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Heteroblasty in *Conchocarpus heterophyllus* (A.St.-Hil.) Kallunki & Pirani (Rutaceae): An approach of leaf development from the unifoliolate leaves

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ABSTRACT

Heteroblasty is a phenomenon marked by abrupt changes in the development of organs of certain species. In our research on *Conchocarpus heterophyllus*, a shrub with palmately arranged leaflets ranging from 3(4) to 5(–7), we observed significant variations in leaf patterns between seedlings, young plants, and plants in the reproductive phase, indicating transition events during plant development. The focus of this study is to thoroughly investigate these events of heteroblasty and heterophylly in *C. heterophyllus*, with new evidence of the unifoliolate condition in Rutaceae. Morphological and anatomical observations were conducted from mature leaves of individuals at different stages of development. The results show that the heteroblasty in *C. heterophyllus* is marked by changes in leaf patterns across three stages. In stage 1, seedlings exclusively produce unifoliolate leaves from the first foliage leaf. After the eighth to tenth node from the cotyledons, heterophylly becomes evident with the emergence of leaves containing 2–3 leaflets, in addition to unifoliolate leaves, characterizing the second stage. The third stage is defined by the onset of the reproductive phase, marked by the presence of inflorescences. In this stage, the leaf complexity varies, with the number of leaflets ranging from 2 to 5, and unifoliolate leaves are lacking. Furthermore, our study identified the occurrence of vascular traces of leaflets in the swollen region located at the apex of the petiole of all unifoliolate leaves, occurring in stages 1 and 2. These vascular traces are interpreted as remnants of the lost lateral leaflets in unifoliolate leaves and represent novel evidence of this special condition in compound leaves. Heteroblasty in *C. heterophyllus* is a genetically controlled phenomenon, and consequently, prospective investigations involving microRNAs, particularly microRNAs 156 and 172, have the potential to provide valuable insights into the genetic mechanisms underlying the transition between different phases.

1. Introduction

Rutaceae occur in temperate, subtropical, and tropical regions, most notably in Tropical America, South Africa, and Australia (Kubitzki et al., 2011; Appelhans et al., 2021). This family exhibits considerable complexity in leaf patterns, including simple, trifoliolate, palmate, pinnate, and unifoliolate leaves. The latter resemble simple leaves morphologically but have a swollen region or constriction at the tip of the petiole, features that lead most authors to classify them as compound leaves (Kubitzki et al., 2011). Cases of heterophylly have also been reported in this family (Pirani, 1999; Kubitzki et al., 2011; Cruz et al., 2015).

Heterophylly, defined by some authors (Arber, 1919; Pigliucci, 2001; Zotz et al., 2011) as changes in leaf shape within a single plant, may be induced by environmental conditions or caused by intrinsic factors (Font Quer, 1979). In some plant species, abrupt transitions in leaf size and shape occur during development, a phenomenon termed heteroblastic, which is widely observed among angiosperms (Zotz et al., 2011). This is observed in *Passiflora* L. (Passifloraceae), where leaves transition from monolobed to trilobed (Cutri et al., 2013; Chitwood and Otoni, 2017; Silva et al., 2019), and in *Eucalyptus grandis* W. Hill ex Maiden (Myrtaceae), which exhibits ovate leaves in the juvenile phase and lanceolate leaves in the adult phase (Boland et al., 1984). The term heteroblastic was first defined by Goebel (1889) to describe “substantial differences between early and later stages” of development. However, most plants undergo small, gradual changes, primarily in the shape and anatomy of their leaves, a process known as homoblastic development (Goebel, 1889; Zotz et al., 2011). In homoblastic development, there are only slight differences between the juvenile and adult leaf forms (Goebel, 1889).

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According to Cruz et al. (2015), Rutaceae has become a highly promising group for studies on leaf development, particularly concerning ontogenetic evidence of simple/unifoliolate leaves. However, the authors describe that little is known about the ontogenetic mechanisms underlying these variations in the group. A recent study involving ancestral state reconstruction showed that compound leaves are likely the ancestral condition within the family, with several transitions to unifoliolate or simple blades (Appelhans et al., 2021). This indicates that the genetic mechanism for the morphogenesis of unifoliolate or simple leaves is a derived condition within Rutaceae. Therefore, understanding the mechanisms behind this diversity will not only provide a better understanding of leaf development from an ontogenetic perspective but will also be valuable from a phylogenetic standpoint, aiming to elucidate the diversity of these processes throughout the evolutionary history of the family.

Conchocarpus J.C.Mikan, the largest genus in the Galipeinae subtribe (tribe Galipeeae, Zanthoxyloideae, Rutaceae), comprises approximately 52 species and exhibits significant diversity in leaf patterns. The leaves are predominantly alternate (opposite only in *C. oppositifolius* Kallunki), and the leaf blade varies from simple (a rare phenotype) to 1–(3–7)–foliolate in a palmate arrangement (Kallunki and Pirani, 1998; Pirani and Groppo, 2020). The genus is distributed from Central America to southern Brazil, with particular diversity in the Brazilian Atlantic Forest (Kallunki and Pirani, 1998; Colli-Silva and Pirani, 2019). *Conchocarpus heterophyllus* (A.St.-Hil.) Kallunki & Pirani is an unbranched shrub found in the Atlantic Forest domain. This species is characterized by alternate leaves clustered at the tips of branches (Fig. 1), bearing leaflets ranging in number from 3 to (rarely) 7 in a palmate arrangement (Kallunki and Pirani, 1998; Pirani and Groppo, 2020).

In this paper, we present data from observations of *C. heterophyllus* individuals at different developmental stages, where we identified polymorphism in leaf patterns (heterophylly), representing the first record of heteroblasty based on ontogenetic data. Additionally, we recorded vascular traces of leaflets in the swollen region, providing novel evidence of the compound nature of unifoliolate leaves.

2. Material and methods

Populations of *C. heterophyllus* (15 to 20 individuals) cultivated in the Department of Botany of the Biosciences Institute at the Universidade de São Paulo (IB–USP) were analyzed from seedlings to reproductive stage. The voucher specimen (*El Ottra 11*) was deposited in the herbarium of the Universidade de São Paulo (SPF). We also investigated variations in the leaf patterns of *C. heterophyllus* using specimens from the SPF herbarium and virtual databases such as Herbário Virtual Reflora (Pirani and Groppo, 2020).

Shoot apices and mature leaves were collected from individuals in different development stages, and fixed in formalin–acetic acid–alcohol 50% (FAA50) for 24 h (Johansen, 1940) and stored in 70% ethanol. Samples were dehydrated in tertiary butyl alcohol series (50–100%) and embedded in Paraplast (Johansen, 1940). Longitudinal and transverse sections of the shoot apex, petiole (base, middle, and apical regions), and leaf blade were cut at 7–10 μm using a rotary microtome (Leica, Germany). The sections were stained with 1% safranin in 50% ethanol and 1% Astra blue in 50% ethanol (Bukatsch, 1972), and mounted with Entellan®.

The venation patterns were classified according to Ellis et al. (2009) from leaf blades following the methodology described by Strittmatter (1973). Anatomical analyses were carried out and documented with digital photographs utilizing a Leica DMLB light microscope (Leica Microsystems, Wetzlar, Germany).

3. Results

3.1. Description of the heteroblastic series

Observations from the living individuals in the early vegetative phase to the reproductive phase, show that it is possible to define a heteroblastic series in three stages (Fig. 2). In the first stage, the plants have two cotyledons and subsequent foliage leaves (until the eighth–tenth node), which develop exclusively single-bladed leaves (Fig. 3A and B). Morphologically, at this stage, the leaves are leathery, oblanceolate, with smooth margins and rounded apex. In the second stage, after the tenth node from the cotyledons, the shoot apical meristem (SAM) produces unifoliolate, bifoliolate and trifoliolate leaves (Fig. 3C). In this stage, the leaves develop obtuse apices. In the third stage, when the inflorescences were observed, the SAM produces leaves with 2–5 leaflets, and there are not unifoliolate leaves (Fig. 3D). Based on consultations of the SPF herbarium and virtual herbaria, individuals with up to seven leaflets have been recorded, though these are quite rare. The most common phenotype in the reproductive adult stage consists of individuals with up to five leaflets.

A swollen region occurs differently among the leaf types: in unifoliolate leaves this swollen region occurs at the distal portion of the petiole, while the bifoliolate, trifoliolate and palmate leaves have a swollen region at the base of each petiolule.

Fig. 1. *C. heterophyllus*. (A) General view of the plants cultivated in the Department of Botany, IB-USP, São Paulo, Brazil. (B) Arrows indicate different leaf patterns observed in the adult phase: compound leaves with 3–4–5 leaflets. (C) Detail of the thyrsic inflorescence (cymose lateral branches).

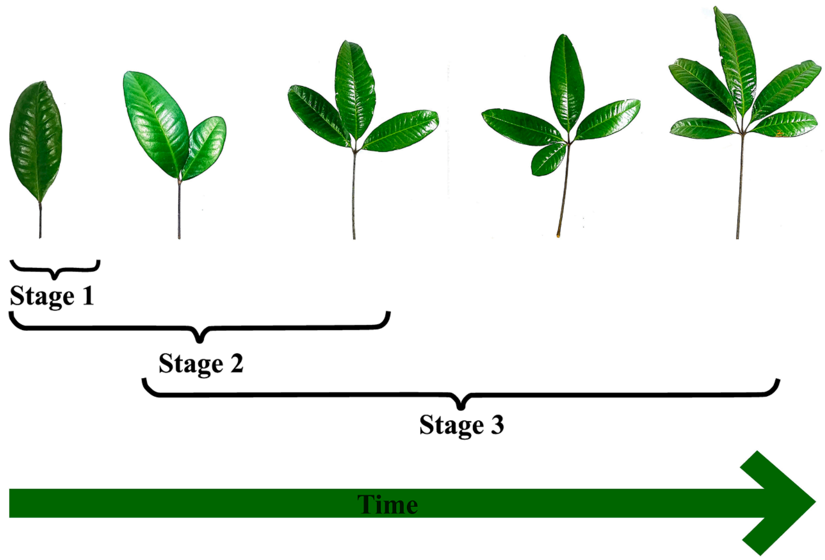

Fig. 2. Sequence of leaves in different stages of the development of *C. heterophyllus*. First stage with only unifoliolate leaves, and in subsequent stages, more complex leaves emerge. This sequence is not strictly linear, and in some cases, there is a direct transition from unifoliolate leaves to trifoliolate leaves. From left to right: Unifoliolate, 2–3–4, and 5 leaflets.

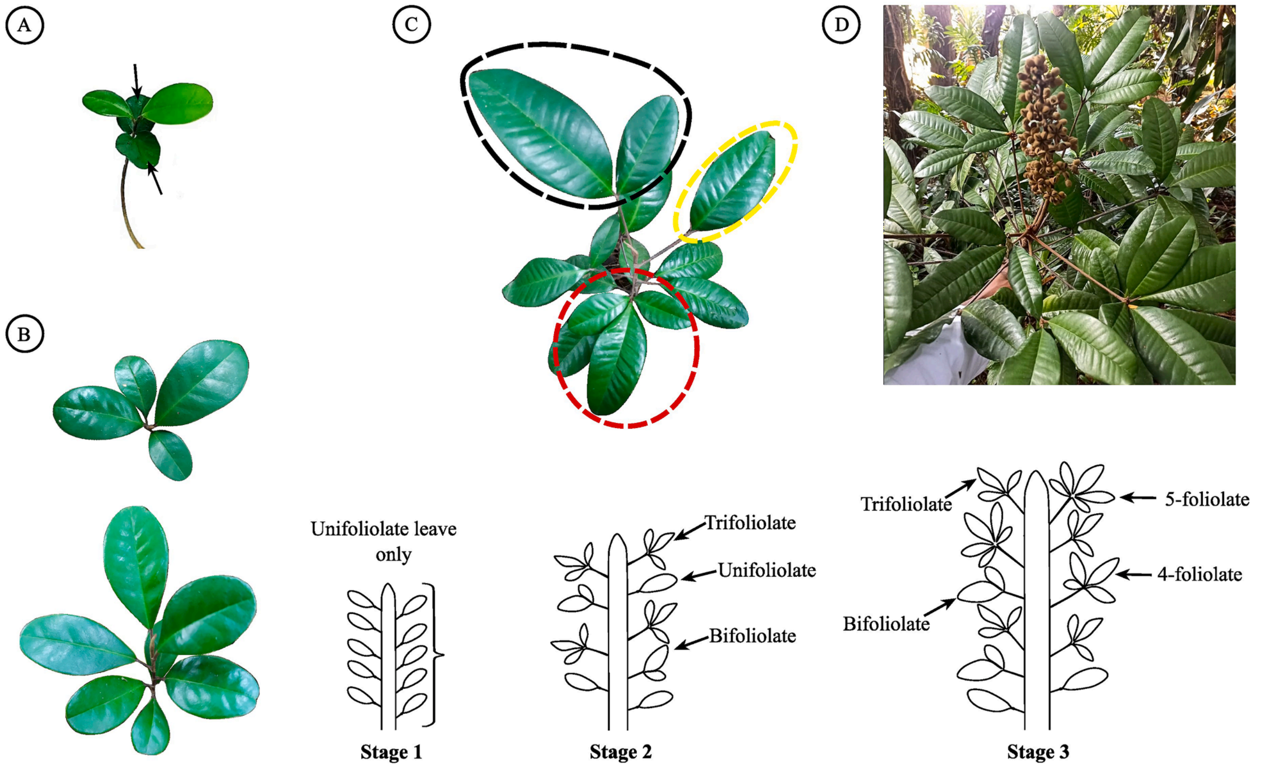

Fig. 3. Stages of heteroblastic transitions in *C. heterophyllus*. (A–B) First stage marked by the exclusive formation of unifoliolate leaves. (A) Plant displaying two cotyledons (black arrows) and the first unifoliolate leaves. (B) Plant showing four and eight nodal regions, with only unifoliolate leaves present. (C) Second stage showing heterophylly with unifoliolate leaves (yellow dashed line), bifoliolate leaves (black dashed line), and trifoliolate leaves (red dashed line). (D) Third stage showing leaves with a range of leaflet numbers (2–3–4, and 5 leaflets).

3.2. Leaf development

In the first stage of leaf ontogeny in *C. heterophyllus*, the shoot apex produces exclusively unifoliolate leaves (Fig. 4A–C). Histologically, the leaf primordia are initially composed of cells with dense cytoplasm and the cells on the abaxial surface undergo structural differentiation, becoming more vacuolated, while cells on the adaxial surface retain meristematic characteristics with dense cytoplasm, indicating a dorsiventral identity in the primordium (Fig. 4B). As development progresses, at the interface between the adaxial and abaxial domains in unifoliolate leaves, the marginal meristem promotes the growth of the leaf blade (Fig. 4C).

The leaf primordia with more than one leaflet begin to develop from the second stage (Fig. 4D–F). In these compound leaves, the lateral

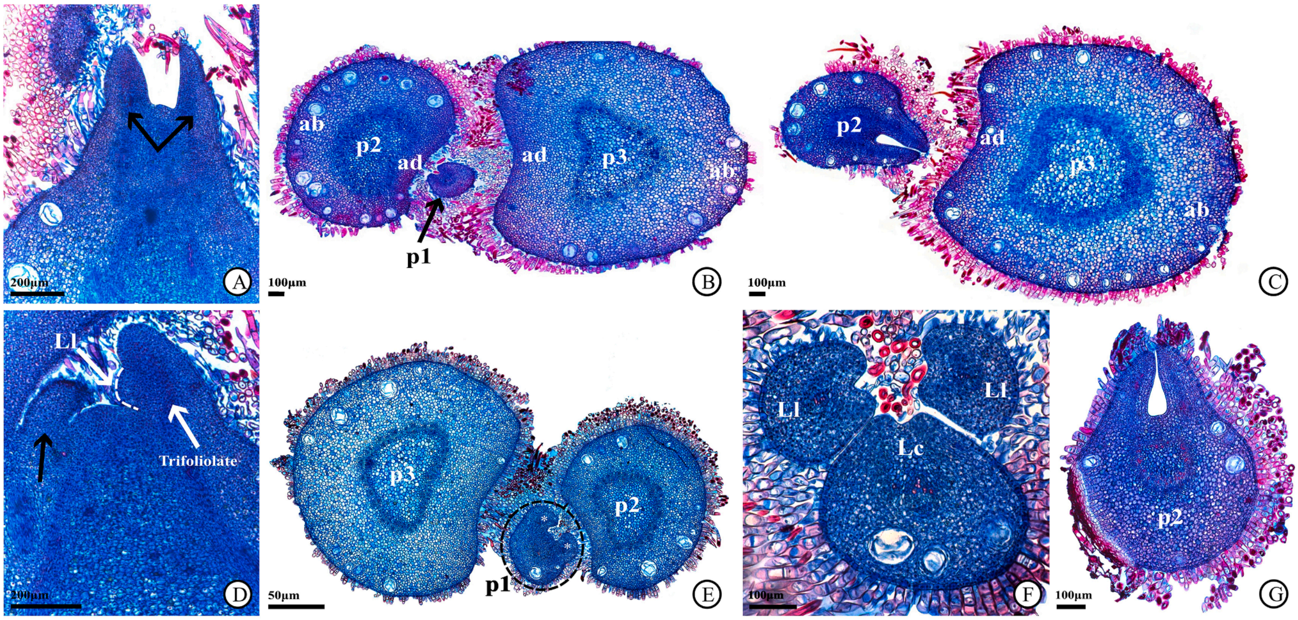

Fig. 4. The ontogenetic development of leaves in the first and second stages of heteroblastic development in *C. heterophyllus*. (A–C) First stage characterized by formation of only unifoliolate leaves. (D–G) Second stage both unifoliolate and trifoliolate leaves begin to emerge. (A) Longitudinal section of the shoot apex with a pair of alternate unifoliolate leaves (arrows). (B and C) Transverse section of the shoot apex from proximal to distal regions shows three unifoliolate leaf primordia. (B) p1 (arrow) is in the blade domain, while p2 and p3 are in the base domain, exhibiting a convex abaxial face and a flat adaxial face. (C) The process of leaf blade formation in p2, through the juxtaposition of the adaxial and abaxial domains, is similar to a simple leaf. (D) Shoot apex with a pair of alternate leaves, one unifoliolate (black arrow) and the other trifoliolate (white arrow), with lateral leaflet primordium forming (white dashed line). (E–G) Transverse sections of the shoot apex, from the proximal to the distal region. (E) Three leaf primordia at different stages of differentiation. p1 demonstrates the formation of lateral leaflets (asterisk), where the primary primordium develops lateral leaflet primordia between the adaxial and abaxial domains. (F) Represents the basic structure of a trifoliolate leaf, consisting of a central leaflet and a pair of lateral leaflets. (G) Leaf blade formation in the unifoliolate leaf, following a similar development process to unifoliolate leaves from the first stage. (ad: adaxial, ab: abaxial, Lc: primordium central leaflet, Ll: primordium lateral leaflet, p1-p3: plastochrons).

leaflet primordia emerge between the adaxial and abaxial domains of the rachis (Fig. 4E and F). Both the central leaflet and the lateral leaflet primordia follow a similar developmental pattern during the growth of the leaf blade (Fig. 4F). The unifoliolate leaf primordia in the second stage develop similarly to those in the first stage, with the leaf blade forming from the adaxial and abaxial domains (Fig. 4G).

In the third stage, heterophyllous sequence forming leaves with 3–4–5 leaflets can be observed in the transverse sections (Fig. 5A–F). In primordia P1, P3, and P5, compound leaves with five leaflets are formed (Fig. 5B, D and F), while P2 and P4 give rise to leaves with four leaflets (Fig. 5C and E). In the axillary bud region of a leaf primordium (P1) two leaf primordia are observed, each featuring a central leaflet and a pair of lateral leaflets at this developmental stage, representing a trifoliate compound leaf (Fig. 5A). After the initiation of the primordia on leaves with 4–5–leaflets, the lateral leaflets emerge from the adaxial/abaxial margins of the central primordium (Fig. 5B–E) and each primordium then forms the leaf blade from its margins, similar to a simple leaf (Fig. 5E and F).

3.3. *Anatomy of the swollen region of the petiole and leaf blade*

The swollen region observed at the distal portion of the petioles of unifoliolate and at the base of the petiolules of palmately compound leaves are morphologically characterized by superficial lenticels, with the swelling being more prominent on the abaxial side of the petiole and petiolules (Fig. 6A and B). Transverse sections of the upper region of the unifoliolate leaf petiole show a closed vascular cylinder (Fig. 6C), while in trifoliolate leaves, at the upper region of the petiole where the leaflets attach, vascular traces diverge, from one another, supplying the lateral leaflets (Fig. 6D). In the swollen region of the unifoliolate leaf (Fig. 6E), there is an increase in cortical parenchyma compared to the non-swollen part. Interestingly, in this swollen region, the vascular traces disrupt the closed formation, giving way to a ring of isolated collateral vascular bundles (Fig. 6E). This is similar to what occurs at the point of leaflet attachment in compound leaves with more than one leaflet (Fig. 6F), where lateral leaflet traces interrupt the closed vascular cylinder (Fig. 6F). The middle region of the petiole in unifoliolate leaves (Fig. 6G) and in leaves with more than one leaflet (Fig. 6H) shows a similar anatomical organization. The leaflet traces merge with the main axis of the petiole, forming a closed vascular cylinder (Fig. 6G and H).

The leaf blade in all observed leaf patterns in *C. heterophyllus* displays the same anatomical features (Fig. 7A–F). In transverse sections, the epidermis is uniseriate, and the mesophyll is dorsiventral, consisting of one layer of palisade parenchyma and about eleven layers of spongy parenchyma, with the presence of secretory cavities (Fig. 7A, C, E). The vascular system in the midrib is a ring formed by a closed cylinder with pericyclic fibers (Fig. 7B, D, F). The leaf venation pattern is classified as camptodromous brochidodromous type (Fig. 8).

4. Discussion

4.1. *Heteroblasty and heterophylly in Conchocarpus heterophyllus*

The sequence of leaf development events originating from the shoot apical meristem, as described in this article, along with the different morphological patterns observed in *C. heterophyllus*, aligns with the original definition of heteroblasty as provided by Goebel (1889), which is based on the occurrence of “abrupt changes in development” within a given species. This is the first detailed description of a heteroblastic series in the genus *Conchocarpus* and the Rutaceae family.

In this study, we demonstrate that heteroblasty in *C. heterophyllus* encompasses a wide range of morphological patterns, from unifoliolate leaves to leaves with up to five leaflets. According to the literature, there are also scattered records of individuals with up to seven leaflets in this

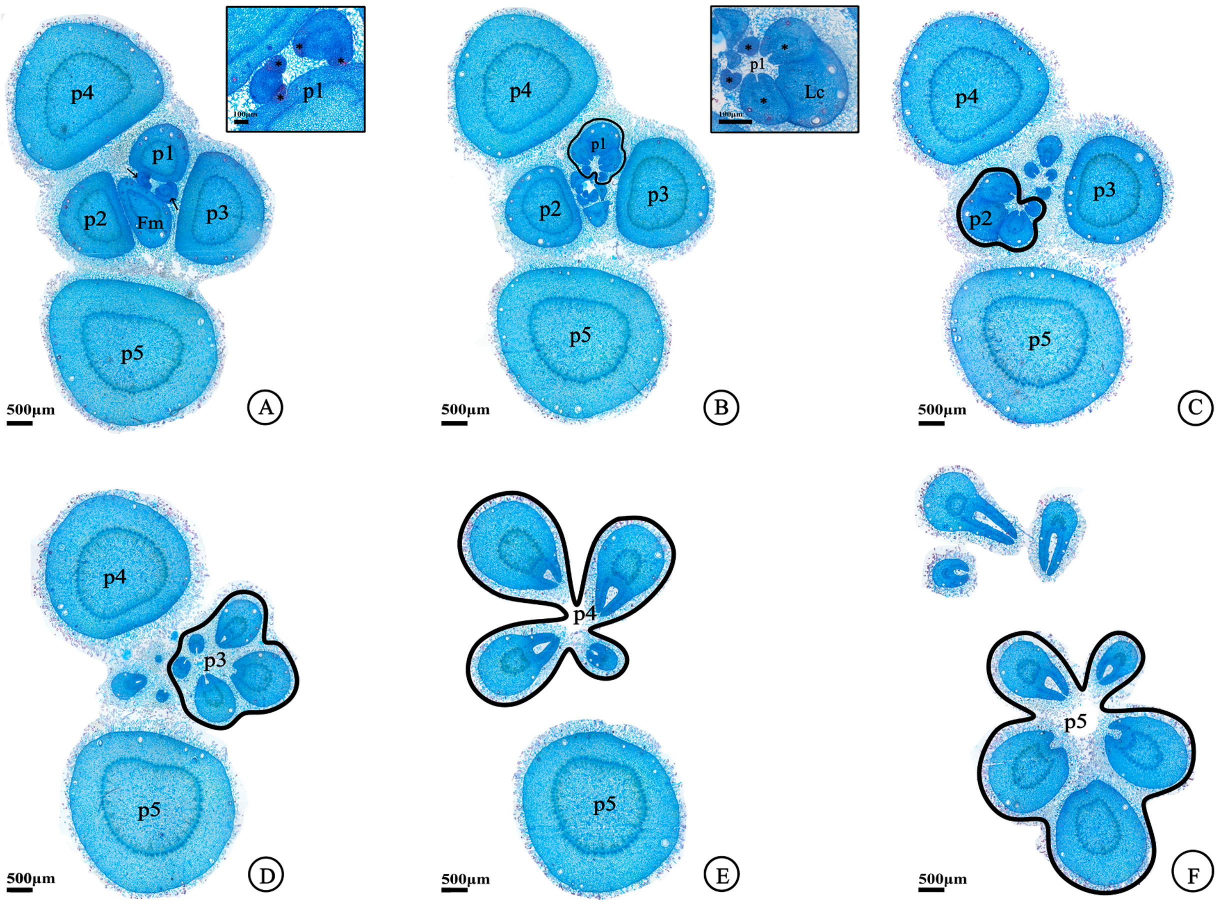

Fig. 5. The ontogenetic development of leaves in the third stage of heteroblastic in *C. heterophyllus*. (A–F) Serial transverse sections of the shoot apex from proximal to distal region. (A) Proximal region showing the petioles from p1–p5. In p1, note the presence of two lateral primordia emerging from the axillary bud (arrow heads), which give rise to trifoliolate leaves (asterisks). (B) Region of p1 showing a leaf with five leaflets, where lateral leaflets (asterisks) are positioned at the juxtaposition of the adaxial/abaxial domains. (C) Region of p2 with four leaflets. (D) Region of p3 with five leaflets. (E) Region of p4 with 4 leaflets. (F) Region of p5 with five leaflets. The leaf blade in all leaflets develops from the marginal meristem (p1–p5). (Lc:primordium central leaflet, Fm: floral meristem, p1–p5: plastochrons).

species (Kallunki and Pirani, 1998; Pirani and Groppo, 2020). We observed that the transition from unifoliolate to bifoliolate and trifoliolate leaves, and in some cases directly to trifoliolate leaves, occurs on average from the eighth to the tenth node, defining the second stage. A similar pattern can be observed in the heteroblastic series of *Leea guineensis* Valeton (Vitaceae), where the authors observed an abrupt transition from simple leaves to multipinnate compound leaves (Gerrath and Lacroix, 1997). In *Berberis* s.l. (Berberidaceae), heteroblasty is evident from the seedling stage (Pabón-Mora and González, 2012), with the first two pairs of leaves exhibiting dentate margins, followed by a sharp shift to trifid spiny leaves on the main axis.

The phenomenon of heterophylly in *C. heterophyllus* has been documented by the variation in the number of leaflets during the second and third stages of development. This is particularly evident in the exclusive occurrence of unifoliolate leaves during the first stage. In a population of *Murraya exotica* L. (Rutaceae), Astuti et al. (2016) identified heterophylly with leaf variation ranging from paripinnate to imparipinnate compound leaves, as well as an intermediate pattern between the two. The occurrence of heterophylly was considered to be influenced by environmental factors or random interactions between genes and the environment (Astuti et al., 2016). Despite heterophylly being induced by various environmental factors such as humidity, temperature, light intensity, photoperiod, and nutrients (Nicotra et al., 2011; Zotz et al., 2011; Nakayama et al., 2017), we believe that in *C. heterophyllus*, this is not influenced by environmental conditions, as all observed individuals are growing in the same environment. Therefore, the changes in morphological patterns in *C. heterophyllus* are likely genetically regulated, rather than driven by environmental factors, as observed in other studies (Gamage and Jesson, 2007; Ostria-Gallardo et al., 2016).

Recent studies with *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) and *Passiflora edulis* Sims. (Passifloraceae) are revealing the genetic basis of heteroblasty, emphasizing the role of microRNAs in conjunction with transcription factors in vegetative development across different plant lineages (Wu et al., 2009; Huijser and Schmid, 2011; Wang et al., 2011; Silva et al., 2019). Among these regulators, miR156 and miR172 play key roles sequentially. miR156 is abundant in the juvenile phase and decreases during the transition to the adult phase, while miR172 exhibits the opposite expression pattern (Wu et al., 2009; Huijser and Schmid, 2011). In this context, future studies on *C. heterophyllus* will help to determine whether the same regulatory mechanisms described in *A. thaliana* and *P. edulis* are operating during the developmental stages in *C. heterophyllus*.

Another case of heterophylly in Rutaceae was described in species of *Metrodorea* (*M. nigra* A.St.-Hil., *M. flavida* Krause, and *M. concinna* Pirani & P.Dias, Pilocarpinae, Rutaceae) by Cruz et al. (2015), who suggested that heterophylly is mediated by a pair of connate stipules at the base of the leaf, which limits the space for the development of primordia. According to the authors, this phenomenon is referred to as “leaf abortion” resulting in variations in the number of leaflets. Although *C. heterophyllus* exhibits a phenotypic pattern during the second stage similar to the *Metrodorea* species mentioned above (1–3 leaflets), it differs in having alternate leaves and the absence of broad stipules that

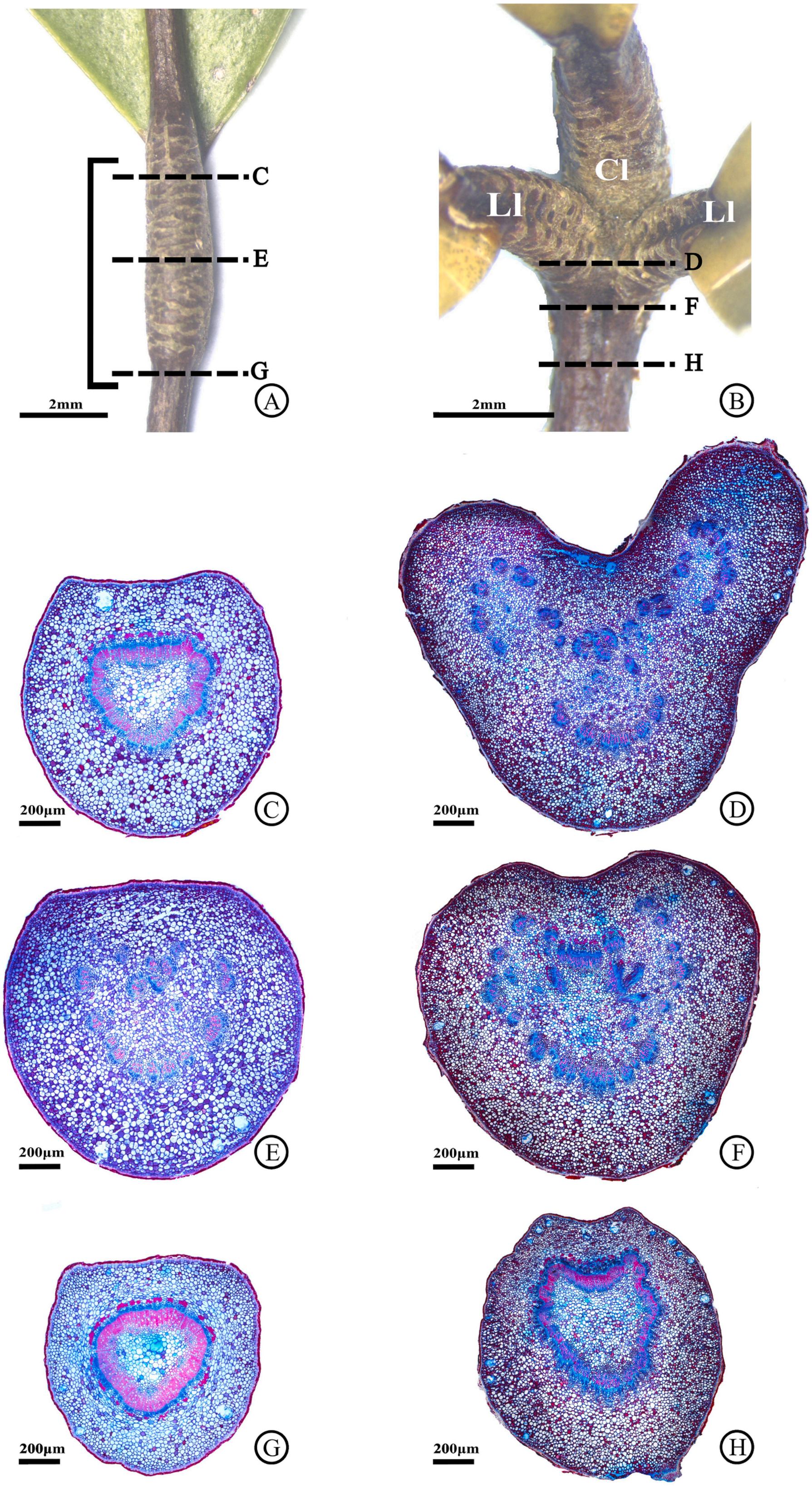

Fig. 6. Similar anatomical organization between the petiole of unifoliolate and trifoliolate leaves. (A, C, E, G) Unifoliolate leaf. (B, D, F, H) Trifoliolate leaf. (A) Shows the non-swollen and swollen regions at the apex of the petiole (bracket). (B) The morphology of the petiole apex with a central leaflet and a pair of lateral leaflets. (C) In the upper region of the unifoliolate leaf petiole with a closed vascular cylinder. (D) In the upper region of the trifoliolate leaf petiole, where leaflets attach, vascular traces diverge to supply the lateral leaflets. (E) Swollen region, showing the expansion of the cortical region and lateral leaflet traces. (F) At the point of leaflet attachment in the trifoliolate leaf. Note the lateral leaflet traces disrupt the closed vascular cylinder. (G–H) Mid-region of the petiole of the unifoliolate and trifoliolate leaves reveals similar anatomical organization. (CL: central leaflet, Ll: lateral leaflet).

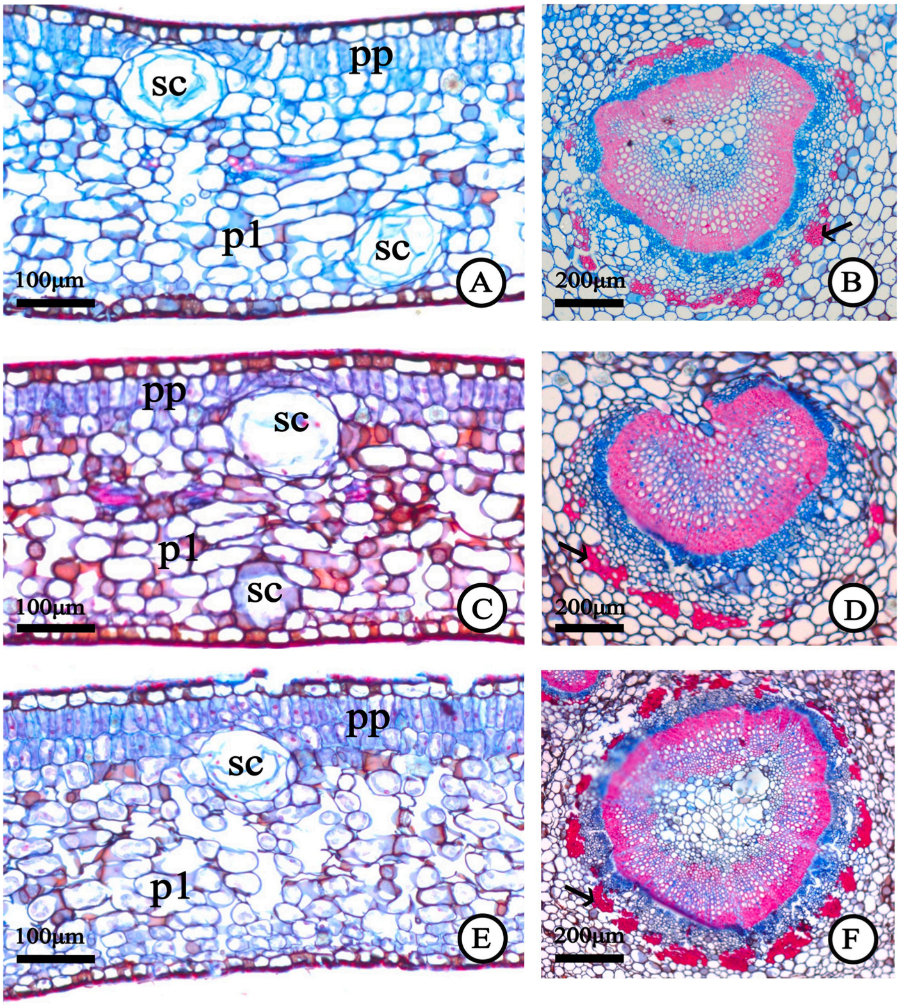

Fig. 7. Leaf anatomy of *C. heterophyllus* in unifoliolate, trifoliolate and palmate leaves. (A–B) Unifoliolate leaf. (C–D) Trifoliolate leaf. (E–F) 5–foliolate leaf. (A, C and E) Transverse section of the leaf blade, showing an uniseriate epidermis, dorsiventral mesophyll, with a single layer of palisade parenchyma, and several layers of spongy parenchyma. Note the presence of secretory cavities in mesophyll. (B, D, and F) Transverse section of the midrib, with a closed vascular cylinder, surrounded by pericyclic fibers (black arrows). (pp: palisade parenchyma, sc: secretory cavity, sp: spongy parenchyma).

act as a limiting factor for the development of lateral leaflet primordia. Therefore, the heterophylly event is different between *C. heterophyllus* and *M. nigra* and *M. flavida*, which belong to sister subtribes within the Galipeeae tribe. Thus, from the absence of aborted leaflets in *C. heterophyllus*, we hypothesize that heterophylly is mediated by the process of heteroblasty, which is responsible for the morphological changes in leaf patterns throughout the plant's life phases.

In the third stage (reproductive phase) of *C. heterophyllus*, there are sequential and progressive changes in each plastochron, resulting in more complex leaves with three to five leaflets. It is interesting to note that unifoliolate leaves are no longer produced in this stage, suggesting that the system for forming more complex leaves, initiated in the second stage, becomes established in the third stage. This indicates that reversion to the unifoliolate state does not occur at this stage of life. Based on these results, we can interpret that in *C. heterophyllus*: 1) unifoliolate leaves represent a phenotype that is only manifested in early developmental stages and can be used as a morphological trait of the juvenile and vegetative adult stages; 2) from the reproductive stage onwards, there is a shift in molecular processes, leading to the production of only compound-palmate leaves.

Morphological features observed in all unifoliolate leaves, as a swollen region at the apex of the petioles (El Hadidi, 1973; Kubitzki et al., 2011) and an articulation between the leaflet and the apex of the petiole (Kubitzki et al., 2011) are derived from the reduction of lateral leaflets. A study conducted by Cruz et al. (2015) documented a similar development pattern to a simple leaf in exclusively unifoliolate leaves of *Metrodorea* A.St.-Hil. (Rutaceae, Pilocarpinae, sister group to Galipeinae) and also reported leaves with one or two aborted lateral leaflet primordia.

Anatomically, in the swollen region, both in unifoliolate leaves and in leaves with multiple leaflets, there is a proliferation of cortical tissue, similar to the swelling described in other Rutaceae species (Cruz et al., 2015; Ferreira et al., 2022). According to Ferreira et al. (2022), this expanded region is similar to pulvini and is referred to as "pulvinus-like thickenings". The authors emphasize the need for studies on leaf movement and ultrastructure to definitively classify this region as a pulvinus.

In Burseraceae (Sapindales), Daly et al. (2011) described the thickening in the petioles and petiolules of pinnate leaves as pulvini and pulvinules in many species. In *C. heterophyllus*, we agree with Ferreira et al. (2022) that studies involving ultrastructural and leaf movement data must be conducted to confirm whether this region can be classified as a pulvinus. Regardless of whether or not it is a pulvinus, our serial anatomical analyses of the entire swollen region of the petiole in *C. heterophyllus* revealed the presence of lateral leaflet traces in unifoliolate and leaves with more than one leaflet. In the case of unifoliolate these traces could be indicating remnants of structures that did not fully develop, as described in carpels, petals, and stamens of Cucurbitaceae,

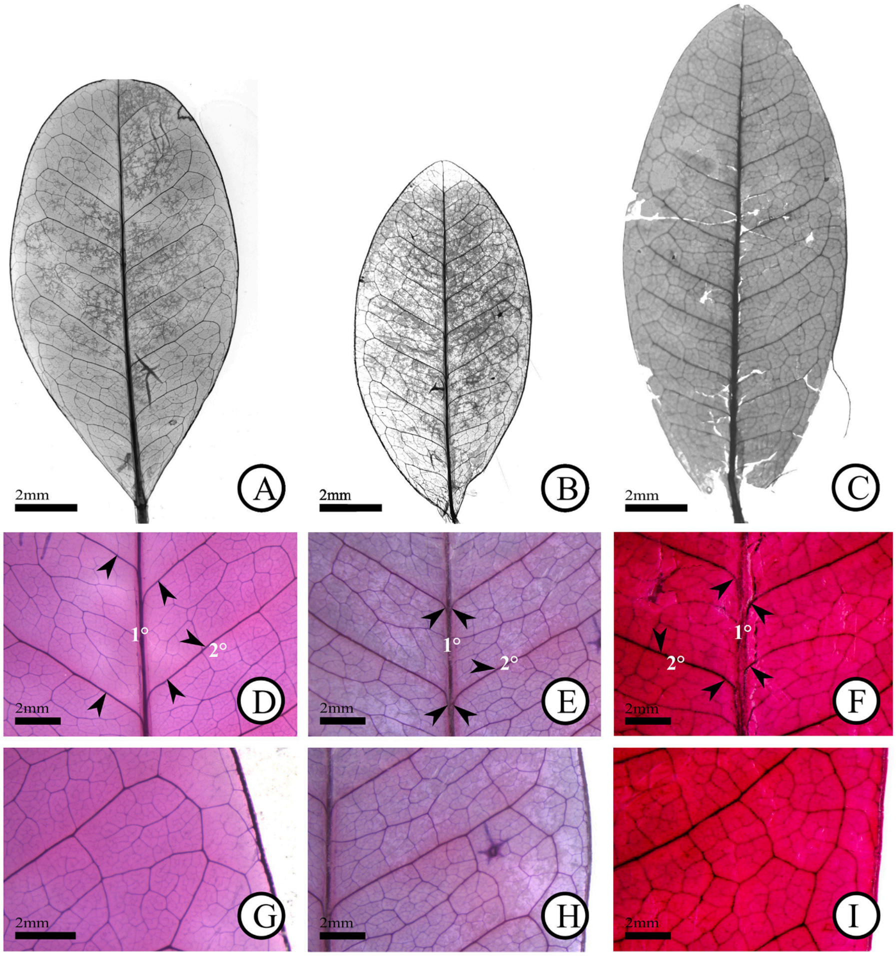

Fig. 8. Vein patterns of *C. heterophyllus* in unifoliolate, trifoliolate and palmate leaves. (A, D, and G) Unifoliolate leaf. (B, E and H) Trifoliolate leaf. (C, F and I) 5–foliolate leaf. All leaves exhibit camptodromous brochidodromous venation (A, B, C, G, H, I). Connections of the secondary veins with the midrib are reticulate in unifoliolate and palmate leaves (D, F), while in the trifoliolate, they are excurrent (F). (1° and 2°: First and second order veins).

Ericaceae, and Rutaceae (Eames, 1961; Fahn, 1982).

4.2. Ontogenetic approach from unifoliolate leaves to compound leaves with more one leaflet

Recent studies on leaf development in species that are exclusively unifoliolate (i.e., not heterophyllous), such as *Metrodorea maracasana* Kaastra and *Esenbeckia grandiflora* Mart. (subtribe Pilocarpinae, Galipeeae), have revealed structural homology between unifoliolate and simple leaves (Cruz et al., 2015), as observed in our study of *C. heterophyllus*. Considering the three continuous and overlapping phases of the leaf development, described in Dengler and Tsukaya (2001) and Melo-de-Pinna and Cruz (2020), we did not observe any differences between unifoliolate leaves and compound leaves during the initiation phase. The divergence occurs during primary morphogenesis, when the leaf sub-regions are established, leading to the formation of lateral leaflets in compound leaves (Dengler and Tsukaya, 2001). In this phase, we observed that the activity of the marginal meristem in unifoliolate leaves produces the leaf blade juxtaposed to the abaxial and adaxial domains of the primordium, similar to what has been described for simple leaves by various authors in leaf development (Avery, 1933; Foster, 1936; Esau, 1977; Waites and Hudson, 1995; Fukushima and Hasebe, 2014; Melo-de-Pinna and Cruz, 2020). On the other hand, as described in our results, in leaves with 2–5 leaflets, the main primordium produces lateral leaflet primordia from the same region, specifically at the interface of the adaxial and abaxial domains. Therefore, we suggest that maintaining the adaxial and abaxial domains is essential for the development of both the leaf blade and lateral leaflets. It is noteworthy that Kim et al. (2003), in their analysis of the expression of *PHANTASTICA* in compound leaves, describe that the *PHAN* expression domain is crucial for establishing the adaxial domain. They also note “that the boundary between adaxial and abaxial domains is required not only for blade formation but also for leaflet formation”.

The presence of both unifoliolate and compound leaves with varying numbers of leaflets on the same shoot of an individual during the same stage of life, suggests that *C. heterophyllus* has different genetic processes occurring during this period. In recent decades, studies on molecular regulation have provided a better understanding of the genetic mechanisms underlying leaf development. The presence of lateral leaflet traces in leaves of *C. heterophyllus* demonstrates that its underlying condition is indeed unifoliolate compound, a state which was previously inferred by phylogenetic reasoning but which is now empirically observed.

Therefore, it would be expected that, at some point in the leaf development, the molecular basis for compound leaves is expressed. This could involve mechanisms similar to those seen in species like *Solanum lycopersicum* L. and *Cardamine hirsuta* L., where Class I *KNOTTED1-LIKE HOMEOBOX* (*KNOX1*) genes are required for the production of compound leaves (Bharathan et al., 2002; Hay and Tsiantis, 2006). Alternatively, it may function differently, as observed in *Pisum sativum* L. (pea), where the formation of compound leaves requires the *UNIFOLIATA* (*UNI*) gene, the orthologue of *Arabidopsis LEAFY* (*LFY*), to reestablish an indeterminate state (Hofer et al., 1997; Bharathan et al., 2002; Champagne et al., 2007). However, so far, little is known about the genetics of leaf development in wild species like *C. heterophyllus*.

Therefore, future studies will be necessary to identify the genes involved in this process and to elucidate the regulatory networks that lead to different levels of leaf complexity.

Despite the ontogenetic and morphological changes throughout the heteroblastic series, we have shown that there are no anatomical differences between the leaflets of unifoliolate leaves and those of other leaf patterns observed in *C. heterophyllus*. This suggests that the leaf histogenesis machinery for all these patterns remains highly conserved.

5. Conclusion

Our data demonstrate the occurrence of heteroblasty in *C. heterophyllus*, encompassing three life phases in which different leaf patterns are formed, ranging from unifoliolate leaves to leaves with up to five leaflets. The novel evidence of the lateral leaflet traces present in the swollen region of the petiole indicates the compound nature of the unifoliolate leaves. Considering the leaf complexity observed in this species and the regulatory mechanisms of leaf development and heteroblasty, several important questions should be addressed in future studies: First, investigate the expression of *KNOX1* in the leaves of *C. heterophyllus* and other Rutaceae species. This can help determine whether unifoliolate leaves are derived from a compound leaf development program or if other regulatory mechanisms are at play. Second, to investigate whether miRNA156 and miRNA172 are present during the transitions from the juvenile to the adult phases, as observed in other species like *A. thaliana* and *P. edulis*. Third, explore other *Conchocarpus* species with varying numbers of leaflets (3–7 leaflets), such as *C. toxicarius* (Spruce ex Engl.) Kallunki & Pirani, *C. transitionalis* (Rizzini) Kallunki & Pirani, *C. mastigophorus* Kallunki, and *C. ucayalinus* (Huber) Kallunki & Pirani, to determine whether they exhibit changes in leaf patterns throughout their life phases, similar to the heteroblastic event described in *C. heterophyllus* in this study.

CRediT authorship contribution statement

Carlos E.V. Raymundo: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **José R. Pirani:** Writing – review & editing, Writing – original draft, Supervision, Data curation, Conceptualization. **Gladys F.A. Melo-de-Pinna:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Data availability

Data will be made available on request.

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