

Plant anatomy: history and future directions

Comparative floral anatomy and evolutionary interpretations of cryptic dioecy in *Ocotea velloziana* (Lauraceae)

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Abstract

Ocotea velloziana (Lauraceae) is a species with cryptic dioecy. The staminate flowers of this species have a pistillode and pistillate flowers have staminodes, which often makes it difficult to describe circumscribe the reproductive system correctly. Therefore, to understand the evolution of dioecy in this species, the morphology of both floral morphotypes needs to be described. We apply traditional and modern approaches to describe the comparative floral anatomy of the two floral morphotypes in *O. velloziana*. We found that the pistillate flowers have fewer (three) vascular traces in the tepals (compared to five traces in the tepals of the staminate flowers). The pistillode and the staminodes in the flowers of *O. velloziana* are smaller and less vascularized than their homologous, fertile counterparts, the pistils and stamens. In addition, even though the nectaries are smaller in pistillate flowers, their basic morphology and vascularization are similar in the two floral morphotypes. Our results suggest androtopaly in *O. velloziana*, however future ontogenetic studies and investigation of the vascularization are necessary in other Lauraceae species to clarify this issue. The nectaries have originated from the hypanthium and may be characterized as nectar-secreting emergencies not homologous to the stamens.

Key words: dicliny, floral vascularization, histochemistry, nectaries, perianth.

Resumo

Em *Ocotea velloziana* (Lauraceae) ocorre a dioécia críptica, na qual flores estaminadas possuem pistilódios e flores pistiladas possuem estaminódios, muitas vezes difíceis de distinguir de estruturas funcionais. A descrição das características anatômicas e morfológicas das flores pistiladas e estaminadas é necessária para compreender completamente a evolução da dioécia nesta espécie. Neste estudo, aplicamos abordagens tradicionais e modernas para o estudo da anatomia floral comparativa dos dois morfotipos florais de *O. velloziana*. Os resultados mostraram que as flores pistiladas de *O. velloziana* possuem menos (três) traços vasculares nas tépalas (versus cinco traços nas tépalas das flores estaminadas). O pistilódio e os estaminódios nas flores de *O. velloziana* são menores e menos vascularizados do que suas contrapartes homólogas e férteis, ou seja, os pistilos e os estames. Além disso, embora os nectários sejam menores nas flores pistiladas, sua morfologia básica e vascularização são semelhantes nos dois morfotipos florais. Nossos resultados sugerem androtopalia em *O. velloziana*, porém futuros estudos ontogenéticos e investigação da vascularização são necessários em outras espécies de Lauraceae para esclarecer completamente esta questão. Os nectários originam-se do hipanto e podem ser caracterizados como emergências secretoras de néctar não homólogas aos estames.

Palavras-chave: diclinia, vascularização floral, histoquímica, nectários, perianto.

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Introduction

Dioecy is characterized as a reproductive system that presents dichlinous flowers on separate plant individuals (Sakai & Weller 1999; Renner 2014; Cardoso *et al.* 2018). The phenomenon is found in about 6% of angiosperm species around the world (Renner & Ricklefs 1995; Endress 1996). Avoiding self-fertilization by dioecy increases the efficiency of nutrient allocation for reproduction (Thomson & Brunet 1990), maintaining genetic variability (Bawa 1980) and can generate plants that produce more than twice as many seeds compared to species with perfect flowers, *i.e.*, flowers that contain both fertile male and fertile female parts (Sakai & Weller 1991).

Cryptic dioecy is predominant in Neotropical dioecious Lauraceae, *i.e.*, staminate flowers have a pistillode and pistillate flowers have staminodes (Mayer & Charlesworth 1991). This often impedes fast identification of the sexual system (Penagos-Zuluaga *et al.* 2021). However, the total number of occurrences of dioecy within the family ultimately depends on verifying the fertility of the ovules in pollen-producing flowers (van der Werff 2017; Penagos-Zuluaga *et al.* 2021). Studying floral anatomy can help to identify floral morphotypes and to determine whether the flowers are perfect or if they are functionally only pistillate or staminate (see Leme *et al.* 2018, 2021).

The taxonomy of Lauraceae has been mainly based on androecium composition (Rohwer 1993). However, in recent phylogenies, several traditional groups have emerged as paraphyletic or polyphyletic (*e.g.*, Chanderbali *et al.* 2001; Rohwer & Rudolph 2005; Alves & Souza 2013; Trofimov *et al.* 2016; Rohde *et al.* 2017; Trofimov & Rohwer 2020; Penagos-Zuluaga *et al.* 2021). Therefore, new attributes need to be identified that could serve to delimit groups and help to understand the evolution of the family (Nishida & van der Werff 2007, 2011; Trofimov *et al.* 2016; Zeng *et al.* 2017).

Over the past years, learning about the evolution of the reproductive system has also become an important matter in understanding the phylogeny of Lauraceae. The reproductive system has been historically used in generic delimitation (Kostermans 1957; van der Werff 1991) and contains a phylogenetic signal in *Ocotea* (Penagos-Zuluaga *et al.* 2022; Trofimov *et al.* 2022). *Ocotea* is the largest Neotropical genus of Lauraceae with about 400 species and it contains species

with dioecious, gynodioecious or perfect flowers (van der Werff 2017, 2022; Penagos-Zuluaga *et al.* 2021). According to the most widely accepted hypothesis, all dioecious *Ocotea* species belong to the same subclade within the genus and have evolved from a cosexual ancestor (Chanderbali *et al.* 2001; van der Werff 2017). Cosexuality is a sexual system, in which individuals of a population present only perfect (cosexual, hermaphrodite, monoclinal or bisexual) flowers, with functional stamens and pistils in the same flower (Cardoso *et al.* 2018).

Studying floral anatomy is important to elucidate Charles Darwin's "abominable mystery" about the origin and evolution of flowers (Pennisi 2000; Buggs 2017). Such studies provide data to answer questions about the origin and the identity of organs, floral developmental patterns, and organ homology within Lauraceae (Reece 1939; Sastri 1965; Buzgo *et al.* 2007; Sajo *et al.* 2016). Consequently, comparative anatomy plays a fundamental role in systematics (Rieppel 1988). However, few studies have been conducted in Lauraceae and they reached different conclusions with regard to the origin of the perianth, some suggesting bracteotepaly *i.e.*, tepals originating from the bracts (Sajo *et al.* 2016; Ronse De Craene 2008), others androtepaly, tepals originating from the stamens (Albert *et al.* 1998; Chanderbali *et al.* 2006; Soltis *et al.* 2009).

Furthermore, lack of knowledge about the anatomical background is limiting our understanding of dioecy at the functional level (Pätzold *et al.* 2023) and only a few studies have been published on dioecious Lauraceae species (Boyle 1980; Souza & Moscheta 1999; Sajo *et al.* 2016). Therefore, the objective of this work was to study the comparative floral anatomy associated with the morphological characteristics of staminate and pistillate flowers of *Ocotea velloziana* (Meisn.) Mez (Lauraceae), a species with cryptic dioecy. We aim to elucidate the evolution of dioecy in *Ocotea velloziana* by answering the following questions: 1) What are the morphological and anatomical differences between homologous floral (perianth, androecium, gynoecium) organs of staminate and pistillate flowers? 2) Are there differences between the paired nectaries of stamens and staminodes? and 3) Can the floral vascular system (*i.e.*, the number and arrangement of vascular bundles) help us understand floral evolution in *Ocotea velloziana*?

Materials and Methods

Plant material

Specimens with staminate flowers (*Saiefert T.M.O. & Alves F.M. 21* [CGMS 74455]) and pistillate flowers (*E.J. Coutinho 01* [CGMS 84591]) were collected in Mato Grosso do Sul state, Brazil and were deposited in the CGMS Herbarium of the Federal University of Mato Grosso do Sul (INBIO/UFMS) (Thiers, continuously updated - <<http://sweetgum.nybg.org/ih/>>).

Flowers in anthesis and buds in several developmental stages were collected, fixed in buffered formalin or FAA (9:1:1 mixture of 70% ethanol, formalin, and acetic acid) for 48 hours, then dehydrated in or transferred to an ethanol series and, finally, stored in 70% ethanol (Kraus & Arduin 1997).

Herbarium samples were rehydrated for one week in a 1:1 glycerin-water mixture (Leme & Scremin-Dias 2014), dehydrated in an increasing ethanol series that reached and stored in 70% ethanol.

Morphological analysis

Floral morphology was recorded using a Leica M205 C stereo microscope with a Leica DFC 420 Coupled camera. For Scanning Electron Microscopy (SEM), the samples stored in 70% ethanol were dried in the open air and mounted on metal stubs with carbon adhesive tape and sputtered with gold in a Denton Vacuum Desk III system. Electron micrographs were obtained using a Jeol JSM-6380LV scanning electron microscope (SEM).

Staminate flowers were measured in 50 flowers (five flowers from each of 10 individuals) and pistillate flowers were measured from 15 flowers (five flowers from each of three exsiccates).

Anatomical and histochemical analysis

Both the fixed and the rehydrated material, stored in 70% ethanol, was dehydrated in an increasing ethanol series (two hours each in 80%, 90% and 95% solutions). For the preparation of the slides, the samples were infiltrated for two days in a pre-infiltration solution (Leica® activated historesin + 95% ethanol, 1:1) and in activated historesin (Leica®) according to the manufacturer's protocol. Some flowers were infiltrated for over two weeks for better infiltration. Then the material was polymerized and sectioned at 5 µm thickness using Leica RM2145 rotary microtome, mounted on slides and stained with toluidine blue in pH 4.7 0.05

M sodium citrate buffer (modified from O'Brien *et al.* 1964). The histochemical analysis of the main compounds was conducted using Sudan IV to identify total lipids (Pearse 1985), Xylidine Ponceau for proteins (Vidal 1970), ferric chloride for phenolic compounds (Lillie 1965), and Schiff's Reagent/Periodic Acid (PAS) for neutral polysaccharides (O'Brien & McCully 1981). The photomicrographs of the sections were taken using a Nikon Ci Eclipse microscope with a coupled digital camera.

Floral vascularization analysis

For vascularization analysis using high-resolution x-ray computed tomography (HRXCT), the flowers in 70% alcohol were treated in a 1% solution of phosphotungstic acid in 70% ethanol for one week (Staedler *et al.* 2013, 2018). The samples were then mounted in tubes and scanned while immersed in 70% ethanol. Herbarium samples were rehydrated using a 10% ammonium hydroxide aqueous solution, then fixed with FAA (5% formalin, 5% acetic acid, and 90% solution of 70% ethanol; Espinosa & Castro 2018). Microtomography was performed using a MicroXCT-200 imaging system (Zeiss Microscopy) with an L9421-02 90kV Microfocus X-ray source (Hamamatsu Photonics, Iwata City, Japan). We used the XMReconstructor 8.1.6599 program (Zeiss Microscopy) for 3D reconstructions and the AMIRA-based XM3DViewer 1.1.6 program (Zeiss Microscopy) to view the generated data and to create 2D and 3D reconstructions.

Results

Floral organization

Ocotea velloziana is a tree with cryptic dioecy, with a raceme-type inflorescence in axillary position (Fig. 1a-b). The staminate and pistillate flowers are white greenish and pedicellate and the perianth is organized in two whorls of three tepals, classified in outer and inner tepals (Fig. 1c-d). The staminate flowers are larger than the pistillate flowers, but the size of the individual tepals is similar in both (Tab. 1). There are no differences between the inner and the outer tepals from the same morphotype.

The androecium of the staminate morphotype has nine fertile stamens, which are organized in three whorls of three stamens. The stamens of whorls I and II are introrse and the stamens of whorl III are latrorse (Fig. 1c). The stamens of whorl III have a pair of naturally yellow and globose nectaries arranged at the base of each filament (Fig.

1c). The pistillate morphotype has three whorls of staminodes (whorls I, II, and III). The staminodes of whorl III have a pair of naturally yellow and globose nectaries at the base of each filament, similar to those of the staminate morphotype (Fig. 1d). The stamens and staminodes of whorl I are located in front of the outer tepals, those of whorl II are located in front of the inner tepals, and those of the whorl III are located in front of the stamens/staminodes of whorl I (Fig. 1c). The stamens have anthers with four sporangia, arranged in two overlapping pairs and each sporangium releases its pollen through

valvate dehiscence (Fig. 2a). There is a poorly developed pistillode in the center, lacking locule and ovule (Fig. 2b-c).

In the pistillate morphotype, the staminodes contain no pollen and the anthers do not dehiscence, although lines of dehiscence are visible on the anthers (Fig. 2d). The gynoecium is well developed, unilocarpellate, unilocular, uniovulate and the ovule is pendant (apical placentation; Fig. 2e-f). Male floral organs are larger in staminate than in pistillate flowers, whereas female organs are substantially larger in pistillate flowers (Tab. 1).

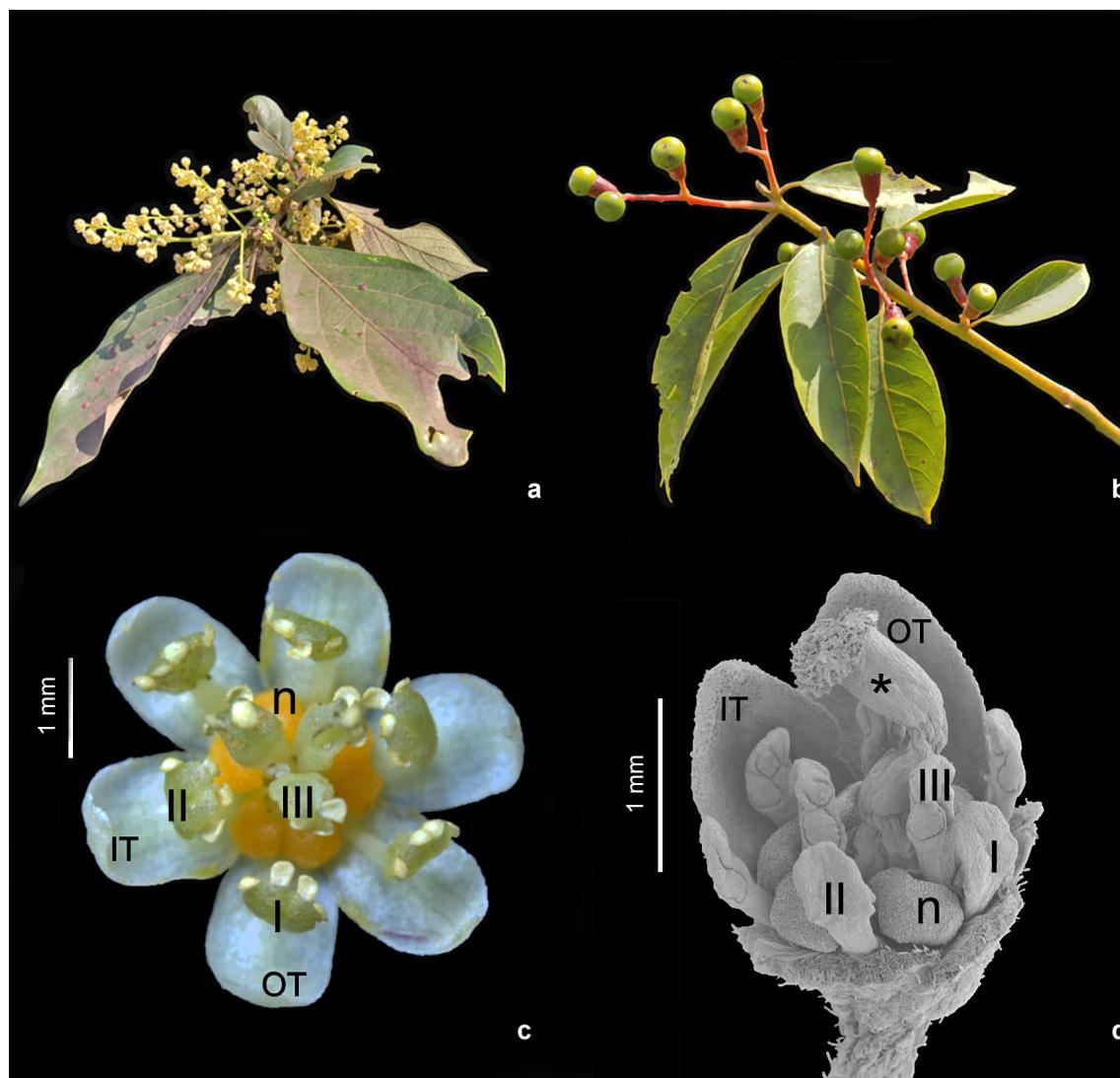


Figure 1 – a-d. *Ocotea velloziana* – a. flowering branch; b. fruiting branch; c. staminate flower showing the nine fertile stamens in three whorls (I, II and III) and the nectaries (n) at the base of whorl III; d. pistillate flower showing the staminodes of whorls I and II (I and II), nectaries (n) at the base of each filament of the staminodes of whorl III (III) and pistil in the center (black asterisk). Symbols: I = whorl I; II = whorl II; III = whorl III; IT = inner tepal; OT = outer tepal; n = nectary.

Table 1 – Comparative floral structure in staminate and pistillate flowers of *Ocotea velloziana*.

Floral structure	staminate	pistillate
Floral diameter	3.5–5.5 mm	2.5–3.5 mm
Floral length	4–4.5 mm	3–3.5 mm
Tepals	3–3.5 mm	3–3.5 mm
Stamens/staminodes (whorls I and II)	1.8–2.2 mm	0.8–0.9 mm
Anther	0.9–1 mm	0.3–0.4 mm
Filament	0.5–0.6 mm	0.4–0.5 mm
% of anther in stamen	65%	40%
Stamens/staminodios (whorl III)	1.8–3 mm	0.9–1.4 mm
Nectaries (base of stamens/staminodes of whorl III)	0.8–1 mm	0.4–0.5 mm
Pistillode/pistil	1.5–2 mm	2.2–3 mm
Ovary width	0.4–0.5 mm	0.8–1 mm
Ovary length	0.5–0.7 mm	0.8–1.2 mm
Stigma	0.2–0.3 mm	0.5–0.6 mm
% of style in pistil	75%	50%
Number of vascular bundles		
	staminate	pistillate
Pediceal	6	6
Outer tepals	5	3
Inner tepals	5	3
Stamen S1/staminode S1	1	1
Stamen S2/staminode S2	1	1
Stamen S3/staminode S3	3	1
Nectaries	numerous	numerous
Pistillode/pistil	1	10

Floral anatomy

The pedicel epidermis is slightly papillose and has non-secreting unicellular trichomes in the staminate and the pistillate floral morphotypes (Fig. 3a-b). The pedicel cortex is constituted of a regular-type parenchyma with rounded cells (Fig. 3a-c). The pedicel has six main vascular bundles (Fig. 3a). In the receptacle, the traces branch towards the center to serve the pistillode or pistil in the staminate and pistillate flowers, respectively (Fig. 3b-c).

In both floral morphotypes, the tepals and the stamens/staminodes are inserted on a hypanthium. The epidermis of the hypanthium is simple and its adaxial surface contains more papillae and non-

secreting unicellular trichomes than the abaxial side (Fig. 3c-f). The multi-stratified mesophyll is composed of homogeneous parenchyma tissue with oil-secreting idioblasts (Fig. 3e-h).

The outer and inner surfaces of the tepals possess papillose epidermis in both floral morphotypes (Fig. 4a-j), with trichomes on the abaxial and adaxial surfaces and stomata on the abaxial surface (Fig. 4c-d). The parenchymatous and homogeneous mesophyll (Fig. 4c-d) is four-to-ten cell layers thick and contains oil idioblasts. The tepals are vascularized by five vascular bundles in the staminate flowers and three vascular bundles in the pistillate flowers at the base (Fig. 4c-d).

The stamens and staminodes in both floral morphotypes possess filament with a papillose epidermis and a parenchymatous ground tissue surrounding the vascular tissue (Fig. 4e). The epidermis of the anthers of the staminate flowers has a simple layer with two subepidermal layers of regular parenchyma (Fig. 4f). The flaps of the anther consist of a layer of strongly lignified endothecial cells (Fig. 4f). The microsporangia contain pollen grains (Fig. 4f). In the pistillate flowers, the staminodes have a continuous epidermis around the entire anther, and a few layers of parenchyma in the subepidermal region (Fig. 4g-h). The anthers remain closed and

consist of weakly lignified endothecium cells and inconspicuous locules with no pollen grains (Fig. 4h).

In the staminate flowers, the pistillode has papillose epidermis mainly in the apical region, which is filled with parenchymal tissue and a vascular bundle in the center (Fig. 4i). Locules and ovules are absent (Fig. 4i). In the pistillate flower, the epidermis of the pistil is cuticularized and the parenchymatous mesophyll is multi-stratified and homogeneous with evident ventral and dorsal bundles and 3–4 adjacent bundles on each side, as well as oil-secreting idioblasts (Fig. 4j). The ovary is unitegmic and contains a single ovule (Figs. 2f; 4j).

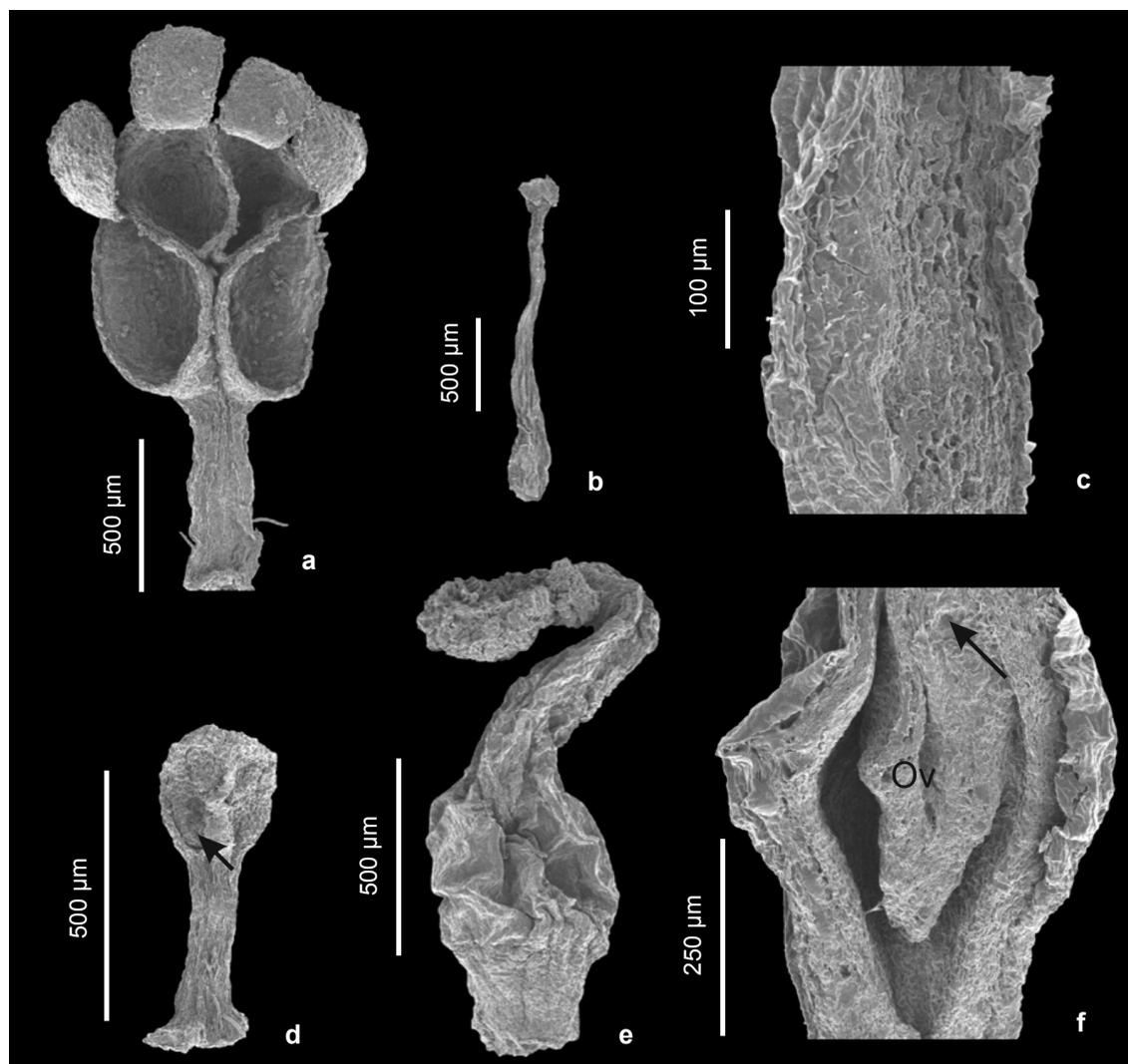


Figure 2 – a-f. Androecium and gynoecium of *Ocotea velloziana*, in SEM – a-c. staminate flower – a. fertile stamens; b. pistillode; c. detail of the ovary region in longitudinal section without ovule; d-f. pistillate flower – d. staminodes with valve remains (black arrow); e. pistil; f. detail of the ovary in longitudinal section showing the single ovule and placenta region (black arrow).

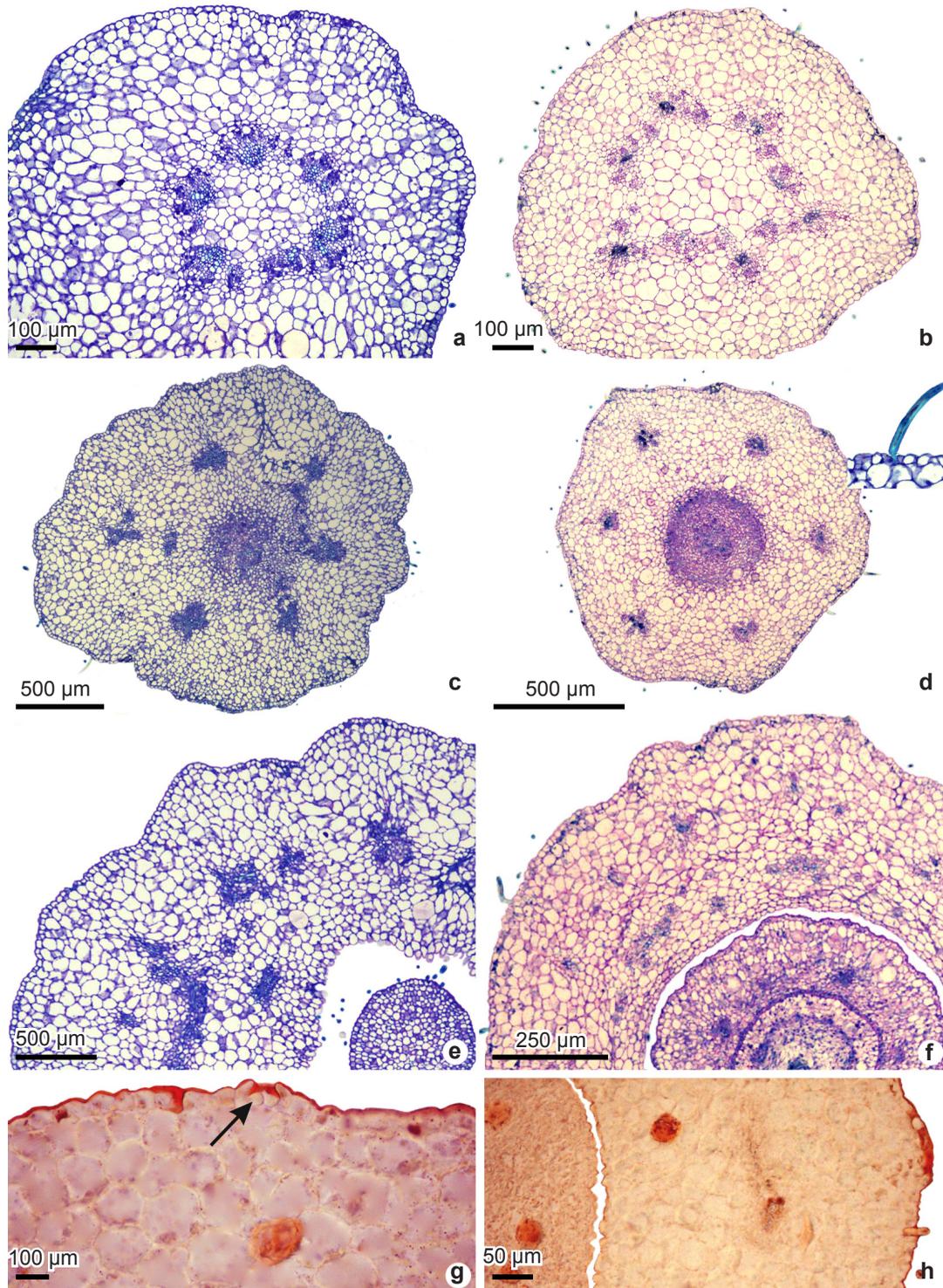


Figure 3 – a-h. Comparative anatomy and vasculature of the flower of *Ocotea velloziana* in cross section (a-f. toluidine blue; g-h. sudan III). a, c, e, g. staminate flower. b, d, f, h. pistillate flower – a. pedicel of the staminate flower; b. receptacle of the pistillate flower; c. floral receptacle, bundles diverging to the pistillode; d-f. hypanthium at base of the ovary in the center and detail of the no secretory trichome; e. hypanthium with pistillode in the center; f. hypanthium with ovary and ovule; g-h. oil-secreting idioblasts in the hypanthium and in the ovary. Note the stomata in the epidermis (black arrow).

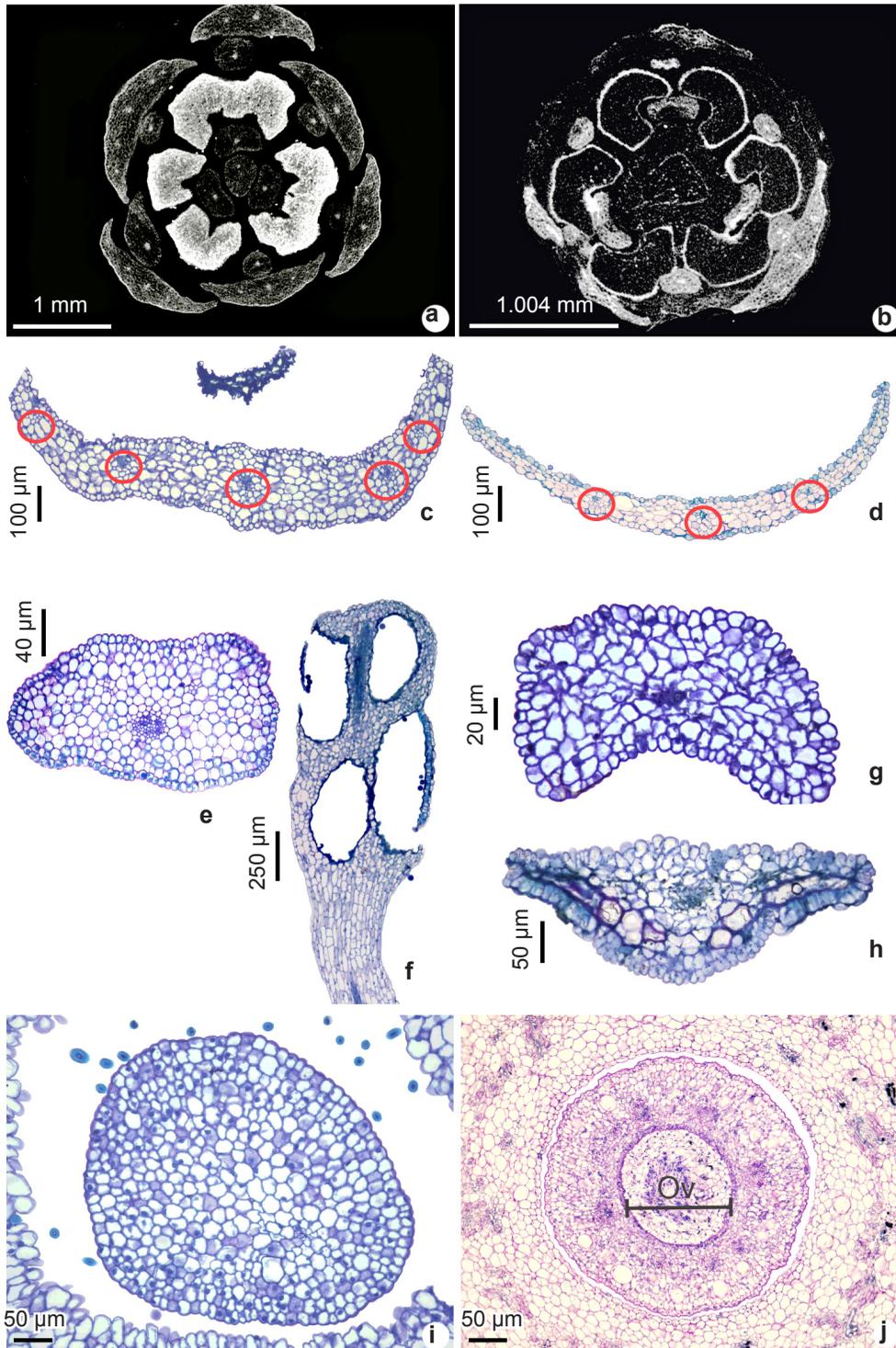


Figure 4 – a–j. Comparative anatomy of the flower of *Ocotea velloziana*. a, b: 2D tomography reconstructions of transverse sections; c–e, g–j: cross sections; f: longitudinal section; c–j: toluidine blue. a, c, e, f, i. Staminate flower. b, d, g, h, j. Pistillate flower – a–b. cross sections in the middle of the flower, note all the floral organs separated; c. outer tepal highlighting the five vascular bundles (red circles); d. inner tepal emphasizing the three vascular bundles (red circles); e. filament of the stamen of whorl III; f. anther with endothecium in the flaps; g. filament of a staminode of whorl III; h. anther of a staminode, without locule and nor pollen; i. pistillode anatomy; j. ovary anatomy with an ovule in the center. Symbols: Ov = ovule.

Floral vascularization

In the staminate and pistillate flowers (Fig. 5a-b), the pedicel has six main vascular bundles represented by green color that remain in the floral base and in the hypanthium (Fig. 5a-d). Some traces are oriented from the vascular bundles of the floral base towards the floral center to supply the gynoecium. The initial vascularization of the hypanthium consists of six vascular traces, which are distributed in two complexes. Each complex vascularizes one whorl of the perianth and one or two whorls of stamens, forming two distinct and alternating vascular complexes (Fig. 5a-b). In the staminate flowers, the gynoecium traces serve the pistillode and in the pistillate flowers, these vascular traces divide into approximately ten vascular traces that supply the pistil (Fig. 3e-f).

Vascular complex 1 gives rise to the vascular traces of the outer tepals, the lateral vascular traces of the internal tepals, the vascular traces of whorl I and III of stamens/staminodes and the nectaries. Vascular complex 2, on the other hand, gives rise to the central traces of the inner tepals and the vascular traces of the stamens/staminode of whorl II (Fig. 5a-d).

Among the vascular traces derived from complex 1, the first vascular trace that splits off vascularizes the stamens/staminodes of whorl III, the innermost whorl of stamens (Fig. 5a-d). Complex 1 is similar in both floral morphotypes, but in staminate flowers the splitting off of the trace leading to whorl III is proximal, located close to the floral base, while in pistillate flower it is located higher up in the distal hypanthium region (Fig. 5a-d). The next branch forms the central trace of the outer tepals (Fig. 5c-d) and the vascular trace of the stamens/staminodes of whorl I (Fig. 5c-d).

The three other vascular traces of the hypanthium (derived from complex 2) branch out and form the central traces of the inner tepals and the vascular traces of the stamens/staminodes of whorl II (Fig. 5c-d).

The vascularization of the tepals differs between staminate and pistillate floral morphotypes. In the staminate flowers, the tepals are vascularized by five vascular bundles at the base in each organ (Fig. 4c). In the pistillate flowers, the tepals are vascularized by three vascular bundles at the base (Fig. 4d). However, the vascularization complex of the two floral morphotypes is similar, the lateral vascular bundles of each tepal (outer tepals + inner tepals) depart from the same vascular trace of the hypanthium, derived from complex 1 (Fig. 5c-f).

In staminate flowers, the lateral vascular traces of each tepal divide again and form two vascular bundles each in the outer and the inner tepals (Fig. 5e). In pistillate flowers, the lateral traces do not divide, forming only one vascular bundle at the lateral side of each tepal (Fig. 5f).

After vascular traces separate, vascular traces split off higher up in the hypanthium and supply the nectaries (Fig. 6a-b). The nectary vascular traces are formed from complex 1 of the hypanthium vascular traces and are vascularized by several vascular bundles (Fig. 6c-d).

In staminate flowers, the stamens of whorl III are vascularized by two additional lateral traces, which are derived from of the vascular traces of the nectaries, arising further up in the hypanthium (Fig. 6a,c).

When fully developed, the outer and inner tepals of the staminate flower have five vascular bundles and the outer and inner tepals of the pistillate flowers have three vascular bundles (Fig. 6e-f). In staminate flowers, stamens of whorls I and II have one vascular bundle, and stamens of whorl III have three vascular bundles at their base, but the two lateral bundles end close to the base of the filament (Fig. 6a,c,e). On the other hand, staminodes in pistillate flowers only have one vascular bundle (Fig. 6b,d,f). The nectaries have more than ten vascular bundles (Fig. 6a-d).

Nectaries

Nectaries have similar structure and are proportional to the size of the flowers in both staminate and pistillate floral morphotypes (Tab. 1). The surface of these round nectaries is covered by papillae epidermis with unicellular secretory trichomes (Fig. 7a-d). Near the base of the nectaries, the epidermis is slightly papillose and produces phenolic compounds (Fig. 7c). The subepidermal region consists of nectary parenchyma containing cells with dense cytoplasm and a large nucleus, which react positively to lipids (Fig. 7d), proteins (Fig. 7e) and neutral polysaccharides (Fig. 7f). The subnectary parenchyma is abundantly vascularized (Fig. 7a-b).

Discussion

Ocotea velloziana has dichlamydeous flowers and both floral morphotypes are superficially similar to perfect flowers. However, the functionally staminate and pistillate flowers have distinct anatomies and considerably different sizes. The main differences

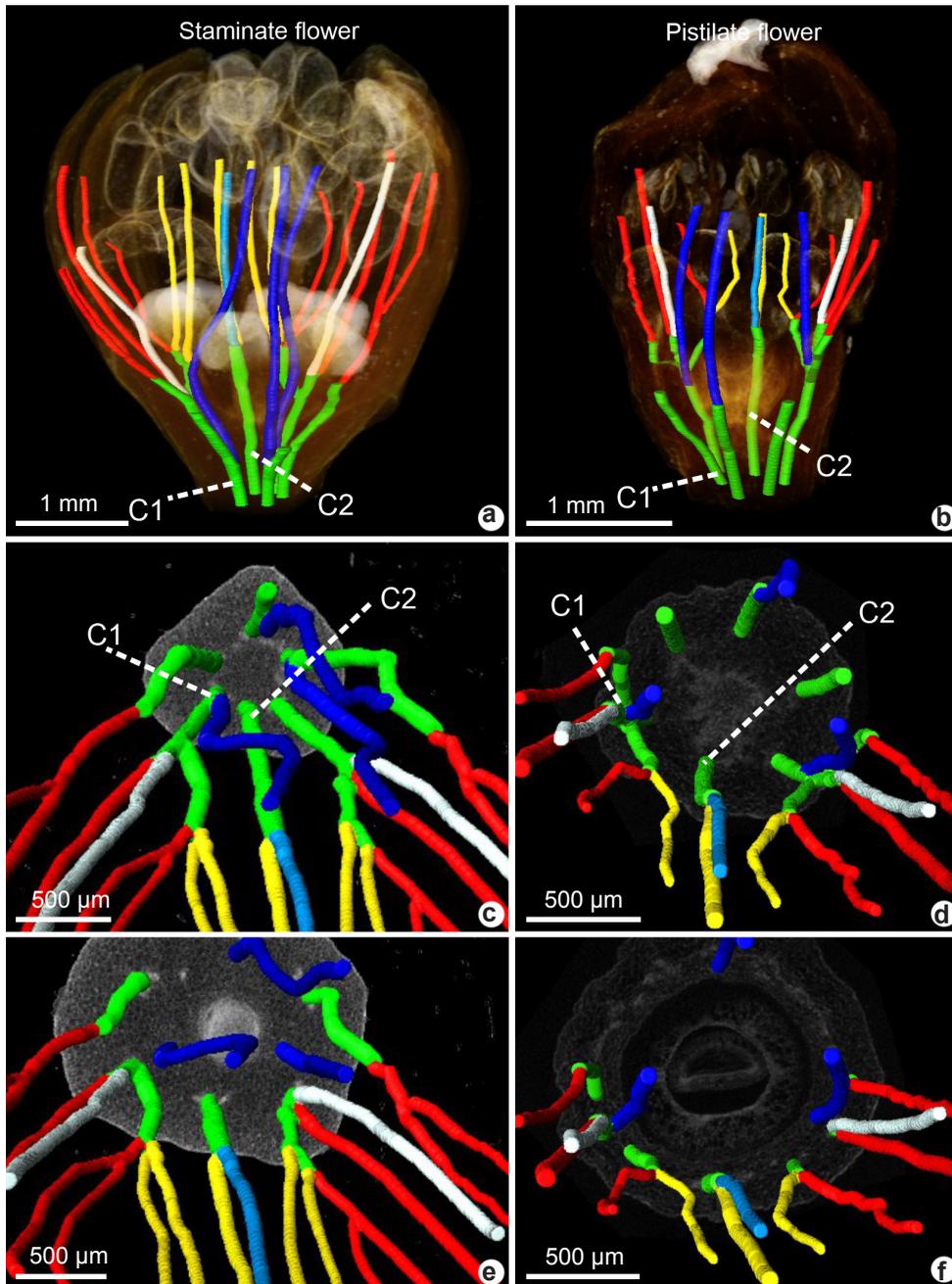


Figure 5 – a-f. Floral vascularization of *Ocotea velloziana* (HRXCT, 3D reconstructions and 2D reconstructions in transverse sections). a, c, e. Staminate flowers. b, d, f. Pistillate flowers – a-b. flowers seen from the side, with the vasculature of the hypanthium and part of the floral organs shown in different colors; c-d. floral vasculature as seen from above; in the center, note that the first vascular traces that split off from the main traces (green) are those of the stamens/staminodes of whorl III, one vascular trace (dark blue) for each stamen/staminode; e-f. the vascular bundles of the hypanthium of the complex one branch higher and give rise to traces of the outer tepals (red) and stamens/staminodes of whorl I (white). The vascular bundles of the hypanthium of the complex two branch and rise to traces of the inner tepals (yellow) and of the stamens/staminodes of whorls II (royal blue). Note that the lateral bundles of the tepals split off higher up in the hypanthium vascular bundles of the complex one. Vasculature colors: green = hypanthium, C1 = complex 1, C2 = complex 2, red = outer tepals, yellow = inner tepals, white = stamens/staminodes of whorl I, royal blue = stamens/staminodes of whorl II, dark blue = stamens/staminodes of whorl III.

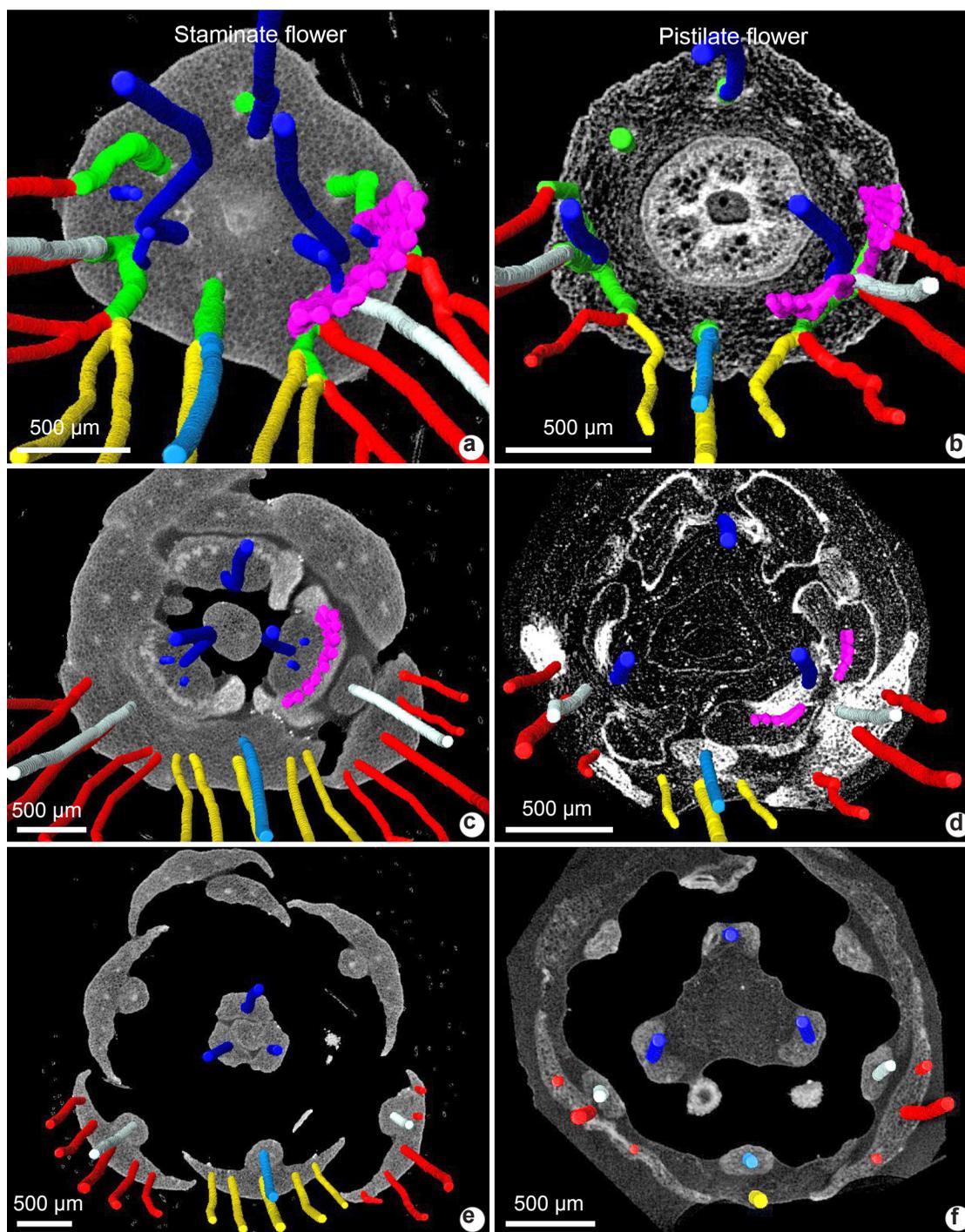


Figure 6 – a-f. Floral vascularization of *Ocotea velloziana* (HRXCT, 3D reconstructions and 2D reconstructions in transverse sections). a, c, e. Staminate flowers. b, d, f. Pistillate flowers – a-b. nectaries vascular bundles (pink) close to vascular bundles of the stamens/staminodes of whorl III (dark blue) – a. in the staminate flower, split off two lateral vascular traces in the hypanthium that extend to the base of the stamens of whorl III (dark blue); c-d. numerous vascular bundles of the nectaries; e. staminate flowers with five vascular bundles in the tepals; f. pistillate flower with three vascular bundles in the tepals. Vasculature colors: green = hypanthium, red = outer tepals, yellow = inner tepals, white = stamens/staminodes of whorl I, royal blue = stamens/staminodes of whorl II, dark blue = stamens/staminodes of whorl III, pink = nectaries.

between the floral morphotypes are the size of the floral structures, the presence or absence of a female gametophyte (embryo sac) or male gametophyte (pollen grains), and the different number of vascular

bundles in the tepals, stamens/staminodes and pistil/pistillode. The histology of the nectary is similar in both floral morphotypes. This is the first study describing the histochemistry and structure of the

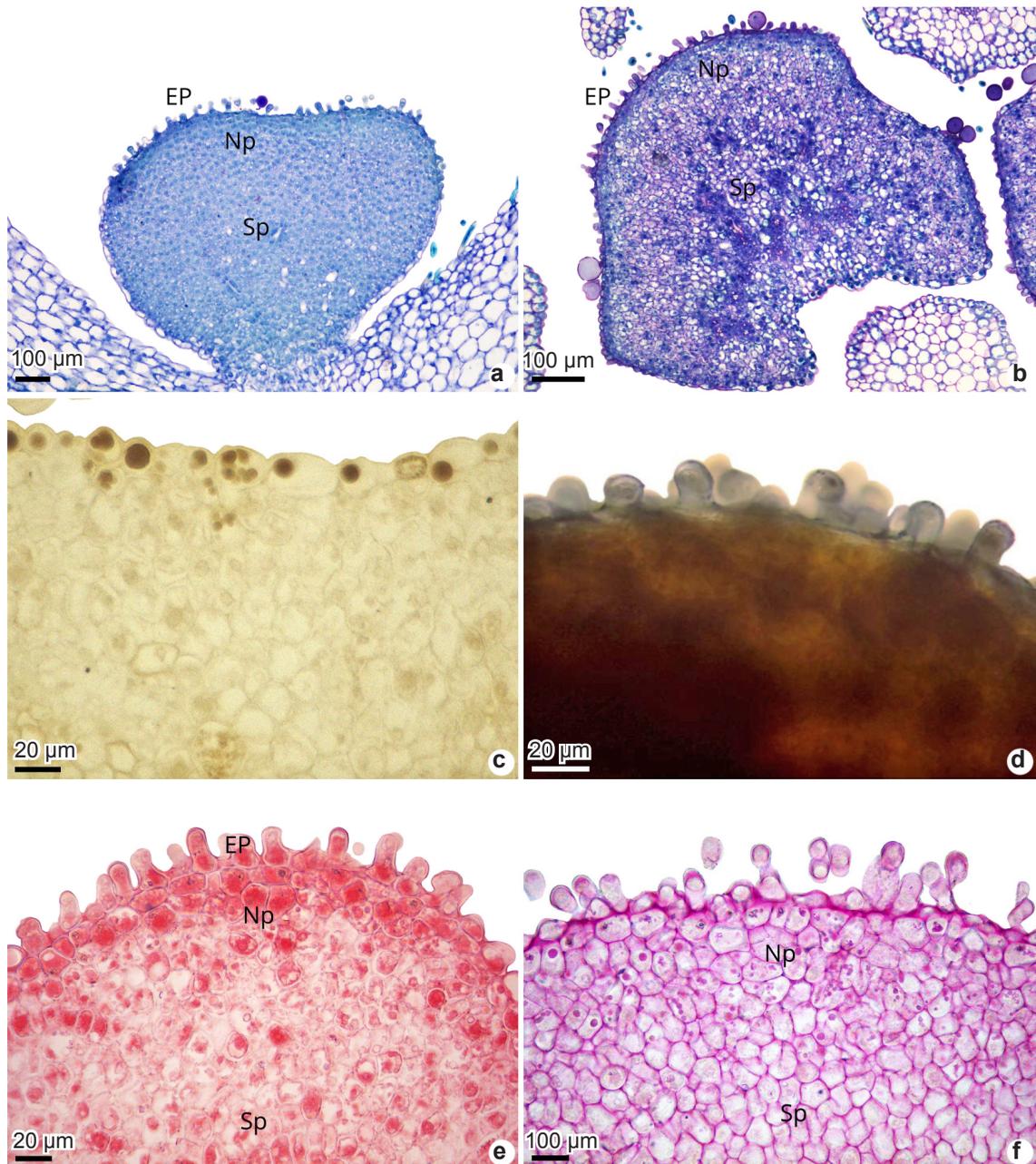


Figure 7 – a-f. Anatomy and histochemical analyses of the floral nectary of *Ocotea velloziana* (nectary of the floral staminate morphotype) – a-b. longitudinal (a) and transverse (b) sections of the nectary, stained with Toluidine Blue; c. nectary with phenolic compounds in the epidermal cells, positive reaction with ferric chloride; d. positive reaction of the papillose cells of the epidermis for lipids with Sudan Black; e. epidermis and nectary parenchyma with positive reaction to proteins (stain: Xylidine ponceau); f. nectary parenchyma with positive reaction to neutral polysaccharides (stain: Schiff reagent). EP = epidermis; Np = nectary parenchyma; Sp = subnectary parenchyma.

nectary of Lauraceae species, in particular nectary emergence, that is, the presence of a nectary with its own vascularization derived from the hypanthium that produces nectar.

Previous studies on floral anatomy have shown a great diversity in the number of vascular traces in floral structures in Lauraceae (*e.g.*, Reece 1939; Kasapligil 1951; Sastri 1952; Boyle 1980; Souza & Moscheta 1999, 2000; Sajo *et al.* 2016). Although in most Lauraceae species both tepal whorls (inner and outer tepals) are vascularized by the same number of bundles (*i.e.*, Souza & Moscheta 1999; Sajo *et al.* 2016), there may be species with more bundles in the outer tepals compared to the inner tepals (Sastri 1952, 1965; Kasapligil 1951). The same number of vascular bundles in the inner and outer tepals is a pattern present in many Lauraceae species. At the same time, the number of vascular bundles per tepal may differ between staminate and pistillate flowers of the same species (Boyle 1980; Souza & Moscheta 1999). Our results corroborate this observation, as we found that each morphotype had the same number of vascular bundles in each tepal, even though the numbers differed between floral morphotypes.

Compared to pistillate flowers, which terminate in three traces, staminate flowers, have an additional branching in the lateral trace of the tepals, thereby terminating in five traces (Figs. 5; 6). Compared to functional organs (stamens and pistils), in non-functional floral organs (*i.e.*, staminodes and pistillode) of *Ocotea velloziana*, there is also a reduction in vascularity. This is similar to what was described for another dioecious species, *Ocotea puberula* (Rich.) Nees (Souza & Moscheta 1999). Therefore, the reduction of floral structures (pistillode and staminodes) is linked to a decrease in vascularization and lower allocation of resources to these structures generating tissue atrophy (*e.g.*, Schmid 1972a, b).

Nutritional conflicts between the male and female functions within a flower can express selective forces that maintain male and female sterility genes in the morphotypes and drive the evolution to floral dichliny (Gibson & Diggle 1997). While hermaphrodite species have a high investment in resources for pollen and ovules, plants dioecious with pistillate flowers are liberated from the cost of the male function and can reallocate resources from pollen to seed formation and vice-versa (Darwin 1877; Ashman 1994). This strengthens the reproduction, since the exclusion of

self-fertilization achieved by dioecy increases the efficiency of nutrient allocation for reproduction (Freeman *et al.* 1997; Thomson & Brunet 1990), maintaining genetic variability (Bawa 1980) and generating plants that produce more than twice as many seeds as cosexual species (Sakai & Weller 1991). The allocation of resources for reproduction and the division of these resources among reproductive structures are of central importance to theories of the evolution of the reproductive system and the stability of hermaphroditism in plants (*e.g.*, Charlesworth & Charlesworth 1978, 1981; Lloyd & Bawa 1984; Charlesworth 1989; Ashman 1994).

In general, the reproductive success of specimens with staminate flowers is limited by access to female gametes, whereas the success of specimens with pistillate flowers is limited by the resources available for ovule production. Consequently, males tend to optimize mating quantity (higher pollen production), while females optimize ovary and ovule quality (Bawa 1980). Pistillate flowers produce smaller flowers (Gibson & Diggle 1997) and less nectar (Ashman & Stanton 1991; Eckhart 1992) compared to staminate flowers (Ashman 1994).

Vascular bundles are responsible for controlling the size and shape of floral structures with large organs having a higher number of bundles and a more branched vascular structure, while small organs have fewer bundles and less branching (Schmid 1972a, b). Therefore, the bundles are functional structures resulting from physiological and fundamental factors as relics exposing the evolutionary loss of organs (Carlquist 1969; Stebbins 1967).

Some authors have argued that gynodioecy is a common evolutionary pathway from hermaphroditism to dioecism in angiosperms (Bawa 1980; Dufay *et al.* 2014). In contrast, recent phylogenetic studies indicate a hermaphroditic ancestry for all dioecious origins in *Ocotea* (Chanderbali *et al.* 2001; Trofimov *et al.* 2019; Penagos-Zuluaga *et al.* 2021). Dioecy would not have arisen as a selection mechanism to avoid inbreeding, but from the differential allocation of resources (Darwin 1877; Bawa 1980; Rohwer 1986). The basic mode of reproduction in Lauraceae is dichogamy (protogyny), a mechanism that avoids self-fertilization (Kubitzki & Kurz 1984; Kubitzki *et al.* 1993).

Lauraceae can have two kinds of glandular organs, *i.e.*, pairs of nectaries usually at the base or along the filament of each stamen/staminode

of whorl III and a glandular head at the tip of the staminodes usually of the whorl IV (Rohwer 2009). Most Lauraceae species have nectaries in whorl III (Kostermans 1957; van der Werff 1991), although they can also be present in all stamens, as in *Chlorocardium*, *Phyllostemonodaphne* and *Urbanodendron* (Rohwer 1993) or be completely absent, as in *Anaueria*, *Mezilaurus* and *Williamodendron* (Rohwer 1993; Alves & Souza 2013). These structures have been interpreted as reduced stamens (Sastri 1965), independent structures (Rohwer 1994), nectar-secreting emergencies not homologous with any organ (Rohwer 1993) or only an attribute of the third whorl (Kasaplilgil 1951; Buzgo *et al.* 2007).

Nectary vascularization originates in the hypanthium in both floral morphotypes of *Ocotea velloziana*, a fact that supports the interpretation that they are nectar-secreting emergencies not homologous with any organ (Kasaplilgil 1951; Rohwer 1993, 1994; Takahashi *et al.* 2014). Their homology with stamens could be refuted by the facts that 1) nectaries have their own vascularization, 2) the lack of transitional forms in living or fossil taxa, 3) stamen initiation occurs earlier in the ontogeny than nectary formation (*e.g.*, Sajo *et al.* 2016; Buzgo *et al.* 2007), and that 4) similar structures are occasionally found in the outermost stamens and tepals (Chanderbali *et al.* 2006; Buzgo *et al.* 2007). Moreover, even though pistillate flowers have a reduced size and lost the functionality of the androecium, the nectaries are associated with whorl III staminodes, remain active, showing vascular independence to nutrient reception.

In spite of the reduced size and lost androecium functionality of the pistillate flower, the nectaries, associated with whorl III staminodes, remain functional indicating that these structures still play an important role in attracting pollinators for reproduction.

We believe that maintaining nectaries in pistillate flowers is likely to be fundamental to flower selection in the synchronization process with staminate flowers and to reward pollinators. Cosexual Lauraceae species present a heterodichogamous protogynous cycle with nectar secretion, *i.e.*, in the female phase by the staminodes of whorl IV (glandular head) and in the male phase by the pairs of nectaries at the base of the filaments in stamens of whorl III (Kubitzki & Kurz 1984; Rohwer 1993; Rohwer 2009). However, dioecious species generally lack

staminodes of whorl IV (glandular head) as well as a heterodichogamous cycle (Kubitzki & Kurz 1984) and the stamens and pistils of different flowers need to mature concurrently. Therefore, the fertility of the pistils in pistillate flowers needs to be synchronized with the pair of glands at the base of the staminodes of whorl III in the same flower, which in turn are already synchronized with the fertility of the stamens in staminate flowers. Consequently, we believe that staminate flowers lost whorl IV given the lack of an active gynoecium, while pistillate flowers lost whorl IV because their pistils had been synchronized with the pair of glands at the base of staminodes of whorl III, making these structures obsolete in both flowers. Therefore, nectaries were responsible for selecting pistillate flowers in synchrony with their nectar secretion. The loss of staminodes of whorl IV, might be due to the fact that their function as nectar-secreting organs during the female flowering phase of heterodichogamous species (Rohwer 2009) became obsolete with dioecy. On the other hand, cosexual species, such as *Persea americana*, have both functional secretory structures (Buzgo *et al.* 2007).

In *Ocotea velloziana*, vascularization of the lateral traces of the stamens of whorl III arises from the nectary vascularization, displaying vasculature complex 1. Unlike in other species, the traces serve different organs, *i.e.*, the tepals and the stamens, reinforcing the role of the nectary as an independent structure. In *Persea americana*, nectaries have been described as emergencies, but from stamens (partial homology) (Buzgo *et al.* 2007), while in *Ocotea prolifera* (Nees & Mart.) Mez and *Cryptocarya moschata* Nees & Mart. the lateral bundles of the staminode and those supplying the nectaries (stamen appendages of the inner androecial whorl) have a common origin in the floral cup (Sajo *et al.* 2016). Nevertheless, as the precise origin of the nectary cannot always be determined with certainty (Rohwer 1993), more studies are necessary on the vascularization of nectaries in other Lauraceae species.

The highly different vascularization between the floral morphotypes complicates the interpretation of the origin of the tepals (androtepaly or bracteotepaly). However, in staminate flowers, only the gynoecium displays structural reduction with fewer bundles in the pistillode compared to the pistil of pistillate flowers. On the other hand, in pistillate flowers, the structure of the androecium is reduced, and

also (or consequently), the vascularization of the tepals. This indicates a strong relationship between stamens and tepals, reinforcing the hypothesis of androtepaly (Albert *et al.* 1998; Chanderbali *et al.* 2001, 2006; Soltis *et al.* 2009), as opposed to bracteotepaly (Sajo *et al.* 2016; Ronse De Craene 2008; Takhtajan 1991; Warner *et al.* 2009). Thus, our results point androtepaly in *O. velloziana*, as suggested by ABC model whose B and C function and floral genes expressed in *Persea* (Chanderbali *et al.* 2006, 2008; Soltis *et al.* 2009), but we suggest that future ontogenetic, expression of floral genes studies, and vascularization work are necessary in other Lauraceae species to confirm this pattern.

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Data availability statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

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