developed stem available, from fully grown plants) were carried out to ensure the cambial variant types studied previously in detail were consistent.

During field work the samples were immediately fixed in FAA 50 (10% formalin, 5% acetic acid, 50% ethanol) for one week and then transferred to a solution of 70% ethanol [44]. Anatomical sections were obtained following two different procedures: i) young and small stems samples were dehydrated in an ethanol series, embedded in Historesin (Leica Mycrosystem, Wetzlar, Germany), sectioned in a rotary microtome (Leica RM2145, Nussloch, Eisfeld, Germany), and stained in 0.05% toluidine blue in glacial acetic buffer at pH 4.7 [45]; ii) adult and large samples were softened in 10% ethylenediamine for up to 2-5 days [46], gradually embedded in polyethylene glycol 1500, sectioned with the help of polystyrene (foam) resins applied upon the stem blocks before sectioning in a sliding microtome with a permanent steel knife sharpened with different grids of sandpapers (Leica SM2010R, Nussloch, Eisfeld, Germany) [47,48] and double stained in 1% astra blue and 1% safranine [49]. Sections were mounted with coverslip in Canada Balsam or Entellan® synthetic resin (Merck KGaA, Darmstadt, Germany) to make permanent slides.

Phylogenetic framework, ancestral state reconstructions

To estimate the evolutionary history of ontogenetic pathways, we applied an ancestral state reconstruction using the same phylogenetic tree applied by Cunha Neto et al. [26] under Maximum Likelihood (ML) assumptions as implemented in Mesquite version 3.5 [50].

Diversification analysis

Divergence times.

To estimate the age of Nyctaginaceae, we conducted a Bayesian inference with BEAST v.2.6.5 [51], using two secondary calibrations derived from a thorough study of the divergence times of the angiosperm families [52]. We applied a uniform prior distribution to calibrate the root of the tree corresponding to the stem age of a group comprising Gisekiaceae and Nyctaginaceae, where the maximum value of the distribution was 83.6 Ma (Million years ago), and the minimum value was 52 Ma. We also applied a uniform prior distribution to calibrate the crown node of Nyctaginaceae, with a maximum value of 47.59 Ma and minimum value of 18.12. In BEAUti, we assigned a molecular substitution model as GTR + G, using empirical base frequencies, molecular clock set as uncorrelated with rates obtained from a log-normal distribution (UCLN; [53]), and a birth-death tree prior. We ran two independent analyses, each with 400 million generations, sampling parameters every 10,000 generations. We corroborated the correct mixing of the Markovian chains in Tracer v.1.6 [54], where the Effective Sample Size (ESS) was equal or higher than 200 for all the parameters. We obtained the Maximum Clade Credibility (MCC) tree with TreeAnnotator v.2.6.5 (beast2.org/treeannotator). The analyses in BEAST2 were performed in the server BEAGLE of the Instituto de Biología (UNAM).

Diversification rate estimation

Using the time-calibrated phylogeny (MCC tree), we evaluated whether there have been changes or shifts in the diversification rate through time and among lineages. The diversification rate corresponds to the

net number of species/lineages generated per time unit (speciation) considering the extinction [55,56]. For this, we implemented a Bayesian analysis of macroevolutionary mixtures (BAMM v.2.5.0; [57]). BAMM estimates diversification rate shifts under a compound Poisson process through time and among lineages, using reversible-jump Markov chain Monte Carlo (rjMCMC) samplers to evaluate models that vary in the number of shifts proposed [57]. We selected a set of priors calculated in the R package BAMMtools [58,59] for the speciation and extinction initial values. We specified a proportion of taxon sampling to consider the missing species of Nyctaginaceae and outgroups. We ran the analysis for 100 million generations. We evaluated the convergence of chains and with the package coda [60] we corroborated that the ESS of the MCMC was 200 or above.

Trait-dependent diversification

To directly evaluate the contribution of characters in the diversification of Nyctaginaceae, we applied the Hidden State Speciation and Extinction method (HiSSE v.2.1.1; [60,61]) for three characters, habit (self-supporting or climbing), eustele type (regular or polycyclic), and secondary growth (regular or variant). Using the dated phylogeny, we tested five models that varied in the relationship of the diversification rate and the observed, focal character. First, we evaluated a model where diversification rates do not change across the phylogeny (Null). The second model tests two regimes of diversification rate that directly depend on the focal trait (BiSSE-like model), similar to the BiSSE method [62]. The third model evaluates the relationship of the focal trait and another unobserved character (HiSSE, with two hidden states). This is to evaluate the relative contribution of the focal trait to the diversification. Finally, following Beaulieu & O'Meara [61], we tested two models of character-independent diversification (CID) allowing diversification rate to vary across the phylogeny, but independently from the focal trait. One of these models has two parameters of diversification rate (CID2), and the other has four (CID4), comparable to BiSSE-like and HiSSE models, respectively. Net diversification rate was obtained through turnover rate (default) and the extinction fraction. In all models, the extinction fraction was constrained to have the same rate for all the character states, the free parameters were the turnover rate and the transitions between character states. Missing species were considered in the models by accounting for the proportion of sampled species relative to the existing species displaying each of the two states. To select models that better explain the variation of the data, we obtained the Akaike weights to compare the relative likelihood of each model. Character dataset (Additional file 2: Table S2) and parameter settings (Additional file 3: R script) can be found in the Supplementary information.

Terminology

As the range of terms related to the vascular system in Nyctaginaceae is highly diverse, we here define the terminology used in the present study (see Glossary in the Additional file 4: Table S3).

Results

Four ontogenetic pathways link procambium to cambium and cambial variants

In Nyctaginaceae, the stems may present two types of eustele, the regular or the polycyclic (with medullary bundles) (Fig. 2). The vascular system is also characterized by a distinguishable pericycle that can be uni- to multiseriate and which is divided into a portion of lignified cells and other that remains parenchymatous (Fig. 2; 4a-d; 9b, d). In mature stem, two types of cambial variants can be recognized, i.e., successive cambia and interxylary phloem, which derive from four different ontogenies (Fig. 2). Interestingly, representatives of all lineages of the family present variant vascular anatomies during secondary growth. Below we detail each of these ontogenies.

Ontogeny 1 **(summarized from Cunha Neto et al.**, [35]**) – Steps: i. Polycyclic eustele, ii. cambium from the continuous cylindrical procambium (CCP), iii. formation of phloem strands, iv. interxylary phloem (**Fig. 2a; 3a-e**).**

This developmental pathway begins with the establishment of a polycyclic eustele – medullary bundles + continuous cylindrical procambium (Fig. 2a and 3a). After vascular bundles are formed from the CCP, a cambial zone is established from the procambium of the bundles (Fig. 3b). At maturity, the cambial zone presents an irregular activity leading to the formation of secondary xylem and phloem derivatives at different rates along the stem circumference, which results in the formation of phloem strands (Fig. 3c). At some points, segments of the cambial zone slightly reduce the production of xylem derivatives concomitantly with the increased formation of phloem derivatives outwards (Fig. 3c). Subsequently, these phloem patches are overarched by cambial segments formed in continuity with the single cambium and originated by differentiation of the axial phloem parenchyma, the coalescent (arching) cambium (Fig. 3c); this cambium produces secondary xylem inwards and phloem outwards enclosing the islands of phloem, constituted mainly by conducting cells and axial parenchyma cells which here we denominate sheathing axial parenchyma (Fig. 3c, d). This process occurs repeatedly in the cambial zone and, as a result, many phloem strands are formed with the original cambial segment embedded within the secondary xylem (Fig. 3e).

The cambial variant described above characterizes in several aspects an interxylary phloem pattern. In Nyctaginaceae, this developmental pathway produces disparate stem architectures ranging from well-defined phloem islands with less sheathing axial parenchyma to long concentric bands of phloem and much sheathing axial parenchyma diffused tangentially (Fig. 2a). Intermediates between these two types also occur, forming phloem strands produced by confluences or patches.

Ontogeny 1 is the most common type within Nyctaginaceae, occurring in genera and species of various growth habits and from five out of the seven tribes, i.e., Boldoeae, Bougainvillea, Colignonieae, Nyctagineae and Pisonieae.

Ontogeny 2 – **Steps: i. Regular eustele, ii. installation of variant cambium (extra-fascicular cambium), iii. successive cambia (**Fig. 2B; 4A-D; 5A-B; 6A-C**).**

This pattern differs from ontogeny 3 (see description below) for not forming a regular cambium, even though their primary vascular system is similarly characterized by a regular eustele (Fig. 4a). The genera under this ontogeny lack medullary bundles. Instead of forming a regular cambium, the first cambium is already the first variant cambium, differentiated from a meristematic zone formed by divisions of the pericyclic parenchyma cells located between the primary phloem and the fibrous pericycle (Fig. 4b-d). This variant cambium differentiates externally to the vascular bundles (i.e., extra-fascicular cambium), giving rise to secondary xylem produced internally and secondary phloem formed to the outside (Fig. 5a-b). Other additional cambia are formed outwards from remaining cells of the previous meristematic zone, whereas some parenchyma cells constitute the tangential conjunctive tissue between the two increments of vascular tissue. In some cases, the first new vascular tissues are initially formed in relatively small tangential segments (patches) (Fig. 4d), while the following cambia are displayed in more continuous and concentric rings (Fig. 5a-b). However, vascular increments forming confluent bands can also be observed in later developmental stages (Fig. 6c), evidencing that the activity of the new cambia do not always form complete concentric rings. Depending on the organization of the conjunctive tissue, the rings of successive cambia can appear forming a wavy appearance (Fig. 6a-b), or more or less concentric rings (Fig. 2c; 6c). Despite that, the sieve-tube elements and their companion cells are formed always at the opposite side of the vessel elements, while conjunctive parenchyma form an intricate network with the vascular rays (Fig. 6b-c).

Ontogeny 2 was observed in the genera *Andradea, Leucaster* and *Ramisia*, which are composed of trees or lianas and all belonging to tribe Leucastereae.

Ontogeny 3 **– Steps**: **i. Regular eustele, ii. regular cambium, iii. installation of variant cambium, iv. successive cambia (**Fig. 2c; 7a-d; 8a-f**).**

This ontogeny initiates with the formation of a regular eustele characterizing the primary vascular system (Fig. 7a-b). Similarly, to species in ontogeny 2, the species under this ontogeny lack medullary bundles. Later, a regular cambium develops from the fascicular and interfascicular cambium and starts to produce secondary tissues in the usual way, i.e., secondary xylem centripetally and secondary phloem centrifugally (Fig. 7a-c; 8a-d). Initially, the interfascicular cambium may produce mostly phloem axial parenchyma to the outside and produce xylem fibres, vessel and ray to the inside (Fig. 7c-d; 8c-d). After some period of regular growth, a new meristematic zone arises through subsequent divisions of pericyclic parenchyma cells outside of the primary phloem (Fig. 8a-c). Then, a new cambium (variant cambium) differentiates in the middle of the meristematic zone (Fig. 8b), whereas some inner layers of cells differentiate into conjunctive tissue (Fig. 8e). This variant cambium produces variant xylem towards the inside and variant phloem towards the outside (Fig. 7c; 8c-d). Subsequently, by the same mechanism of the first variant cambium, new cambia arise successively in centrifugal, concentric order, each originating from the outer derivatives of the preceding meristematic zone (Fig. 8e).

The cambial variant presented here is characterized as successive cambia and give rise to a stem architecture formed by concentric continuous increments of variant xylem and phloem (Fig. 2c). In mature stems, the tangential conjunctive tissue forms a network arrangement with narrow and wide vascular rays (Fig. 8e). The sieve-tube elements and their companion cells form mostly strands surrounded by the tangential conjunctive parenchyma, located at an opposite pole of the radially arranged vessel elements (Fig. 8e-f).

Ontogeny 3 was observed exclusively in the shrubby species of genus *Reichenbachia* (tribe Leucastereae).

Ontogeny 4 – **Steps: i. Polycyclic eustele, ii. cambial zone derived from the continuous cylindrical procambium (CCP), iii. installation of variant cambium, iv. successive cambia (**Fig. 2d; 9a-b; 10a-e**).**

This ontogeny differs from ontogeny 2 for having a polycyclic eustele in the primary vascular system, constituted of medullary bundles and the continuous cylindrical procambium (CCP) (Fig. 9a). The CCP is constituted by the fascicular procambium that originates the vascular bundles and the interfascicular procambium that gives rise to fibres (Fig. 9b). Subsequently, a vascular cambium develops from the procambium and interfascicular procambium; this cambium produces regular secondary xylem and phloem in the usual way, but this activity is maintained for a relatively short period of time (Fig. 10a-b). Later, divisions of the pericyclic parenchyma cells (Fig. 10b) form a meristematic zone, more conspicuous in *Allionia* than in *Okenia*. New segments of cambia develop within the meristematic zone producing new vascular increments composed of variant secondary xylem and phloem in the usual polarity (Fig. 10c-e). This pattern of secondary growth is characterized as a successive cambia system.

Ontogeny 4 was found only in two small genera from tribe Nyctagineae, which are constituted of herbaceous species, i.e., *Allionia* and *Okenia.* Although successive cambia establish in an early period of their lifespan, these species show relatively little amount of secondary tissues even in the most developed stems, as seen in *Allionia incarnata* (Fig. 2d, 10e) and *Okenia hypogaea* (Fig. 2D, 9C). In *Allionia*, one or two complete rings of vascular increments are possible, whereas in *Okenia hypogaea* only small cambial segments of first order were seen in the most developed stems (Fig. 2D; 10a).

Character mapping and ancestral state reconstruction

To assess the evolution of stem development in Nyctaginaceae, the ontogenies were mapped onto the current phylogeny of the family. Each of the four ontogenetic pathways were delimited as character states. The phylogenetic analysis showed that ontogeny 1 (polycyclic eustele + interxylary phloem) is the most common and was reconstructed as the ancestral state (79% presence), with a few secondary losses (Fig. 11, e.g., Leucastereae, Nyctagineae). Ontogeny 4 (polycyclic eustele + successive cambia) evolved at least twice, being found in genera belonging to crown clades (i.e., Nyctagineae) that includes the majority of the herbaceous species, whereas ontogeny 2 (regular eustele + regular cambium + successive cambia) and ontogeny 3 (regular eustele + extra-fascicular cambium + successive cambia) evolved each only

once, occurring in genera of tribe Leucastereae (the clade sister to the rest of the family) which is formed by shrubs, trees and/or lianas.

Successive cambia and interxylary phloem occur in species with distinct habits (Fig. 11), which has an ambiguous ancestral reconstruction for the ancestral node of the family.

Divergence times and diversification rate estimation

We estimated the age of divergence of Nyctaginaceae and close relatives with BEAST2 (Fig. 12). We obtained the Maximum Clade Credibility tree (Fig. 12, Additional file 5: Nexus tree), which shows the mean age for the main nodes in the phylogeny. Table 1 shows the mean crown age estimates for the major clades and its associated credibility interval represented by the 95 % Highest Posterior Density (HPD). Nyctaginaceae probably diverged from its sister group (stem age), Phytolaccaceae, 48 Ma (HPD: 38.97-55.56 Ma) and diversified (crown age) 42.3 Ma (HPD: 32.82-47.59 Ma), both events occurring at the Middle Eocene.

Table 1
Divergence-time estimation of mean crown ages in Million years (Ma). Highest posterior density interval (95% HPD).

Family	Crown age (Ma)	95 % HPD
Nyctaginaceae	42.3	32.82-47.59
Phytolaccaceae	40.91	26.45-53.95

The estimation of diversification rate shifts implemented in BAMM resulted in a set of configurations, each configuration represents a group of possible rate shifts occurring in the phylogenetic tree at different times. The Maximum a posteriori probability (MAP) configuration was obtained in the R package BAMMtools and is observed in the phylorates plot shown in Figure 13, where the estimated rate at each segment of the branches is the mean of the marginal posterior density of the diversification rate, and the shifts are marked by a green circle. The MAP includes the most frequent configuration with two shifts in the diversification rate (i.e., speciation minus extinction; green circles in Fig. 13; check Additional file 6: Figure S1 for all most credible shift sets recovered by BAMM). The results indicate one diversification shift derived from a rise in the speciation rate alone in the clade that includes Pisonieae-Bougainvilleae-Nyctagineae, and another diversification increase derived from rises in both speciation and extinction rates in bulk of *Commicarpus*, as observed in the speciation rate and in the extinction rate separately.

Table 2 shows the results from the trait-dependent diversification analysis and model comparison. For habit and secondary growth, the preferred model was the character-independent diversification (CID2). In turn, for eustele type, the preferred model was a trait-dependent diversification with two diversification

regimes (BiSSE-like). This indicates that eustele type had a relationship with diversification rate, being the polycyclic eustele associated to elevated diversification rate compared to regular eustele (Fig. 14).

Table 2
Model comparison from the trait-dependent diversification analysis. Log-likelihood (loglik); corrected Akaike score (AICc); Akaike weight (AICw). Bold rows indicate the preferred model for each evaluated character.

	Habit			Eustele type			Secondary growth		
Model	**loglik**	**AICc**	**AICw**	**loglik**	**AICc**	**AICw**	**loglik**	**AICc**	**AICw**
Null	-366.72	741.84	0.00	-322.19	652.79	0.02	-319.67	647.75	0.00
BiSSE-like	-366.65	743.91	0.00	**-316.99**	**644.58**	**0.91**	-317.88	646.38	0.00
HiSSE	-350.85	724.07	0.32	-315.52	653.41	0.01	-309.27	640.98	0.05
CID2	**-354.99**	**722.85**	**0.60**	-318.70	650.28	0.05	**-311.29**	**635.47**	**0.71**
CID4	-354.70	726.91	0.08	-317.77	653.05	0.01	-310.04	637.64	0.24

Discussion

The evolution of cambial variants in Nyctaginaceae represents an example of continuum morphology

Because evolution can be seen as the transformation of ontogenies, many authors have argued that plant morphology is better understood under more dynamic and process thinking than the typological view of classical morphologists (e.g., Wilhelm Troll, Donald Kaplan) [63, 64]. One of the alternative worldviews is based on processes and continuum morphology which has been built upon the works of several botanists from the 20th century such as Agnes Arber [65] and Rolf Sattler [3, 66, 67]. This approach implies that the static view of plants having structures (such as organs or the different variants here) with clear cut boundaries seems no longer sufficient to explain plant morpho-anatomical diversity [64]. In contrast, the process and continuum morphology recognize plants as combinations of developmental processes or as dynamic continua, based on the acceptance of the partial homology concept [65, 67], which reiterates the blurry boundaries (mixed identities, fuzzy morphology) between what used to be considered as separate structural categories. In other words, plant organs are no longer seen as structural categories, but they are likely to intergrade to a considerable degree [65, 64, 67]. This means that diversity is more likely to result from quantitative rather than from qualitative differences in development, which leads to the identification of several intermediate forms between two categories [63]. Although these concepts are not new, they have recently inspired many developmental evolutionary biologists to look at morphology and anatomy in a more holistic way [63, 64]. The process thinking and the continuum approach may be as well the better way to look at the evolution of successive cambia from the interxylary phloem in Nyctaginaceae.

Although successive cambia and interxylary phloem are recognized in the literature as two types of cambial variants, their occurrence in Nyctaginaceae indicates that in some cases there is a blurry boundary delimitation between these two patterns – which are represented in two levels. First, plants with ontogeny 1 that are characterized with interxylary phloem may present phloem strands immersed within the xylem with different arrangements (i.e., phloem islands, patches or bands). These arrangements result from different extensions of the coalescent cambium which is formed in continuity with the single cambium and encloses the phloem strands. Given that in plants with bands the stem initiates forming phloem islands followed by patches and then bands, the development of this ontogeny itself indicate the existence of a continuum between these different stem macromorphologies. Second, the ontogenies characterized with successive cambia shows that the independent cambia arise and form usually long tangential bands of vascular tissues similar to the phloem strands in bands of some species with interxylary phloem (the type of cambial variant present in the ancestor of the family). In this sense, in the context of continuum morphology, we can propose that the successive cambia arise from interxylary phloem, by topological change of the new cambia differentiation migrating from secondary phloem parenchyma to pericycle (heterotopy), as a homologue of the coalescent cambium, but with a larger extension, so large that they start constituting completely independent additional cambia. In addition, the diverse topologies observed in plants with successive cambia which has received numerous attempts of subcategorization is another evidence of these blurry categories (Additional file 7: Table S4).

The fuzzy and continuum worldview has been used to distinct morphological systems especially in organ identity [68–71]. Here, for the first time, the diversity of cambial variants is interpreted under the concept of continuum morphology. These observations for Nyctaginaceae enrich our understanding of these complex vascular morphologies, as it gives us the notion of how ontogenies changed across evolutionary time producing intermediate forms that at some point can be distinguished as discrete categories.

The origins and developments determining the cambial variants in Nyctaginaceae

Different developmental pathways accounts for the disparate secondary vascular architectures observed in Nyctaginaceae. The recognition of interxylary phloem along with the occurrence of successive cambia is based primarily on their differences in development, i.e., single vs. multiple cambia respectively [35], but these developmental pathways result in architectonically similar anatomies and to a certain level can be considered to integrate into intermediate forms in Nyctaginaceae.

Here we identified that to the four ontogenies, all events share an origin to their secondary growth internally to the pericyclic fibres. These findings contradict that the cambial variants in Nyctaginaceae are formed from a meristem arising in the cortex as previously suggested [29, 39–41, 72]. Instead, all cambial variants originated from procambial-derived cells (i.e., either pericycle or arching cambia induced in phloem axial parenchyma), corroborating previous findings [37, 38]. Therefore, multiple origins (e.g., primary phloem, secondary phloem, cortex - [32, 73–76]) and developmental trajectories, as we discuss below, can lead to the formation of successive cambia, unlike the idea of a universal phenomenon for the formation of this cambial variant across different plant groups [39, 72, 77]. Indeed, successive cambia

can be established from different stem regions, such as primary phloem [73], secondary phloem [74], pericycle [32, 70], or cortex [76]. Nevertheless, except for the cortex and secondary phloem, in all other cases the origin of the new cambium can be considered developmentally linked with a pre-existing vascular meristem because both pericycle and primary phloem are produced by the procambium. A similar developmental parallel can be established for the origin of interxylary phloem and successive cambia in Nyctaginaceae, because both the cambium giving rise to phloem strands within the secondary xylem (interxylary phloem) and the meristematic zone producing a *de novo* cambium (successive cambia) may be traced back to the procambium at some point in stem development. Therefore, successive cambia and interxylary are evolutionarily and developmentally linked in Nyctaginaceae.

Although the cambial variants in Nyctaginaceae present similar origins at the cell lineage level (i.e., procambium-derived cells), the eustele types and subsequent events in their development are diverse, leading to four distinct ontogenies. In a previous work we showed that the origin and development of interxylary phloem in Nyctaginaceae is similar to that shown in other groups, except for the fact that the single cambium is originated from the continuous cylindrical procambium (CCP), which is part of the polycyclic eustele, that also includes the medullary bundles [35]. Although ontogenies 2, 3 and 4 are characterized as successive cambia at maturity, they are built upon three different step-wise developmental pathways. The developmental steps in the formation of successive cambia in *Reichenbachia* (ontogeny 3) is the same as described in most families with this cambial variant, i.e., a new cambium is formed *de novo* (mostly but not always) from the pericycle in stems with regular eustele; this is the case of species both in the gymnosperms (e.g., *Gnetum, Cycas* – [39]) and several families of angiosperms (e.g., Menispermaceae [78]; Convolvulaceae [32]; Sapindaceae [75]). On the other hand, successive cambia, as described in ontogenies 3 and 4, differ from the taxa mentioned above because they either do not produce a regular cambium, forming an extra-fascicular cambium (ontogeny 2), or because the stem begins with a polycyclic eustele and the first cambium is derived from the CCP instead of a regular cambium (ontogeny 4). It is important to highlight that the appearance of the extra-fascicular cambium, which is independent from the primary vasculature, corroborates the potential of perivascular tissues (i.e., the pericycle) to produce new meristems, as it is observed in species with successive cambia following ontogeny 3 or 4. In addition, all developmental pathways in Nyctaginaceae include a *de novo* formation of new cambia, either entirely autonomously formed in the pericycle generating successive cambia or as short to long coalescing, arching cambia connected to the original single cambium generating interxylary phloem.

Besides the presence of distinct developmental pathways, Nyctaginaceae stands out for having all extant lineages characterized by some type of variant anatomy (see discussion on ‘evolution of development’ below). Regular vascular growth with eustele and a regular cambium forming xylem centripetally and phloem centrifugally, as observed in most eudicots, is observed only during the initial developmental stages of *Reichenbachia* (ontogeny 1), which later develops successive cambia. Species with ontogeny 4 also have a short period of secondary vascular tissues being produced by a bifacial cambium in the central cylinder, but it is developed from a polycyclic eustele [38].

Our observations on the origin and development of vascular meristems in Nyctaginaceae, as presented here and in previous studies [26, 35, 38], challenge a number of interpretations raised in previous investigations: (1) Cambial variants in Nyctaginaceae arise in the cortex [29, 39, 72] – we showed that the origin of cambial variants is from the pericycle (which is procambium-derived) [38]. (2) Successive cambia are the only type of cambial variant in Nyctaginaceae [29, 39, 58, 68] – two types of cambial variants occur in Nyctaginaceae, interxylary phloem and successive cambia [35]. (3) The existence of an 'extra-fascicular cambium' forming vascular bundles [27] – the extra-fascicular cambium produces secondary vascular tissues, observed exclusively in ontogeny 2 [this study]. (4) The presence of an unidirectional cambium forming both secondary xylem and secondary phloem to the inside [80] – instead of an unidirectional cambium we found that a single (bidirectional) cambium acts in the formation of interxylary phloem [35]. (5) The presence of a "Primary Thickening Meristem" similar to the monocots [81–83] – the presence of medullary bundles was probably the main reason for this interpretation, and we have demonstrated that these vascular structures arise from a continuous cylindrical procambium, different from what is observed in the monocots [26, 38].

In addition, because the vessel elements and sieve-tube elements are usually restricted to short tangential areas resembling discrete "vascular bundles", authors have used different terminologies to describe the secondary vascular tissues in stems of Nyctaginaceae, such as "secondary medullary bundles" [84], "vascular strands" [85], "vascular bundles" [28, 86] or "secondary bundles" [68], but in the secondary body this is a misnomer.

The evolutionary history of the diverse cambial variants in Nyctaginaceae

The ancestral state reconstruction presented here demonstrates that the ancestor of Nyctaginaceae already had cambial variant, with interxylary phloem (ontogeny 1) reconstructed as the most likely character state for the ancestral node of the family. This observation is remarkable because it indicates that Nyctaginaceae is one of the few examples where the cambial variants are present in all members and is shown to be shared with the other members of the phytolaccoid clade, being therefore plesiomorphic for Nyctaginaceae. In most families with cambial variants they appear only in one group, mostly in clades containing lianas or descending from lianas (e.g., Bignoniaceae, Convolvulaceae) [11, 12, 31, 87]. Contrary to expectations, interxylary phloem (ontogeny 1) is also the most common type of cambial variant in the family, occurring in five out of seven tribes, inclusive in those most studied genera such as *Bougainvillea, Boerhavia, Mirabilis* and *Pisonia*, which used to be classified as having successive cambia (reviewed by [35]).

The ontogenies are not evolutionary labile since each of them appeared only once, except for ontogeny 4 that evolved twice. The evolution of ontogenies 2 and 3 in members of Leucastereae is interesting because the tribe has other morphological (e.g., type of trichomes, pollen and fruit - [42, 76, 77] and vascular anatomical characters (e.g., type of stele, [26]) that are exclusive or unusual if compared to other lineages of the family. Curiously, from all stem ontogenies of Nyctaginaceae, *Reichenbachia* (ontogeny 3)

is the only taxon following the commonly described development for successive cambia, i.e., regular cylinder + successive cambia [29, 39, 72].

Ontogeny 4 is the only type with more than one evolution with two transitions in tribe Nyctagineae. The evolution of successive cambia in *Allionia* and *Okenia* is noteworthy for the fact that they are both small herbs with limited secondary growth in the regular cylinder, but the successive cambia still develop in some way. However, this might not be surprising since the presence of cambial variants in other herbs (annuals or perennials) is reported in many sister-related families across the Caryophyllales [72, 90]. This observation suggests that if given time to grow, most herbaceous plants in this lineage can form new additional rings of successive cambia or as presented here, forming new phloem islands/patches/bands as in species with interxylary phloem. Nevertheless, the occurrence and diversity of variant anatomies in Nyctaginaceae seem to be not contingent on specific habits, since both cambial variants occurs in species with all the range of growth forms present in the family.

The development and evolution of distinct patterns of cambial variants in Nyctaginaceae is remarkable because successive cambia has been reiterated as the only cambial variant in the family [29, 41, 72, 90]. Although we observed multiple ontogenetic pathways resulting in distinct cambial variants in Nyctaginaceae, similar patterns may also be present in other caryophyllalean families [35]. Comparably to other traits that seem to represent apomorphic tendencies (e.g., floral morphology [91–93]), the presence of cambial variants likely share developmental and genetic programmes (deep homology) triggering the recurrent evolution of this morphological feature in multiple Caryophyllales lineages.

Evolution of development: how different ontogenies generate similar stem macromorphologies

Because ontogeny is a linear process and given that primary and secondary vascular tissue may have intrinsic developmental relations, the investigation of the diversity and evolution of vascular anatomies in Nyctaginaceae needs to include the products of procambium, cambium and cambial variants to thoroughly comprehend the anatomical and developmental shifts in stem ontogeny. Here the integration between ontogeny and phylogeny showed that adult stems with distinct cambial variants evolved from different eustele types. Therefore, the organization of the primary vascular system is not a prerequisite for the evolution of patterns of secondary growth in Nyctaginaceae, achieved through distinct ontogenetic pathways. This scenario diverges from the evolution of secondary growth patterns in stems of Bignoniaceae [11] or *Paullinia* from the Sapindaceae [12] where cambial variants trace back to stems with regular growth in previous stem developmental stages.

For the evolution of interxylary phloem in the ancestor of Nyctaginaceae, several developmental modifications were needed in stem development of the putative ancestor of inner nodes of the phylogeny of the Caryophyllales, which likely had a regular anatomy (Fig. 15). Considering that some of the anatomical changes include: i) the decrease in the formation of xylem derivatives at specific locations, ii) the increased phloem production at the same location and iii) the development of the coalescent cambium. Similarly, considering that the ancestor of Nyctaginaceae had interxylary phloem (ontogeny 1), the appearance of each new ontogeny requires a set of transformations in the developmental pathway of

its ancestor. First, in the case of ontogeny 2, the main steps include the loss of the regular cambium and the appearance of the extra-fascicular cambium, which is probably regulated by the same mechanism leading to the evolution of the new cambium from the pericycle in ontogeny 3, that result in the formation of successive cambia. The difference from ontogeny 3 to ontogeny 2 is that the later regained the regular cambium and form regular xylem and phloem for some period. For the evolution of ontogeny 4, the evolution of new cambia from the pericycle as in ontogeny 2 and 3 is also observed, but this time the primary vascular system is characterized by the polycyclic eustele, while the main cambium produces regular tissue for a shorter period of time compared to ontogeny 3. Surprisingly, at maturity, the stem macro-anatomy of all ontogenies in Nyctaginaceae are alike, especially the ones with successive cambia (e.g., *Reichenbachia* – ontogeny 3) or interxylary phloem forming bands (e.g., *Bougainvillea, Colignonia* – ontogeny 1). Species with interxylary phloem forming only phloem islands are more easily distinguished in stem topology, although the development follow virtually the same steps in species with patches/bands, except for the length of the coalescent cambium.

Evolutionary mechanisms are hard to be interpreted for the evolution of secondary vascular patterns in Nyctaginaceae due to multiple and complex developmental transitions (Fig. 15). Here, three processes are inferred to generate the stem diversity found in the family (Fig. 15): homeosis, heterochrony, and heterotopy. (1) The formation of interxylary phloem (ontogeny 1) in relation to a putative ancestor with regular anatomy seems likely to represent a case of homeosis, since the unusual activity of the cambium leads to the presence of phloem strands in the place of secondary xylem. Similar cases of homeosis in woody plants has been hypothesized for example in species with parenchymatized xylem, that is, in cases where non-lignified parenchyma occur where fibres, vessels and lignified axial parenchyma would be present (e.g., lianas, succulents) [10, 11]. (2) In the evolution of ontogeny 2 from ontogeny 1, the extra-fascicular cambium appeared, suggesting a case of heterotopy since the first vascular cambium arises in a different position from that present in the ancestor. In addition, at the structural level the development of ontogeny 2 is based on the earlier onset of formation of the cambial variant by suppressing one of the ontogenetic stages (i.e., formation of a cambium from the primary vascular system), therefore, it may also illustrate for the first time a case of predisplacement, a form of peramorphosis (heterochrony). In wood anatomy, most cases of heterochrony suggest the occurrence of prolonged juvenile characteristics into adult forms (paedomorphosis) [10, 94, 95], and a case of peramorphosis (hypermorphosis - evolution by developmental additions) is also suggested for the origin of successive cambia in *Paullinia*, Sapindaceae [12]. Moreover, because ontogeny 2 evolved from ontogeny 1, this transition also requires modifications in the primary vascular system which indicates developmental changes that are regulated by an independent developmental module [96]. Thus, modularity may also be a source for anatomical diversity in this group. The evolution of ontogeny 3 from ontogeny 2 implicates in the appearance of a regular cambium. This transition indicates that a partial regression to the state of the ancestor of the family occurred in this lineage, if we consider that the regular cambium occurs in the same position of the single cambium generating interxylary phloem. Similarly, the evolution of ontogeny 4 requires a reversion from the cambium with unusual activity to the regular cambium, and then a new cambium is formed

constituting the successive cambia, which suggests an additional developmental event. Whether these interpretations would hold under a genetic developmental approach is something yet to be explored.

In the challenge to understand developmental changes during evolution, one can notice that evolutionary changes such as heterochrony and heterotopy are likely to involve fewer processes than other changes such as homeosis or evolutionary novelty [97]. Regardless, these combinations of developmental processes as observed in Nyctaginaceae may be under complex gene regulation, given that multiple cellular and tissue processes are involved in the formation of each cambial variant [35] and that morphological fuzziness results from overlapping developmental programs [64]. For example, it is likely that the loss-of-function related to receptor-like kinases (*PHLOEM INTERCALATED WITH XYLEM* - PXY), which positively regulates the WUSCHEL-RELATED HOMEOBOX4 (WOX4), and III HD ZIPs genes (e.g., *PtrHB4*) implicated in regulating the rate of cambial cell division and development of interfascicular cambium [98–102] could be involved in the development of interxylary phloem macromorphologies. In contrast, the superexpression of other class III HD ZIPs orthologous to *Populus* (e.g., *popREVOLUTA*) may result in formation of ectopic cambia [102, 103] which may be similar to the formation of de novo cambium in patterns of cambial variants with multiple cambia, as in cases of successive cambia. In addition, it is likely that the formation of the primary vascular system function as a module independent from the establishment of secondary growth since different secondary architectures can evolve from distinct pre-vascular conditions in the primary stem.

Sheathing axial parenchyma vs. conjunctive tissue: origin, classification, and functional significance.

The term conjunctive tissue has been applied predominantly in the context of cambial variants, particularly for successive cambia [18, 22, 29, 39, 68], but also in cases of interxylary phloem (=included phloem, [104]). In the context of cambial variants, conjunctive tissue is described as the parenchymatous or fibrous tissue between vascular increments (rings) derived from the meristematic zones that produce the new cambium in the successive cambia system. This interpretation has been maintained for cases of successive cambia in Nyctaginaceae [38]. However, to describe the parenchymatous tissue bordering the conducting cells of phloem strands in species with interxylary phloem a different name (i.e., sheathing axial parenchyma) has been applied because this tissue originates from the phloem axial parenchyma formed by the main cambium [35], while conjunctive tissue is formed from remaining cells of the meristematic zone that originates the variant cambium [38, 61, 67]. As indicated in relation to the origin of the cambial variants, the sheathing axial parenchyma and conjunctive tissue as products of these two systems are also regarded as developmentally linked.

The spatial distribution of conjunctive tissue and sheathing axial parenchyma is one of the main aspects resulting in the diversity of stem macromophologies in Nyctaginaceae. Given their structural and organization similarities, these tissues are likely to develop the same functions indicated to wood axial parenchyma (e.g., storage, involvement in mechanical strength, defence against pathogens and in hydraulic maintenance - [87, 88, 89]). In addition, the abundant parenchyma present in some plants with cambial variants may also represent adaptive advantages for increased flexibility, mechanical strength

and injury repair as suggested to climbing plants [20–22, 68]. Other functions have also been attributed to species with successive cambia (e.g., salt sequestration, xylem and phloem three-dimensional network – [68]). Curiously, successive cambia and interxylary phloem, are two types of cambial variants that can be now observed in species with distinct habits, from herbs to lianas and large trees [32,68,90,this study]. In any case, the capacity of producing multiple cambia or secondary phloem within the secondary xylem, in such intricate organization, might represent a beneficial physiological alternative to the typical regular growth of woody plants [29, 39, 86], given the multiple evolution of these cambial variants across angiosperms in both scandent and self-supporting plants. Experimental work is still needed to substantiate these hypotheses.

The impact of transitions in habits, habitats and cambial variants in the diversification of Nyctaginaceae

The diversification of Nyctaginaceae was most probably in the Middle Eocene (~48 Ma), when most of the extant angiosperm families were already established forming the contemporaneous tropical biomes [52]. Other estimates for the split between Nyctaginaceae and close-related families assumes an interval lying between 13 and 33 Ma [109], as inferred for the divergence from Aizoaceae + Phytolaccaceae (e.g., 26 Myr, [110]).

Speciation/diversification rate increased in Nyctaginaceae 28.62 Ma, at the time of emergence of a group comprising the Bougainvilleeae and Pisonieae (“B&P”) clade + the Nyctagineae (“NAX”) clade [42], and has been maintained since then. There is not an apparent unique characteristic for this group that could explain its increase in diversification. However, different hypotheses have been pointed out for the high number of species in each tribe individually. For instance, a remarkable radiation of genera from the NAX clade occurred in deserts of North America, and they are associated with multiple evolutions of cleistogamy and edaphic endemism to grow on gypsum soils (Douglas & Manos, 2007); the B&P clade stands out by having most of the neotropical and large, woody species of the family, which include both the *Guapira/Neea/Pisonia* trees, as well as the shrubby-scandent or tree species of *Bougainvillea* [42]. In addition, the evolution of fleshy anthocarps (the fruits of Nyctaginaceae) in the *Guapira/Neea* lineage and the likely appearance of endozoochory, seems to be one of the possible explanations for the rapid radiation of taxa of this lineage [42, 43].

Commicarpus has experienced a high turnover of species, where many species have been generated but also went extinct, as observed by the rise in both speciation and extinction rate that ultimately involve an increase in the diversification rate. This genus is one of several lineages of Caryophyllales where a diversification rate shift has been detected, indicating a very recent and rapid radiation [111]. In some other caryophyllalean lineages, genome duplications (polyploidy species) were associated with diversification shifts, which was not identified in *Commicarpus* in that study sampling. Within the NAX clade, *Commicarpus* stands out for having few American species and being mostly diverse in Africa, with several species showing restricted distributions (endemics) in tropical regions, some of them also growing on gypsum or limestone [42, 112, 113].

Our results also suggest that there was no increase in diversification in the lineages containing lianas (e.g., *Colignonia*), therefore, contradicting previous hypotheses [33, 34]. This observation is noteworthy because it indicates for the first time with an explicit analysis that higher speciation rates correlated to the evolution of lianas seem not to hold, at least when the whole group has a cambial variant, as it is the case of Nyctaginaceae. On the other hand, we found out that there is a transition from regular to polycyclic eustele in the clade comprising the tribes Boldoeae+Colignonieae+Pisonieae+Bougainvillea+Nyctagineae, which is probably the main vascular character associated to an increase in diversification rate (Fig. 14). However, diversification rate shift results obtained with BAMM (Fig. 14) show that a slight increase in diversification rate occurred in a clade comprising Pisonieae+Bougainvillea+Nyctagineae, a less inclusive group compared to Boldoeae+Colignonieae+Pisonieae+Bougainvillea+Nyctagineae observed in the HiSSE results, thus possibly the shift to a polycyclic eustele (medullary bundles) type was a precursor, or a background variable, for diversification rate shift instead of a trigger [114]. This may suggest that other factors can be involved in the diversification rate shifts within Nyctaginaceae, such as extrinsic variables like habitat occupation.

Conclusions

By comparing the stem developments in all main lineages of Nyctaginaceae, we discovered that the mature vascular architectures range from typical successive cambia to interxylary phloem, following four disparate ontogenies. These ontogenies share developmental stages and thus may contain intermediate forms between the typical state of these two cambial variants. This way, the stem diversity in Nyctaginaceae, which is driven by developmental changes triggered by heterochronic, heterotopic and homeotic processes, may represent a strong case of continuum morphology represented by the evolution of successive cambia from interxylary phloem. Nyctaginaceae is also one of the first groups to show cambial variants in all members of the family, whose ancestor was reconstructed as having interxylary phloem instead of the most endorsed type, successive cambia. These cambial variants are built upon two dissimilar primary vascular organizations, the regular or polycyclic eustele, suggesting that distinct developmental modules are present in the stem ontogeny of these plants. We also presented that high species richness in Nyctaginaceae has probably not been driven by transitions in habits or cambial variants, which indicates that other functional traits, such as the acquisition of medullary bundles, may have been more important in their diversification. Medullary bundles may be advantageous in xerophytic plants, being present in other groups such as Amaranthaceae and Cactaceae. The complex and diverse developmental pathways shown by Nyctaginaceae may be present in close-related families and be of important phylogenetic significance within Caryophyllales, given the likely potential for convergent evolution in this group. Further investigations in the evolution of development of other caryophyllalean families remain essential in our desire for a better interpretation of the morphological evolution in this important lineage. In addition, understanding the genetic regulatory network underlying stem development in Nyctaginaceae seems to be the next step, since it will be easier to identify the role of

genes once it is investigated in plants where the developmental and evolutionary patterns are further comprehended.

Declarations

Availability of data and material

The dataset(s) supporting the conclusions of this article are available in the Figshare repository [https://figshare.com/s/dd889591a9537277b4a2; https://figshare.com/s/d4a00ec93a97478f7891; https://figshare.com/s/238dfc9fc2d302a9df4f; https://figshare.com/s/d3f30d2a302afe185a33; https://figshare.com/s/85ee9518509374521510; https://figshare.com/s/9925d77ea2cd5cccdc7c; https://figshare.com/s/d854203e22fc50fca327].

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Authors' contributions

ILCN, MRP and VA designed the study. ILCN, MRP, RHG organized the methodology. ILCN performed the investigation, data curation and wrote the first version of the manuscript. ILCN and RHG prepared data presentation. All authors read and approved the final manuscript.

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Availability of supporting data

All supporting data are available in Additional files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have provided consent for publication.

Competing interests

The authors declare that they have no competing interests.

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Figures

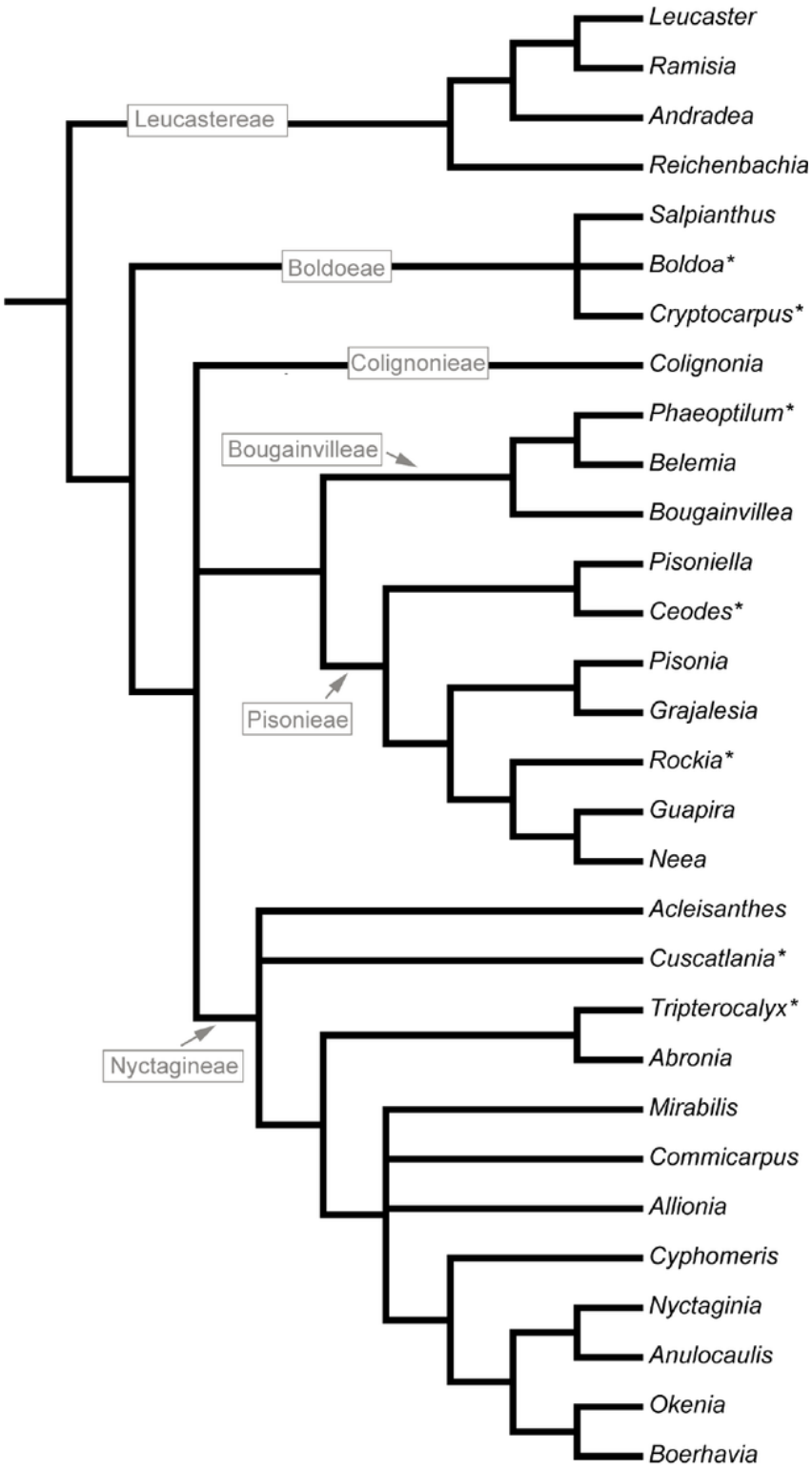

Figure 1

Phylogenetic relationship of major Nyctaginaceae lineages, based on the most recent data (Douglas & Manos 2007; Douglas & Spellenberg, 2010; Rossetto et al., 2019). In the present study, mature stems of all genera in the tree were analyzed (except for Tripterocalyx, Cuscatlania and Boldoa), and ontogenetic studies were carried out for all genera except for the genera indicated with asterisk.

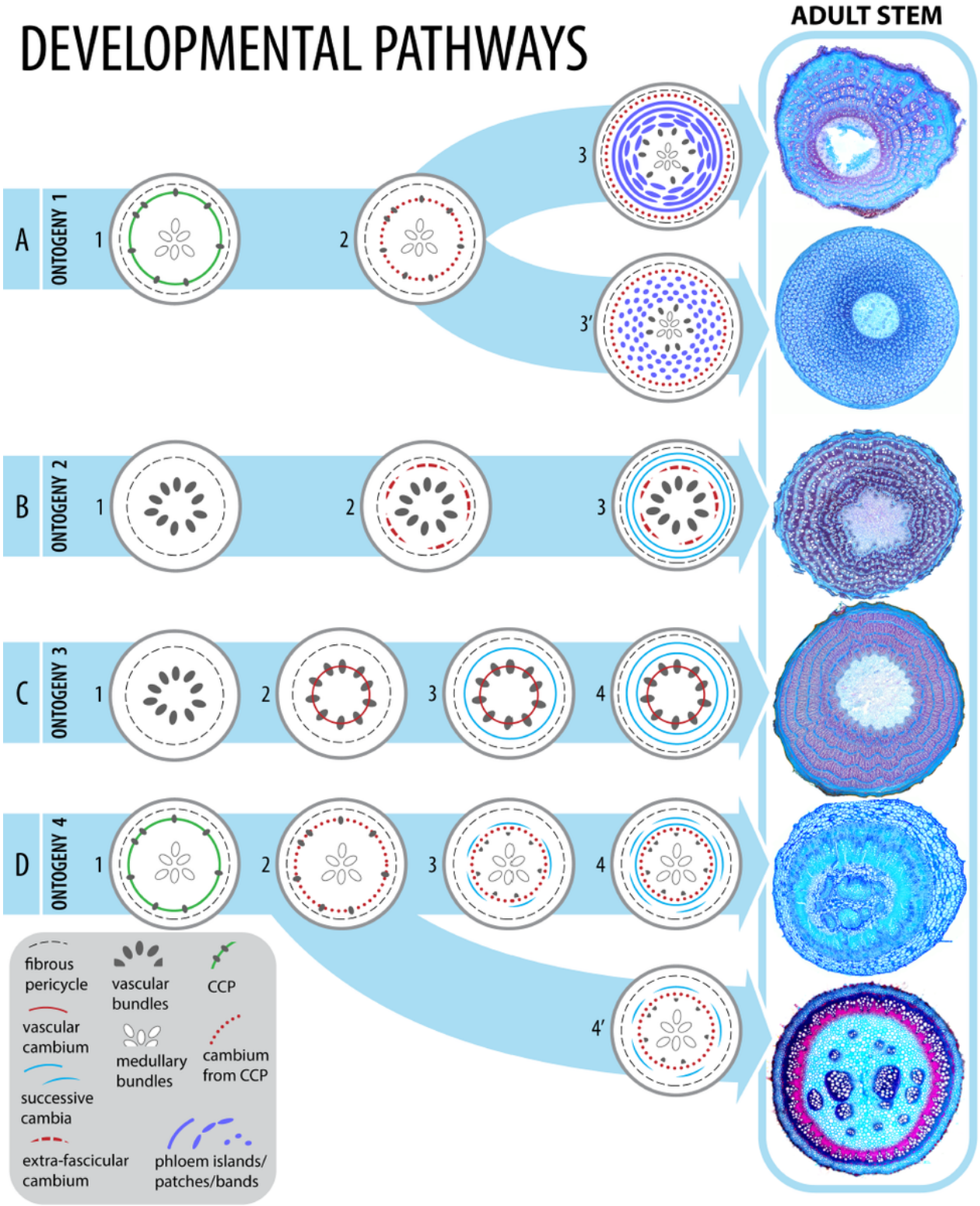

Figure 2

Diversity of stem ontogenies in Nyctaginaceae, illustrating developmental steps from eustele types to cambial variants. a. Ontogeny 1 (interxylary phloem): polycyclic eustele, cambium derived from the CCP, phloem strands with different arrangements, illustrated by Colignonia glomerata (upper) forming bands and Pisonia aculeata (lower) forming phloem islands. b. Ontogeny 2 (successive cambia): regular eustele, extra-fascicular cambium derived from the pericycle, additional successive bands or rings;

Leucaster caniflorus. c. Ontogeny 3 (successive cambia): regular eustele, regular cambium, new cambium formed de novo from the pericycle, additional successive bands or rings; Reichenbachia hirsuta. d. Ontogeny 4 (successive cambia): polycyclic eustele, regular cambium derived from the CCP, new cambium formed de novo from the pericycle, additional small bands or rings of successive cambia; Allionia incarnata (upper) and Okenia hypogaea (lower). Drawing: Marcelo Kubo.

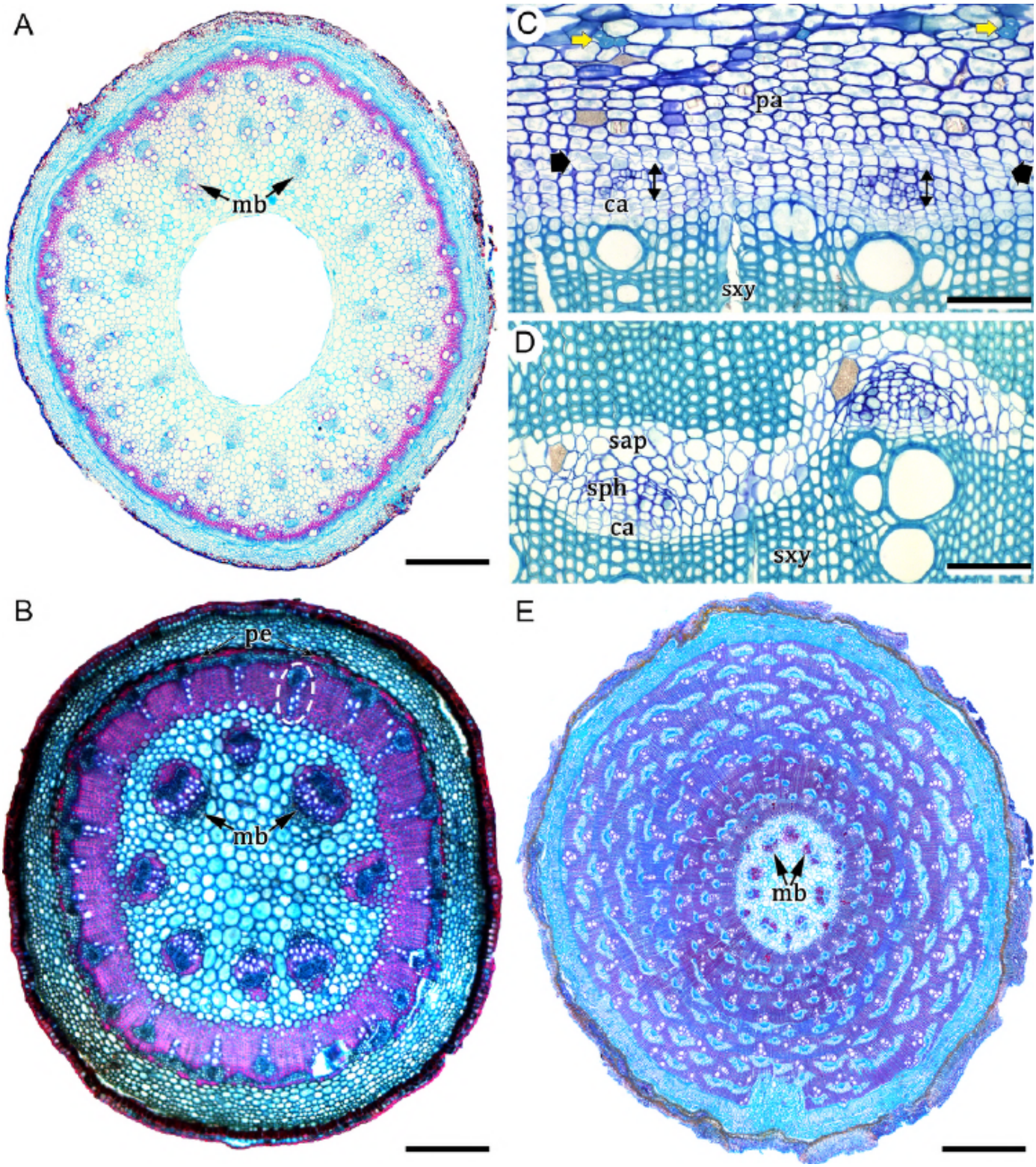

Figure 3

Development of interxylary phloem in stems of Colignonia glomerata and Guapira pernambucensis (ontogeny 1). a. Colignonia glomerata, young stem showing polycyclic eustele and the transition from primary to secondary growth. b-e. Guapira pernambucensis. b. Stem in transition from the activity of the CCP to the cambium; note the vascular bundles (ellipse) formed by the CCP, whose phloem will be the first phloem island. c Irregular activity of the cambium which results in phloem islands (double arrows) after the development of the coalescent (arching) cambium (thick arrows). Note that the coalescent cambium is originated from the axial phloem parenchyma. Yellow arrows indicate pericyclic fibers. d. Two phloem islands which are formed by secondary phloem and sheathing axial parenchyma. e. Mature stem with phloem islands and some patches. Scale bars: 200 μm (a-c); 100 μm (d-e). Abbreviations: ca, cambium; mb, medullary bundles; pa, axial phloem parenchyma; pe, pericycle; sap, sheathing axial parenchyma; sph, secondary phloem; sxy, secondary xylem. (a-b, e) Stained with astra blue and safranin. (c-d) Stained with toluidine blue.

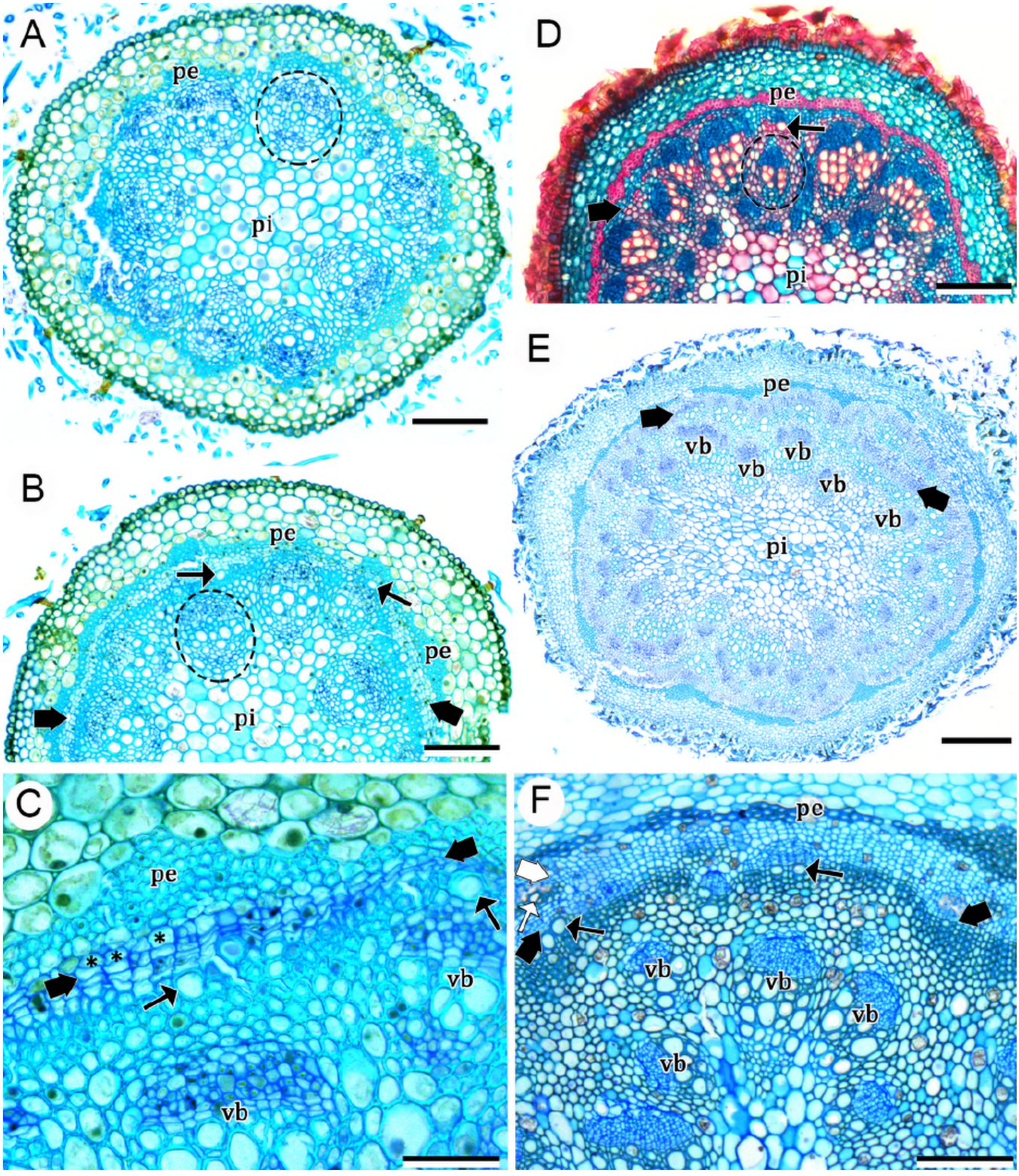

Figure 4

Development of successive cambia in stems of Andradea floribunda, Leucaster caniflorus and Ramisia brasiliensis (ontogeny 2). a-c. Leucaster caniflorus. a. Young stem showing vascular bundles (dashed ellipse) forming a regular eustele. b. Initiation of secondary growth through the formation of the extra-fascicular cambium (thick black arrows) and first formed vessels of the secondary xylem (thin black arrows). c. Detail of the extra-fascicular cambium (thick black arrows) and first formed vessels (thin black

arrows); note the pericycle parenchyma cells (asterisks), and the extra-fascicular cambium (variant) d-f. Andradea floribunda. d and e. Note the extra-fascicular cambium and their vascular products (thick black arrows) with the first formed vessels (thin black arrows), externally to the vascular bundles (dashed ellipse in d). f. Detail of the extra-fascicular cambium (thick black arrows) and first formed vessels of the secondary xylem (thin black arrows) and the subsequent ring of successive cambia and their products (thick white arrows), with the first formed vessels of the successive cambia (thin white arrows). Scale bars: 100 µm (a-b, d); 50 µm (c); 400 µm (e); 200 µm (f). Abbreviations: pe, pericyclic fibres; pi, pith; vb, vascular bundles of regular eustele. (a-d, e-f) Stained with toluidine blue. (c) Stained with astra blue and safranin.

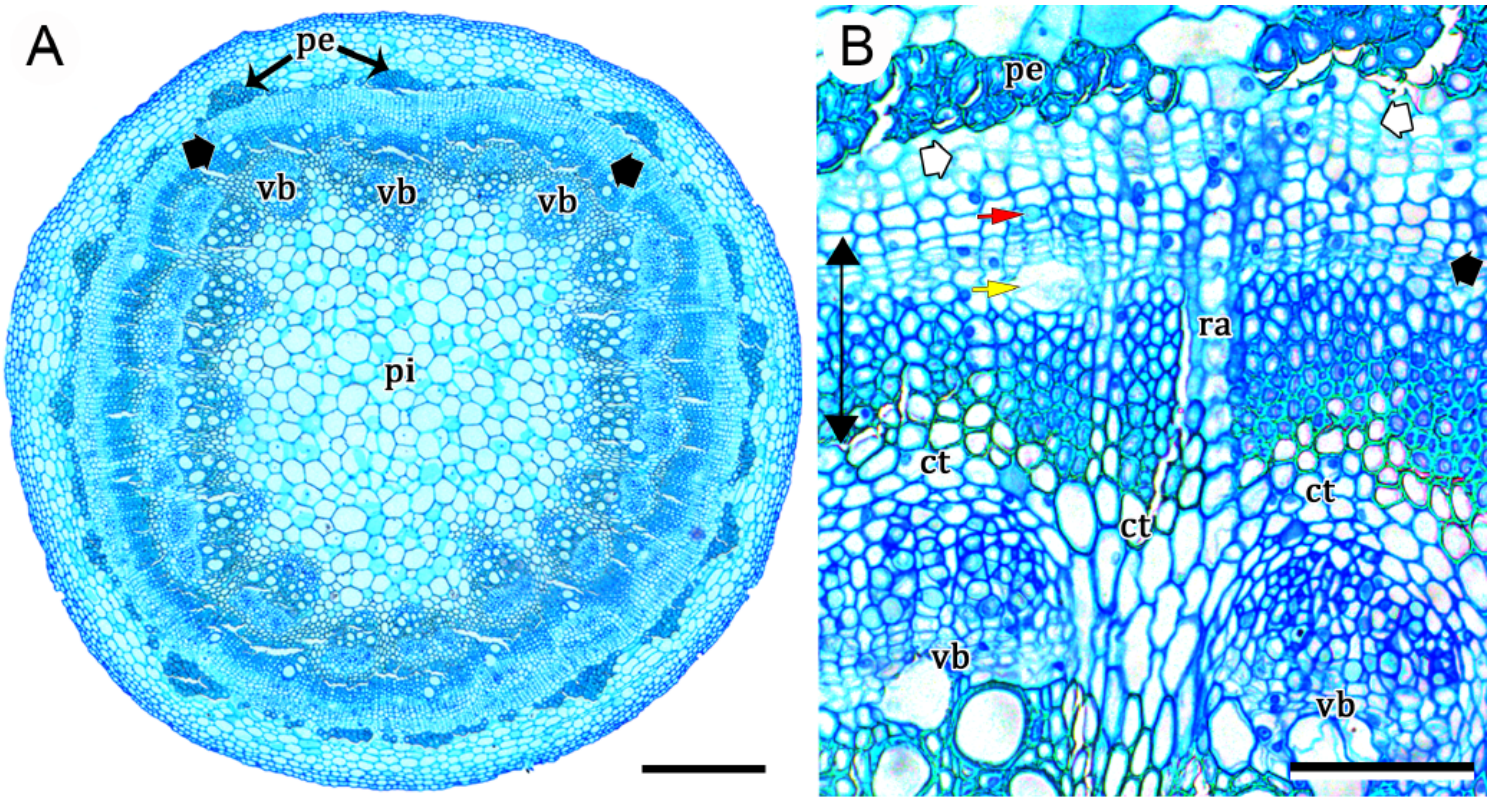

Figure 5

Details of the extra-fascicular cambium and first successive cambia in Ramisia brasiliensis (ontogeny 2). a. General view of the stem during the establishment of the extra-fascicular cambium (arrows) outside the vascular bundles of the eustele. b. Secondary vascular tissue (double arrow) originated from the extra-fascicular cambium (black arrow). The yellow arrow indicates a vessel element, and the red arrow indicates a sieve-tube element and its companion cell. Note the developing meristematic zone (white arrows), still with little layers of cells, and that will give rise to the subsequent ring of successive cambia; also note the conjunctive parenchyma cells between the vascular bundles and the first ring of successive cambia. Scale bars: 400 µm (a); 100 µm (b). Abbreviations: ct, conjunctive tissue; pe, pericyclic fibres; pi, pith; ra, ray; vb, vascular bundle of the eustele. (a, b) Stained with toluidine blue.

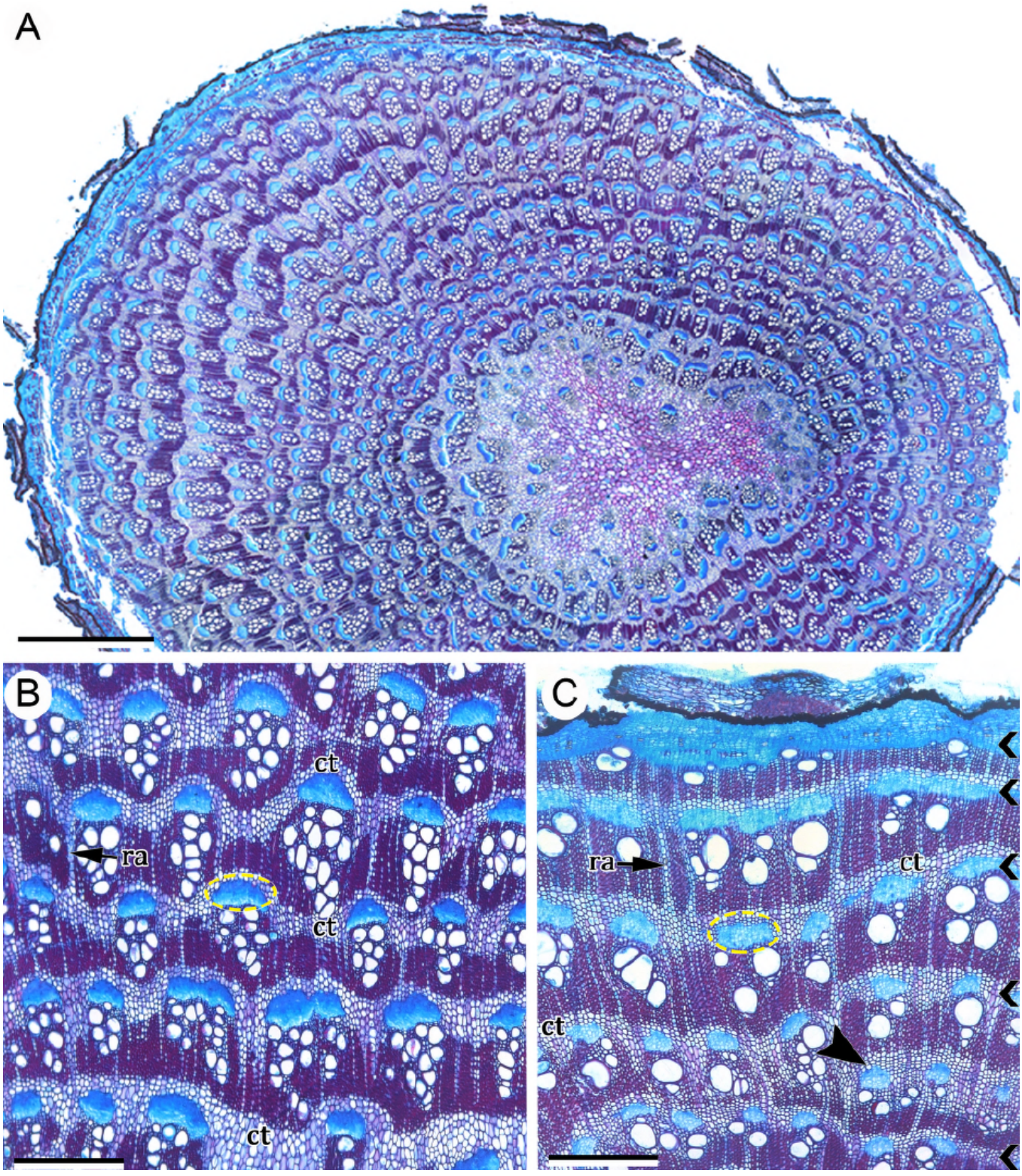

Figure 6

Cross view of developed stems with successive cambia (ontogeny 2). a and b. Andradea floribunda. C. Leucaster caniflorus. a. Adult stem showing several rings of successive cambia. b. Detail of the successive cambia and their vascular products; the phloem form small strands (yellow ellipse) and are composed mainly by sieve-tube elements and their companion cells bordered by the conjunctive tissue; note the wavy appearance of the new increments caused by the conjunctive tissue. c. Rings of successive

cambia in more or less regular concentric arrangement (pointers); the arrowhead indicates an incomplete segment between the other upper and lower rings; note the phloem forming small strands (yellow ellipse). Scale bars: 2000 µm (a); 500 µm (a-b). Abbreviations: ct, conjunctive tissue; pe, pericyclic fibres; pi, pith; ra, ray. (a-c) Stained with astra blue and safranin.

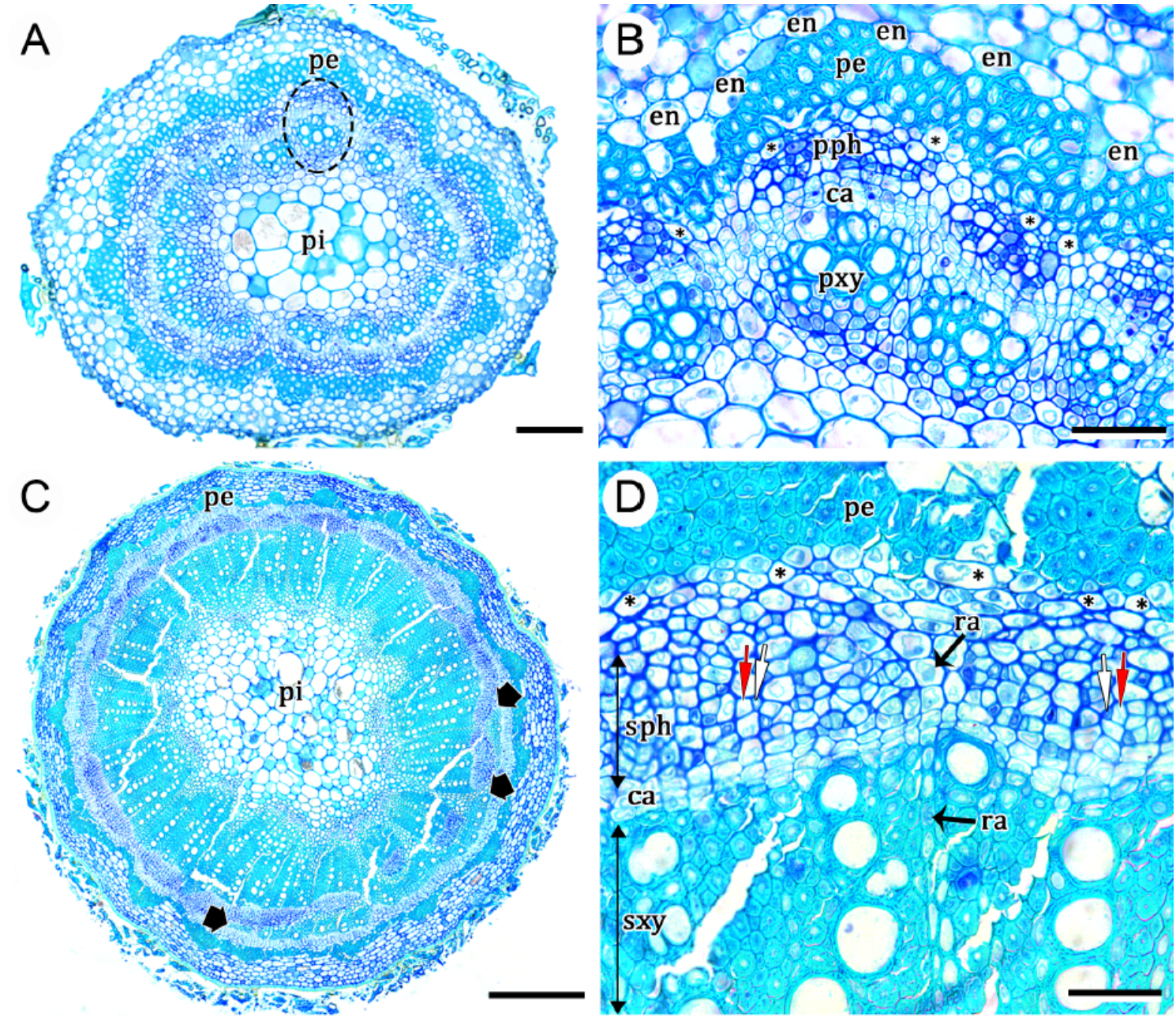

Figure 7

Stem development in Reichenbachia hirsuta (ontogeny 3). a. Young stem showing early secondary growth derived from a regular eustele; the dashed ellipse indicates the position of a vascular bundle. b. Detail of regular cambium and its derivatives, primary vascular tissues, fibrous pericycle and parenchymatous pericyclic cells (asterisks). c. Stem during regular secondary growth. Note the appearance of the first new cambium and its derivatives (thick arrow); see details in next figures (8a-d). d. Detail of previous image showing regular cambium producing secondary xylem and secondary phloem.

Scale bars: 50 µm (a-c); 400 µm (d). Abbreviations: Arrow (white), companion cell; Arrow (red), sieve-tube element; Asterisks, pericyclic parenchyma cells; ca, regular cambium; en, endodermis; mz, meristematic zone; pe, pericyclic fibres; pi, pith; pph, primary phloem; pxy, primary xylem; ra, ray; sph, secondary phloem; sxy, secondary xylem. (a-d) Stained with toluidine blue.

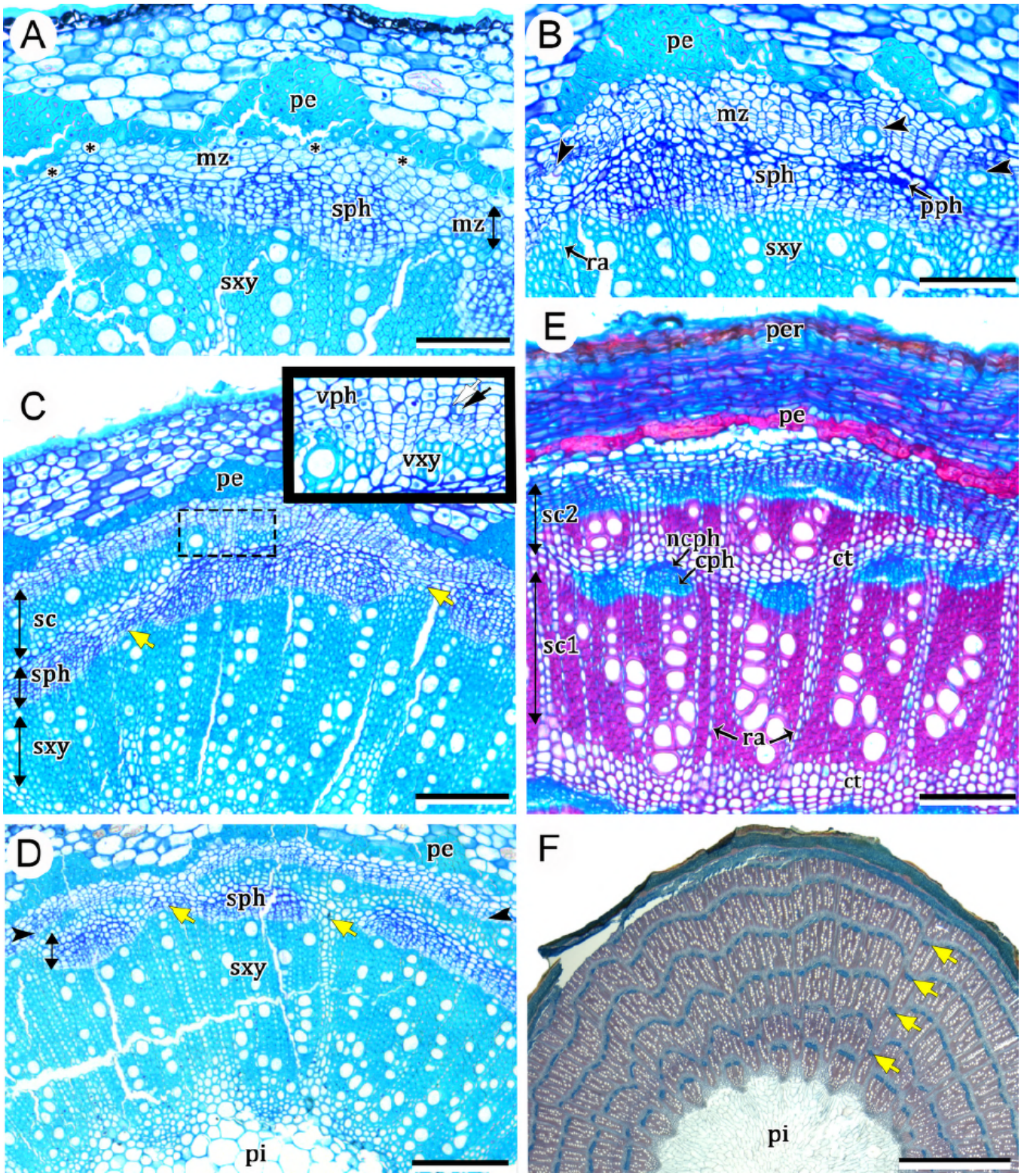

Figure 8

Development of successive cambia in stems of Reichenbachia hirsuta (ontogeny 3). a. Meristematic zone; asterisks indicate pericyclic cells that remain parenchymatous. b. Development of the first new cambium within the meristematic zone. c. First ring of successive cambia with their variant xylem and variant phloem (sc). Dashed rectangle indicates the inset. d. Initially, the interfascicular cambium (yellow arrow) produces mostly phloem axial parenchyma to the outside; the vascular tissue may be produced at unequal rates which contribute to irregular bands of vascular tissue (double arrow). e. Detail of developed stem showing two rings of successive cambia with conjunctive tissue between them. f. Cross view of adult stem showing several rings of vascular tissue; note the irregular regions resulted from differential activity at some regions of the new cambia (yellow arrows). Scale bars: 100 µm (a-c); 200 µm (d-e); 500 µm (f). Abbreviations: Arrow (black), sieve-tube element; Arrow (white), companion cell; Arrowhead, variant cambium; cph, conducting phloem; ct, conjunctive tissue; ncph, non-conducting phloem; mz, meristematic zone; pe, pericyclic fibres; per, periderm; pph, primary phloem (crushed); ra, ray; sc, sc1 e sc2, increments of successive cambia; sph, secondary phloem; sxy, secondary xylem; vph, variant phloem; vxy, variant xylem. (A-D) Stained with toluidine blue. (D-E) Stained with astra blue and safranin.

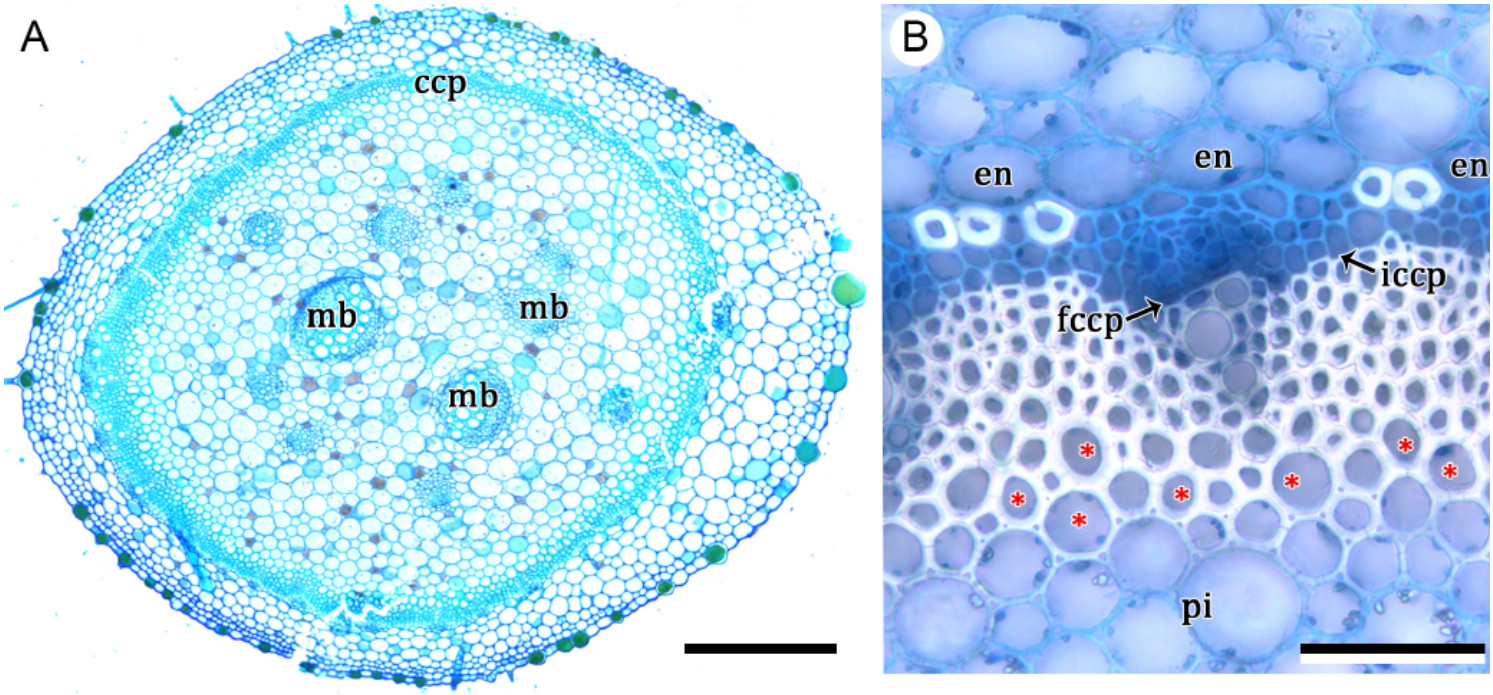

Figure 9

Cross sections of Okenia hypogaea (ontogeny 4) in primary growth. a. Note the polycyclic eustele with medullary bundle and continuous cylindrical procambium. b. Detail of fascicular procambium (fccp) forming the vascular bundles and interfascicular procambium (iccp) forming mostly xylem fibres; note also pericyclic fibres (large fluorescent cells beneath the endodermis) and lignification of peripheral pith cells (red asterisks). Scale bars: 200 µm (a); 100 µm (b). Abbreviations: ccp, continuous cylindrical procambium; en, endodermis; fccp, fascicular CCP; iccp, interfascicular CCP; mb, medullary bundles; pe, pericyclic fibres; pi, pith. (a) Stained with toluidine blue; (b) Stained with aniline blue.

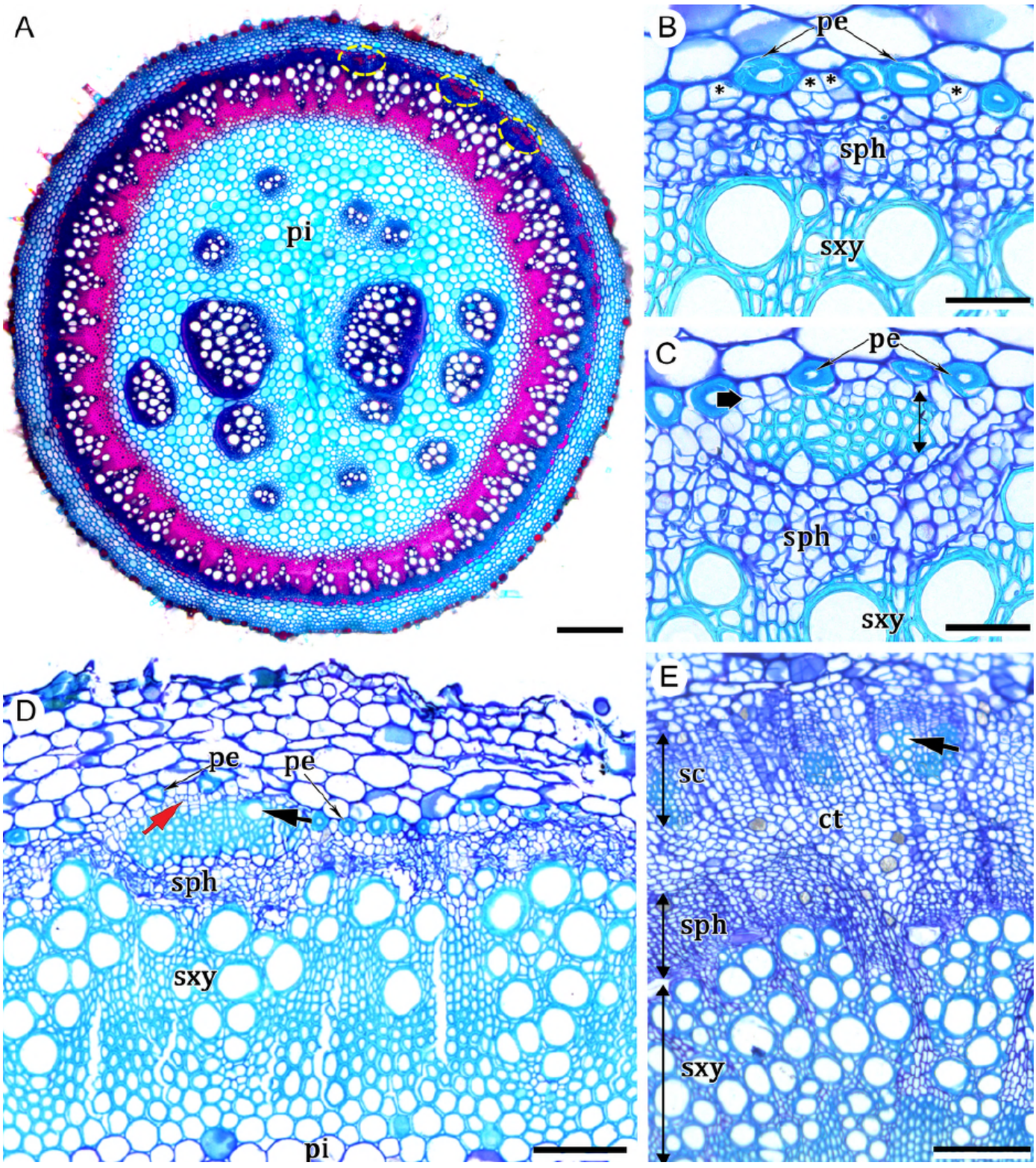

Figure 10

Development of successive cambia in stems of Okenia hypogaea and Allionia incarnata (ontogeny 4). a-d. Okenia hypogaea. e. Allionia incarnata. a. Stem with cambial variant. Dashed ellipses indicate the successive cambia; see detail in figures 10d; note the medullary bundles in the pith. b. Secondary xylem and secondary phloem derived from the cambium of the CCP; note pericycle parenchyma undergoing cell division (asterisks) to form a meristematic zone where new cambia will be formed. c. Developing variant

cambium (thick arrow) and variant xylem formed mostly by fibres (double arrow). d. Developing arc of successive cambia showing variant xylem with first formed vessel (black arrow) and secondary phloem with sieve-tube elements (red arrow). e. Secondary xylem and secondary phloem of the central cylinder separated from the first ring of successive cambia by conjunctive tissue. Black arrow indicates vessel from the variant xylem. Scale bars: 400 µm (a); 50 µm (b, c); 100 µm (d, e). Abbreviations: ct, conjunctive tissue; pe, pericyclic fibers; pi, pith; sph, secondary phloem; sxy, secondary xylem. (a) Stained with astra blue and safranin. (b-e) Stained with toluidine blue.

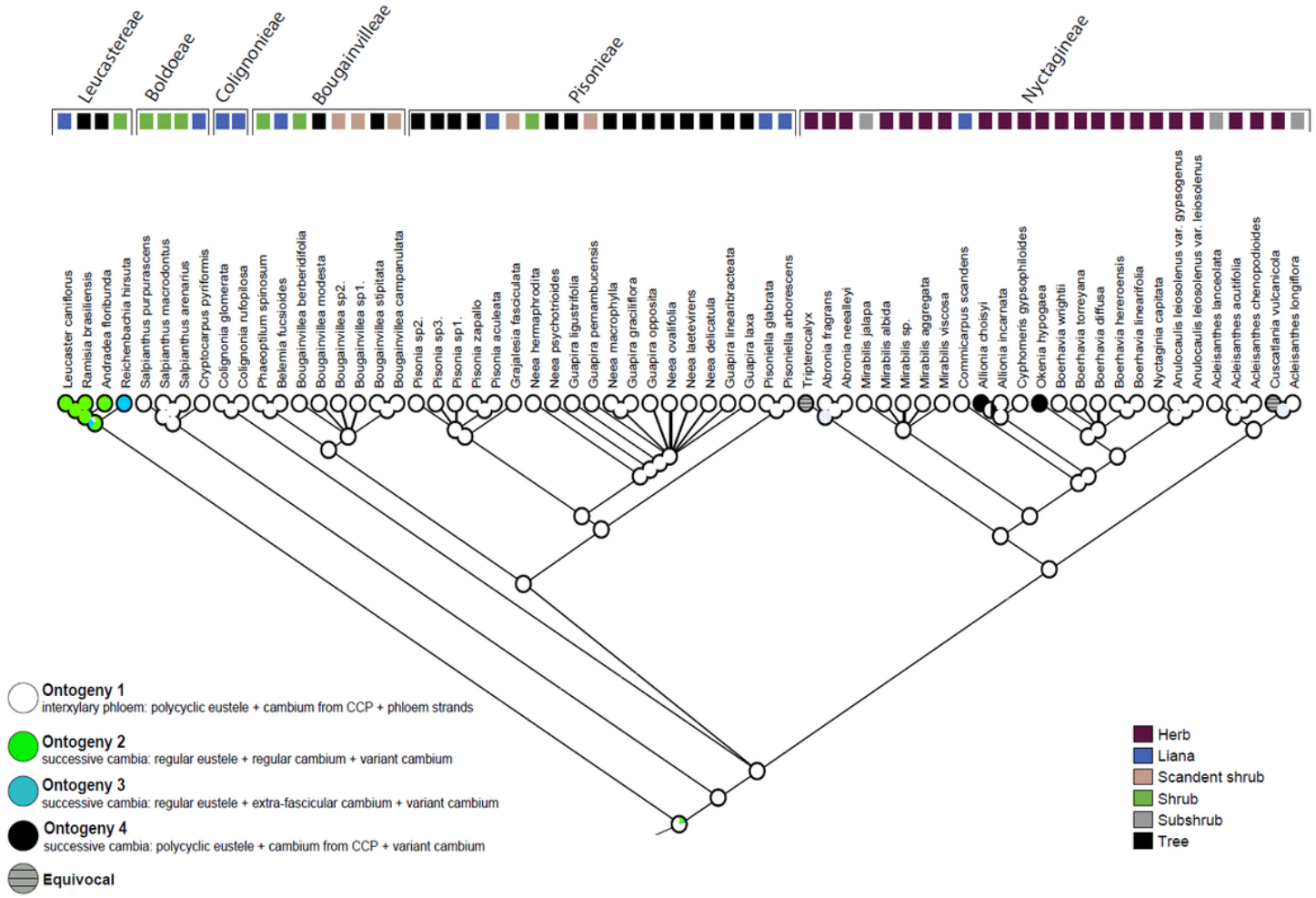

Figure 11

Maximum likelihood reconstruction of ontogenetic pathways mapped with Mesquite on a phylogenetic tree of Nyctaginaceae following the same topology used by Cunha Neto et al. (2020).

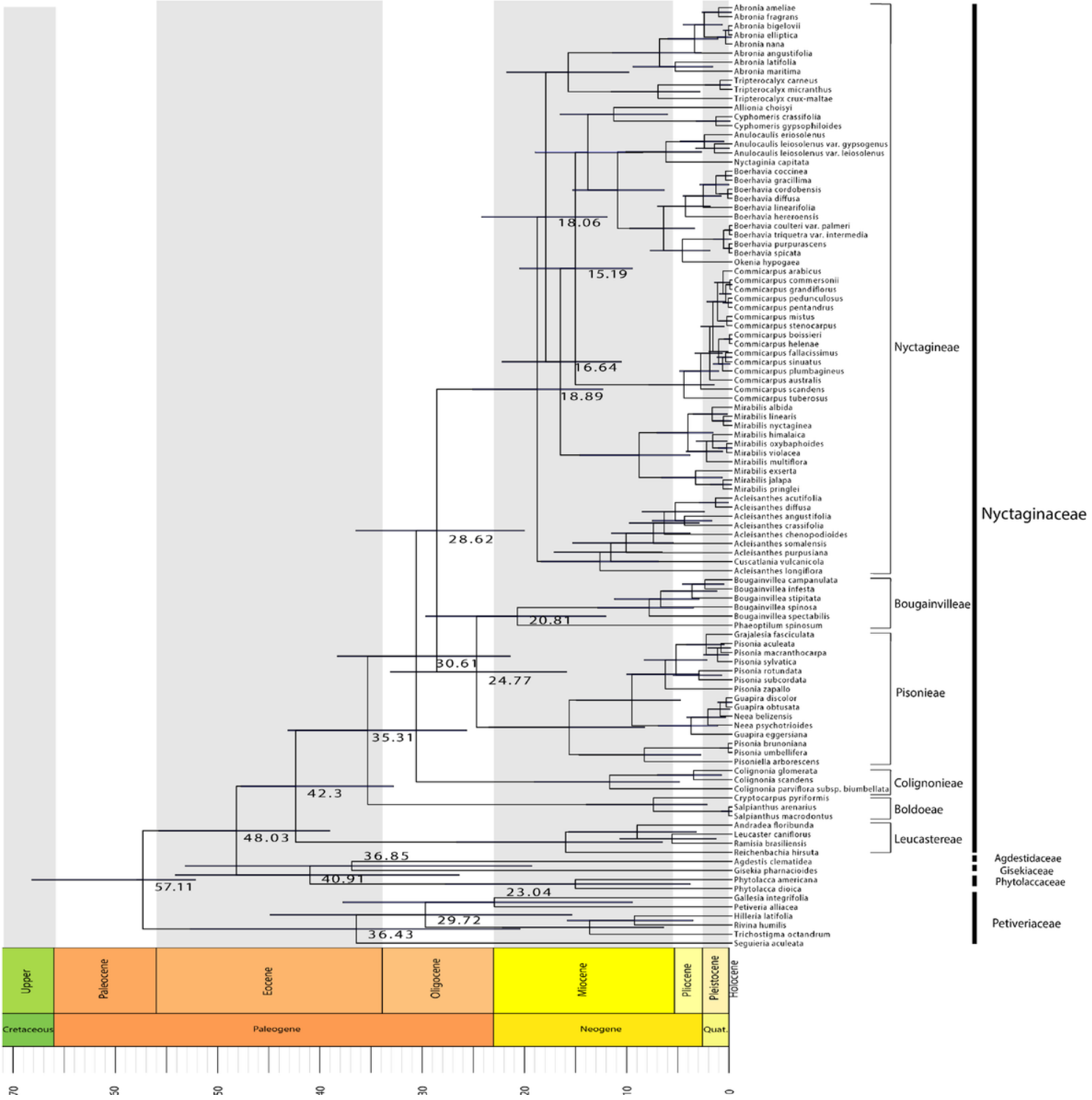

Figure 12

Maximum clade credibility tree (MCC) with divergence time estimates for Nyctaginaceae and related families. Numbers correspond to mean age estimates (in million years). Bars indicate age confidence intervals from a 95% Highest posterior density (HPD).

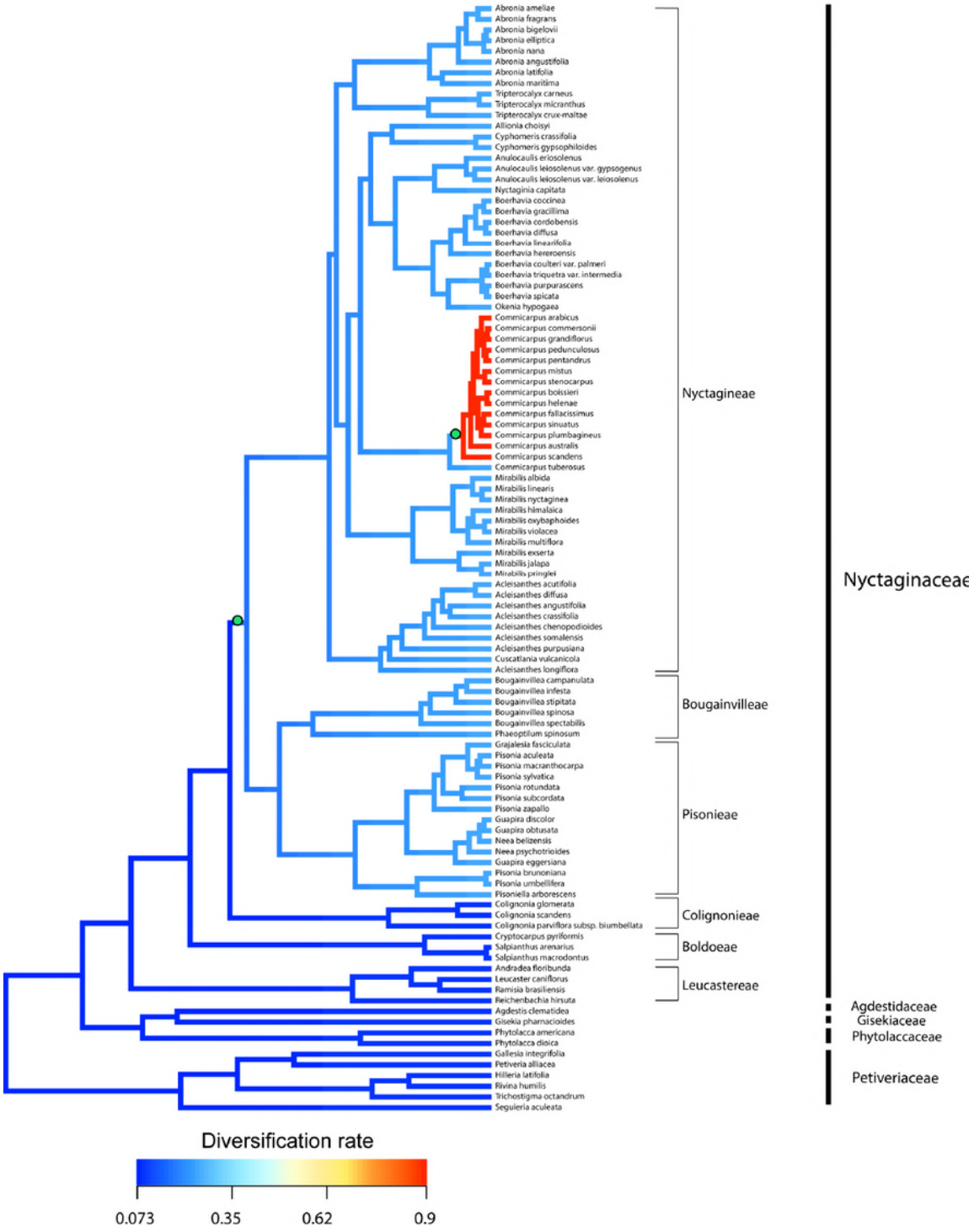

Figure 13

Net diversification rate dynamics in Nyctaginaceae and most-closely related families estimated by BAMM. Branch color reflects the mean of the marginal posterior density of net diversification rates for each segment of the branches, with rates increasing from blue to red. Two green circles indicate the most probable rate shift configuration found using BAMM.

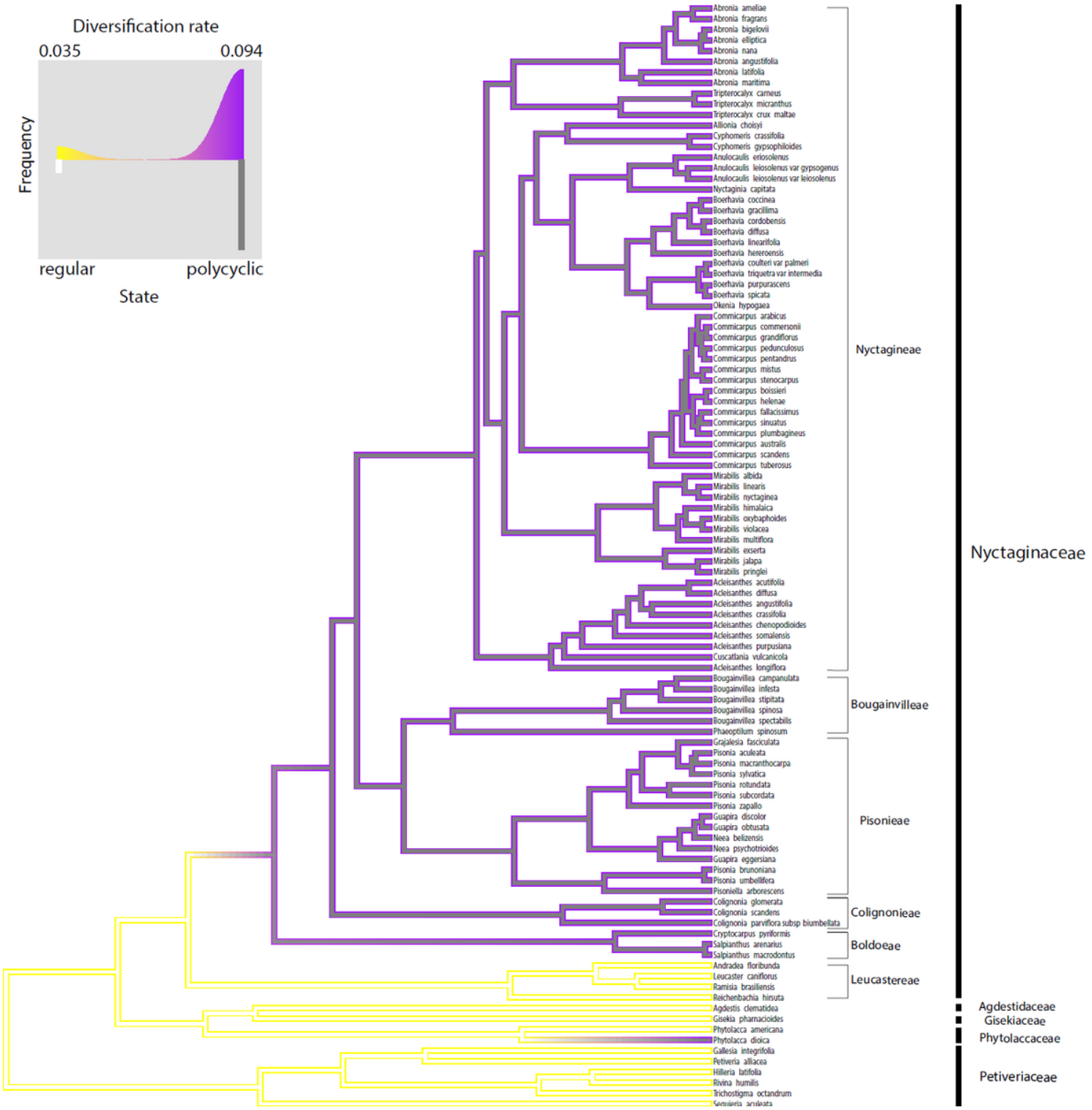

Figure 14

Diversification rate associated to eustele type. Diversification rate and character state transition correspond to the preferred model, BiSSE-like. Diversification rate is represented by branch contour color: yellow = lower rate, purple = higher rate. Character states are represented by branch filling color: withe = regular, gray = polycyclic. Legend shows the histograms of the frequency of species with one of the two states and their association with diversification rate.

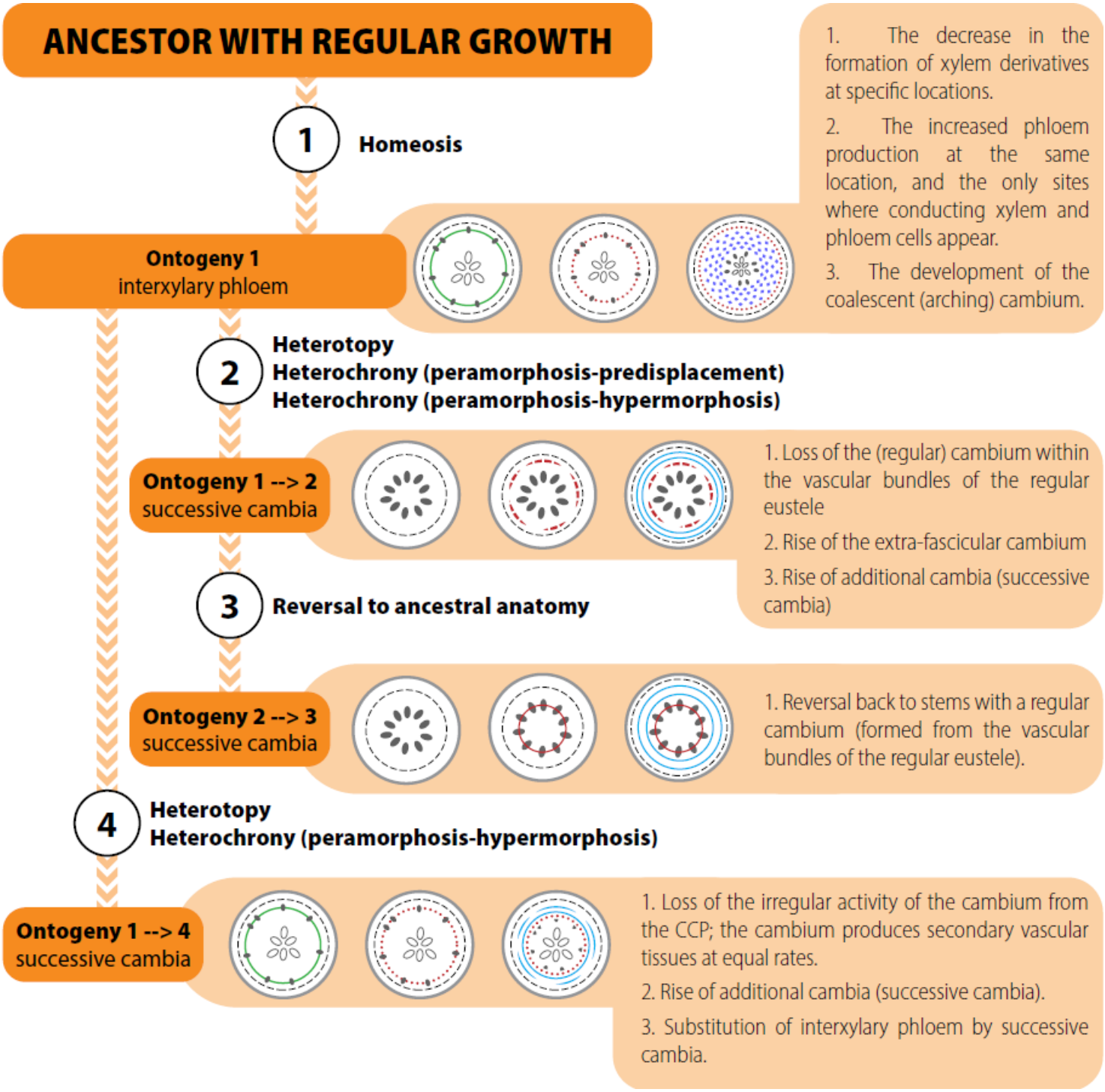

Figure 15

Overview of the anatomical modifications across evolutionary time in the stem vascular system of Nyctaginaceae and the evolutionary mechanisms generating their complex morphological diversity. Drawing: Marcelo Kubo.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile2Tables2Characterdataset.pdf
- AdditionalFile6FigureS1.pdf
- AdditionalFile5Nexusmcc.tree
- Additionalfile1TableS1Listofspecies.pdf
- Additionalfile3HiSSEscript.r
- Additionalfile4TableS3Glossaryofterms.pdf
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