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ORIGINAL ARTICLE

Phylogenetic analysis and evolution of morphological characters in *Metrodorea* and related species in Rutoideae (Rutaceae)

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Abstract The aim of the study is to compare distinct morphological characters of *Metrodorea* (Rutaceae) with those of related genera, under a phylogenetic approach. Morphoanatomy of leaves was analyzed, and data concerning reproductive structures were compiled from the literature. We used available molecular sequences to recover a maximum likelihood tree, and we present a reconstruction of ancestral morphological character states made with the maximum likelihood criterion. The topologies obtained retrieve as synapomorphic characters of *Metrodorea*: aborted leaflets, vascularized intrapetiolar stipules, sinuous cell walls contour in the surface view of the epidermis, glandular trichomes on the adaxial and proximal region of the petiole, good development of areoles, muricate fruits, and valvate estivation of the corolla. The specific relationships within *Metrodorea* and between *Metrodorea* and other genera of Rutoideae are supported by characters as the location of crystals, type of adherence at leaf bases, and presence/absence of trichomes on the blade. The close relationship of *Metrodorea* and *Raulinoa* is strongly supported by several synapomorphies. Our data are mostly consistent with recent phylogenies, showing that the monophyly of *Esenbeckia* cannot be confirmed and that the clade *Helietta-Balfourodendron* (formerly included in another subfamily) is closely related to *Raulinoa* and *Metrodorea* (both part of Pilocarpinae) than to *Pilocarpus* itself. Possible relationships between these genera are discussed based on the morphological states found, and most of them supporting the hypothesis that *Metrodorea* is the sister group of *Raulinoa*.

Keywords "*Balfourodendron* alliance" · "*Esenbeckia* alliance" · Galipeeae · Leaf anatomy · Reconstruction of ancestral character states · Sapindales

Introduction

Rutaceae comprises 154 genera and approximately 2100 species distributed worldwide, but mainly in tropical and subtropical regions (Kubitzki et al. 2011). Members of the family are characterized by the presence of secretory cavities or oil glands within the tissues of the shoot system that appear as pellucid dots on the leaves (Engler 1931; Metcalfe and Chalk 1950; Kubitzki et al. 2011). Phylogenetic and morphoanatomical studies within the family are of particular importance in order to better target the exploitation of the group (Groppo et al. 2008).

The traditional classification of Rutaceae was proposed by Engler (1931), who recognized six subfamilies according to the degree of connation of carpels, the structure of fruits, and anatomic features of oil glands. Engler's taxonomic treatment is the most extensive ever performed on Rutaceae and includes detailed information about the morphology and geographical distribution of all groups. However, the classification has been questioned on the basis of evidence obtained from studies on chemosystematics (Silva et al. 1988) and, more recently, molecular phylogenetics (Chase et al. 1999; Poon et al. 2007; Groppo et al. 2008, 2012; Bayly et al. 2013; Morton and Telmer 2014). It is important to accurately investigate morphological features of members of Rutaceae, looking for

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further evidence toward a better understanding of the relationships within the family. Thus, it is necessary to find morphological synapomorphies supporting the clades recently recognized using molecular data.

Metrodorea A.St.-Hil. is a Neotropical genus within Rutaceae that contains six described species that are distributed mainly in the Brazilian rainforests and seasonal forests, but also in Suriname and Bolivia (Kaastra 1982; Kubitzki et al. 2011; Dias et al. 2013, 2015). The phyllotaxy is decussate with peculiar intrapetiolar stipules, which are united by intertwined hairs, and so cover the shoot apex, thus protecting developing leaf primordia (Cruz et al. 2015). Most species of the genus have leaves comprising one to three leaflets on the same plant. Cruz et al. (2015) observed that heterophylly results that even though all leaves start out as trifoliate, one or two leaflets may abort during development, probably associated with the reduction in space caused by the tight structure covering the shoot apex. Such limitation of space may have also have led to the selection of individuals that produce a single terminal leaflet rather than more leaflets, generating a strictly unifoliolate architecture, as observed in *M. maracasana*.

Metrodorea has been classified with *Esenbeckia* Kunth, *Raulinoa* R.S.Cowan, and *Pilocarpus* Vahl, which together made up the subtribe Pilocarpinae of the tribe Galipeeae in subfamily Rutoideae (Engler 1931; Kaastra 1982; Pirani 1999). This subtribe is characterized by globular floral buds, and yellowish or violet petals that are nearly regular and free (Kaastra 1982). However, phylogenetic analyses based on molecular data (Groppo et al. 2008, 2012) indicate that the clade formed by *Balfourodendron* Méllo ex Oliv. and *Helietta* Tul., traditionally included in the subfamily Toddalioideae (Engler 1931; Pirani 1998) is strongly supported as the sister group of a clade containing *Metrodorea* and *Esenbeckia*. These two clades form a polytomy with *Pilocarpus* and other genera, while *Raulinoa* was not present in those analyses.

A recent molecular phylogeny of *Metrodorea* based in the ITS nuclear ribosomal spacers and *trn*S-G intergenic spacer (Dias et al. 2015) revealed that the sister group of *Metrodorea* could be *Raulinoa* and/or the clade *Helietta* + *Balfourodendron*, depending on the use of parsimony or bayesian criteria. Strong floral similarities between *Helietta* and *Esenbeckia,* which belong to different subfamilies according to Engler's classification, have been highlighted by Kaastra (1982) and Pirani (1998). Kaastra (1982) suggested that it might be appropriate to remove all genera but *Pilocarpus* from the Pilocarpinae because this genus is distinctly different from the other in its morphological and chemical aspects. Kubitzki et al. (2011) proposed a

number of informal alliances, based on the phylogeny of Groppo et al. (2008), which include the "*Esenbeckia* alliance" (*Metrodorea*, *Raulinoa*, and *Esenbeckia*) and the "*Balfourodendron* alliance" (*Balfourodendron* and *Helietta*, already transferred to the subfamily Rutoideae). Furthermore, these authors stated that the current phylogenetic evidence makes it very difficult to establish associations between *Pilocarpus* and the other genera.

Previous studies on the leaf anatomy of members of *Pilocarpus* and *Raulinoa* have identified a wide array of structures potentially useful for the understanding of the relationships between the members of Rutoideae especially those belonging to Galipeeae (Arioli et al. 2008; Muntoreanu et al. 2011; Cruz et al. 2015). However, additional information concerning the stipules of *Metrodorea* together with other novel anatomical features may constitute key characters for elucidating the species relationship within this genus and might also assist in understanding its relationships with other genera.

Within this context, the aims of the present study are: (1) to provide a detailed examination of leaves and reunite available reproductive structures data of *Metrodorea* and closely related genera; (2) to perform a combined analysis of morphological and available molecular sequences from databases in order to analyze whether morphological data can add resolution to previous phylogenies, contributing to elucidate the phylogenetic relationships within this group; and (3) to get insights on the structural evolution based on molecular trees and possible interpretations.

Materials and methods

Taxon sampling

Twenty species belonging in Rutoideae were analyzed (Table 1), including all the six described species of *Metrodorea*, five species of *Esenbeckia* [5/30 spp. of the genus, according to Kubitzki et al. (2011)], two species of *Pilocarpus* (2/17 spp.), two of *Helietta* (2/8 spp.), *Raulinoa echinata* (monospecific genus), *Balfourodendron riedelianum* (1/2 spp.), *Conchocarpus heterophyllus* (1/45 spp.), and *Galipea trifoliata* (1/15 spp.). All these species are representative from the "American Clade" of Rutoideae (Groppo et al. 2008, 2012). As an outgroup, we used *Zanthoxylum rhoifolium*, a representative from "Old World and Oceania Clade" (Groppo et al. 2008, 2012) that share many chemical similarities with some of the Australian genera (Kubitzki et al. 2011). Voucher specimens (Table 1) are deposited in the Universidade de São Paulo herbarium (SPF).

Table 1 Classification of analyzed species and their voucher numbers

Classification		Species	Voucher number at SPF Herbarium
Engler ([1931](#))	Kubitzki et al. ([2011](#))		
Subfam. Rutoideae Tribe Galipeae Subtribe Pilocarpinae	Subfam. Rutoideae "*Esenbeckia* alliance"	*Metrodorea concinna* Pirani & P.Dias	*Dias & Udulutsch 318*
		Metrodorea flavida K.Krause	*Dias 229*
		Metrodorea maracasana Kaastra	*Cruz 21*
		Metrodorea mollis Taub.	*Cruz 25*
		Metrodorea nigra A.St.-Hil.	*Groppo 1111*
		Metrodorea stipularis Mart.	*Pirani 3847*
		Esenbeckia grandiflora Mart.	*Duarte 3*
		Esenbeckia irwiniana Kaastra	*Duarte 1, 2*
		Esenbeckia leiocarpa Engl.	*Pirani 6497*
		Esenbeckia pilocarpoides Kunth subsp. *pilocarpoides*	*Dias 249*
		Esenbeckia pumila Pohl	*Dias 225*
		Raulinoa echinata R.S.Cowan**	*Dias 275*
	Subfam. Rutoideae Lacking close relatives	*Pilocarpus jaborandi* Holmes	*Dias 252, 253, 254*
		Pilocarpus microphyllus Stapf ex Wardlew.	*Dias 235, 237, 238*
Subfam. Toddalioideae Tribe Toddalieae Subtribe Pteleinae	Subfam. Rutoideae "*Balfourodendron* alliance"	*Balfourodendron riedelianum* (Engl.) Engl.	*Dias 217, 345*
		Helietta apiculata Benth.	*Dias 344*
		Helietta puberula R.E.Fr.	*Dias 278*
Subfam. Rutoideae Tribe Cusparieae Subtribe Cusparinae*	Subfam. Rutoideae "*Angostura* alliance"	*Conchocarpus heterophyllus* (A.St.-Hil.) Kallunki & Pirani	*El Ottra 11*
		Galipea trifoliata Aubl.	*Dias 230, 231*
Subfam. Rutoideae Tribe Zanthoxyleae	Subfam. Rutoideae "*Euodia* alliance"	*Zanthoxylum rhoifolium* Lam.	*Pirani 4423*

* The valid name of Tribe Cusparieae and Subtribe Cusparinae are Galipeeae and Galipeinae, respectively (Kallunki and Pirani [1998](#))

** *Raulinoa echinata* was described after Engler's classification, but recognized as a close relative of *Metrodorea* and *Esenbeckia* by Kaastra ([1982](#))

Morphological and anatomical studies

Leaf characters were directly examined by us. Five leaves between the fifth and tenth nodes of different branches were collected from plants of each species, fixed in formalin-acetic acid–ethanol 50% (FAA) for 48 h and stored in 70% ethanol. Samples selected based on their structural integrity were submitted to conventional ethanol/*tert*-butanol gradient (50–100%) dehydration and embedded in paraffin (Johansen [1940](#)). Longitudinal and transverse sections were obtained with a rotary microtome, stained with Safranin and Astra Blue dyes according to the method of Bukatsch ([1972](#)), and mounted permanently on slides with synthetic resin. Leaves epidermis were dissociated for surface viewing following Franklin ([1945](#)). Scanning electron microscopy (SEM) was used to observe trichomes on the leaf surface and in the inner region of protective intrapetiolar stipules. For SEM analysis, shoot apices and leaves fragments fixed in FAA were dehydrated through a gradient series of ethanol, critical point dried, mounted on metal stubs, and sputter-coated with gold as described by

Silveira ([1989](#)), and then analyzed on a Zeiss DSM 940 microscope. Leaves were cleared and stained according to Strittmater ([1973](#)), and the venation pattern was classified according to the guidelines established by Ellis et al. ([2009](#)).

All reproductive structures characters used in the analyses were compiled from Kaastra ([1982](#), for former Pilocarpinae); Pirani ([1998](#), for *Helietta* and *Balfourodendron*), Pirani ([1999](#), for all species, except *Metrodorea concinna*); Kubitzki et al. ([2011](#), for all genera), El Ottra et al. ([2013](#), for *Conchocarpus heterophyllus*), and Dias et al. ([2015](#), for *Metrodorea*).

Forty-five morphological characters (see Table [2](#)) showed some variation and were used for the construction of a matrix of taxa and morphological characters (Table [3](#)), following the guidelines of Sereno ([2007](#)).

Retrieving of molecular sequences and alignment

We used the nuclear ribosomal spacers ITS1 and ITS2, and the gene 5.8S (here referred as ITS/5.8S region) and

Table 2 Characters and their states used in the compilation of the matrix

Character	Character states
Leaf morphology	
1. Leaves: simple x number of leaflets at the beginning of development	Simple (0), 3-foliolate (1), 4 or 5-foliolate (2), pinnate (3)
2. Leaves: phyllotaxy	Alternate or subopposite (0), opposite (1)
3. Leaflets: macroscopic distinction of the base	Sessile to subsessile leaflets (0), base well distinct from the leaflet blade forming an evident petiolule (1)
4. Leaf base: intrapetiolar stipule	Absent (0), present (1)
5. Leaf base: elongated petiole distally to stipule	Absent (0), present (1)
6. Domatia	Absent (0), present (1)
7. Constriction between the leaf base and blade	Absent (0), present (1)
8. Swelling between the leaf base and blade	Absent (0), present (1)
9. Early trifoliolate leaves: loss of leaflets during ontogenesis	Absent (0), present (1)
Anatomy of the leaf base	
10. Petiole: epidermal cells in transverse section	Polygonal (0), papillose (1)
11. Petiole: non-glandular trichomes	Absent (0), present (1)
12. Petiole: long non-glandular trichomes concentrated in the adaxial-proximal region	Absent (0), present (1)
13. Petiole: glandular trichomes	Absent (0), present (1)
14. Petiole: glandular trichomes concentrated in the adaxial-proximal region	Absent (0), present (1)
15. Petiole: secretory cavities	Absent (0), present (1)
16. Petiole: perivascular fibers	Absent (0), present (1)
17. Leaf base: adaxial bundles in the intrapetiolar stipule	Absent (0), present (1)
18. Intrapetiolar stipules: margins	Glabrous (0), interlocking cells (1)
Dermal system of the leaf blade	
19. Uniseriate non-glandular trichomes	Absent (0), present (1)
20. Uniseriate non-glandular trichomes: number of cells	One (0); more than one (1)
21. Glandular trichomes	Absent (0), present (1)
22. Glandular trichomes: types	Capitate (0), peltate (1)
23. Contour of the anticlinal cell walls of the epidermis: frontal view	Straight (0), sinuous (1)
24. Stomata: location	Abaxial only (0), both surfaces (1)
Mesophyll and midrib region	
25. Palisade parenchyma: number of layers	One (0), more than one (1)
26. Secretory cavities	Absent (0), present (1)
27. Extensions of the bundle sheath	Absent (0), present (1)
28. Midrib: medullary arch	Absent (0), present (1)
29. Crystals: types	Prismatic (0), druses (1), raphides (2)
30. Crystals concentrated in compartments of the palisade parenchyma	Absent (0), present (1)
31. Crystals in the hypodermis of the midrib region	Absent (0), present (1)
Venation	
32. Connections of the secondary veins larger than that of the central vein	Totally decurrent (0), decurrent only at the leaf base(1), totally excurrent (2)
33. Intersecondary veins	Absent (0), present (1)
34. Category of quaternary veins	Freely branched (0), irregular reticulate (1)
35. Development of the areoles	Poor (0), good (1)
36. Vein termination	Incomplete (0), looped (1)
Reproductive structures	
37. Flower: basic number of parts	Tetramerous (0), pentamerous (1)
38. Corolla: color	White to cream (0), red to purple (1)
39. Corolla: estivation	Imbricate (0), valvate (1)

Table 2 continued

Character	Character states
40. Carpels: connation	Fully connate (0), partially connate (1), free (2)
41. Fruits: ornamentation	Smooth (0), muricate (1), echinate (2)
42. Fruits: dorsal expansions	Absent (0), present (1)
43. Fruits: type of dorsal expansions	Apophyses (0), winged structures (1)
44. Seeds: number per locule	One (0), two (1)
45. Seeds: polyembryony	Absent (0), present (1)

All enlisted leaf characters were directly observed by us, while reproductive structures characters were retrieved from the literature as cited in the methodology

Table 3 Matrix of taxa and characters

	01–05	06–10	11–15	16–20	21–25	26–30	31–35	36–40	41–45
Balfourodendron riedelianum	1110-	11000	100-1	0--10	10000	10001	02100	00000	01100
Conchocarpus heterophyllus	2010-	000-0	100-1	1--11	10000	10011	00101	11001	00-00
Esenbeckia grandiflora	0000-	011-0	10101	1--10	0-000	10001	02101	01000	20-?1
Esenbeckia irwiniana	1010-	00000	10101	1--10	10010	11101	00111	11000	110?0
Esenbeckia leiocarpa	0000-	001-1	10100	0--10	10000	00001	00001	01000	01010
Esenbeckia pilocarpoides	0000-	01000	10101	1--0-	10100	10020	00111	11001	11001
Esenbeckia pumila	1000-	01000	10101	1--10	10001	11010	02111	01000	10-?0
Galipea trifoliata	1000-	00000	10001	0--10	10100	10000	02101	11001	00-??
Helietta apiculata	1100-	01001	10101	0--10	11001	10001	00100	01000	01100
Helietta puberula	1100-	01000	10101	1--10	11001	10000	02100	01000	01100
Metrodorea concinna	11011	01010	10111	1110-	11100	10001	11101	11111	11010
Metrodorea flavida	11111	01010	11111	1110-	11100	10001	10101	11010	11010
Metrodorea maracasana	01111	010-0	01111	1100-	11100	10001	10101	11010	110?0
Metrodorea mollis	11011	01011	11111	01110	11100	10000	01101	11010	10-?0
Metrodorea nigra	11111	01010	11111	1000-	11100	10001	11101	11110	11010
Metrodorea stipularis	11110	01010	11111	1100-	11100	10001	01101	11010	11010
Pilocarpus jaborandi	3010-	010-0	100-1	1--10	11000	10011	00110	01101	00-00
Pilocarpus microphyllus	3000-	010-0	100-1	1--10	10000	10011	00100	01001	00-00
Raulinoa echinata	0100-	000-0	110-1	1--0-	11000	10001	01100	10100	01000
Zanthoxylum rhoifolium	3010-	010-0	1-1-1	1--10	0-000	10100	00110	1100-	10-00

"-" is used for non-applicable character

"?" for unknown data

the cpDNA *trn*S-G intergenic spacer obtained by Dias et al. (2015) for their *Metrodorea* phylogeny (accession numbers for sequences in GenBank available in their article). We aligned the sequences using the same method used by them (ClustalX software, Thompson et al. 1997) and trimmed the alignment based on the shortest sequence as they did.

Phylogenetic trees and reconstruction of ancestral character states

The matrix of taxa and morphological characters (Table 3) was added to the molecular alignment. For species with just morphological data available, we considered each molecular state of character as missing ("?"). Models and

partitions were detected in PartitionFinder 2.1.0 (Lanfear et al. 2016) for molecular data and used for Bayesian analysis (BA) together with morphological data. We treated ITS/5.8S, *trn*S-G, and morphological data as three different partitions, with the models GTR + Gamma, HKY + Gamma, and Standard Discrete Model + Gamma, respectively. The analysis was conducted with MrBayes 3.2 (Ronquist et al. 2012), with 100 million generations and 0.25 burn-in fraction, and aims to detect whether adding morphological data with new taxa will improve the resolution of the BA tree of Dias et al. (2015) that could not solve the sister group of *Metrodorea*.

For the reconstruction of ancestral character states in *Metrodorea*, we used the molecular data (alignments of ITS/5.8S and *trn*S-G) for the 13 species studied by Dias
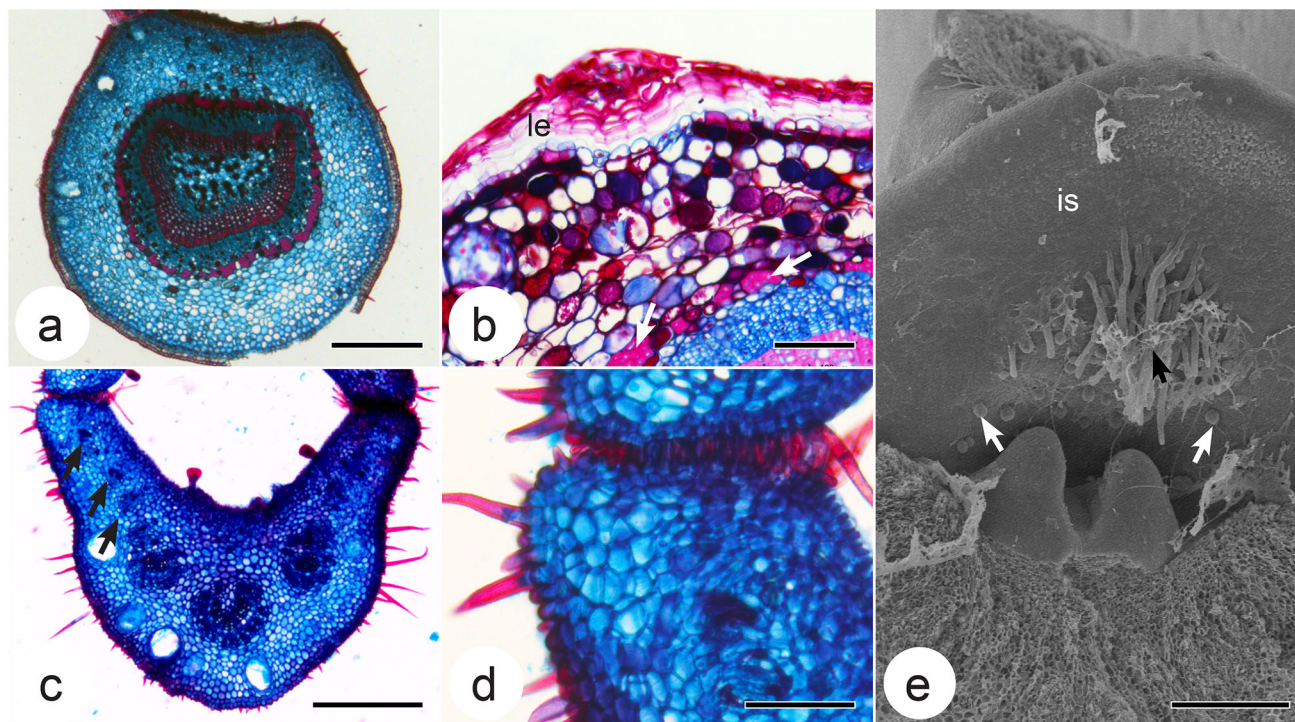
Fig. 1 Leaf base. **a** *Metrodorea nigra*. Distal cross section of petiole. Closed vascular cylinder surrounded by perivascular fibers and periderm. **b** *Esenbeckia grandiflora*. Cross section of petiole. Lenticel and perivascular fibers (*arrows*). **c** *M. mollis*. Proximal cross section of petiole. Vascular bundles (*arrows*) are associated with intrapetiolar stipule. **d** *M. mollis*. Proximal cross section of stipules. Interlocking cells in margins. **e** *M. maracasana*. SEM. Inner region of intrapetiolar stipule with glabrous margin protecting a pair of leaf primordia. Close to the margin, long intertwined non-glandular trichomes (*black arrow*) and below them, two of the glandular trichomes pointed by *white arrows*. *Bars* **a**, **c** 400 µm; **b**, **d** 100 µm; **e** 200 µm; *le* lenticel; *is* intrapetiolar stipule

et al. (2015) and the same model and partitions used by them (GTR-Gamma-I, two partitions). We performed a maximum likelihood (ML) analysis using the software RAxML 8 (Stamatakis 2014) to obtain an ML tree. The morphological data concerning these species were optimized in this ML tree using the Mesquite software (Maddison and Maddison 2015), with the maximum likelihood criterion and the Markov k-state one-parameter model. Character states that appeared in nodes with proportional likelihood ≥0.65 were interpreted as likely (as in Castroviejo-Fisher et al. 2014) and considered a potential synapomorphy.

Results

The matrix (Table 3) of morphological characters and states (Table 2) is descriptive and provides a summary of the examined characters. Below, we give more details for some of the characters that may be of difficult interpretation (e.g., constrictions and swellings) or that deserve better details, with supporting images.

Leaf morphology (characters 1–9)

Leaves of the studied species are simple in *Esenbeckia grandiflora*, *E. leiocarpa*, *E. pilocarpoides*, and *Metrodorea maracasana*; pinnate in *Pilocarpus* spp. and *Zanthoxylum rhoifolium*; and trifoliolate or seldom bifoliolate (some *Metrodorea* samples) in the remaining species. There are constrictions between the base and the blade in *Metrodorea* spp., *Esenbeckia grandiflora*, *E. pilocarpoides*, E. *pumila*, *Pilocarpus* spp., *Balfourodendron riedelianum*, *Helietta* spp.¸ and *Zanthoxylum rhoifolium*. Swellings (tumescences) are present on the leaf bases of *E. grandiflora* and *E. leiocarpa*. The presence of such constrictions and swellings at the base of species with a non-divided blade usually imply the reference to them as "unifoliolate leaves," but they are identical to simple leaves with respect to the development and blade morphology. The only remarkable exception is the mostly trifoliolate species of *Metrodorea* (all but *M. maracasana,* which is consistently simple-leaved), whose leaf primordia bear three leaflet primordia each, but some of them may be aborted during development resulting in mature leaves bearing only one or two leaflets.
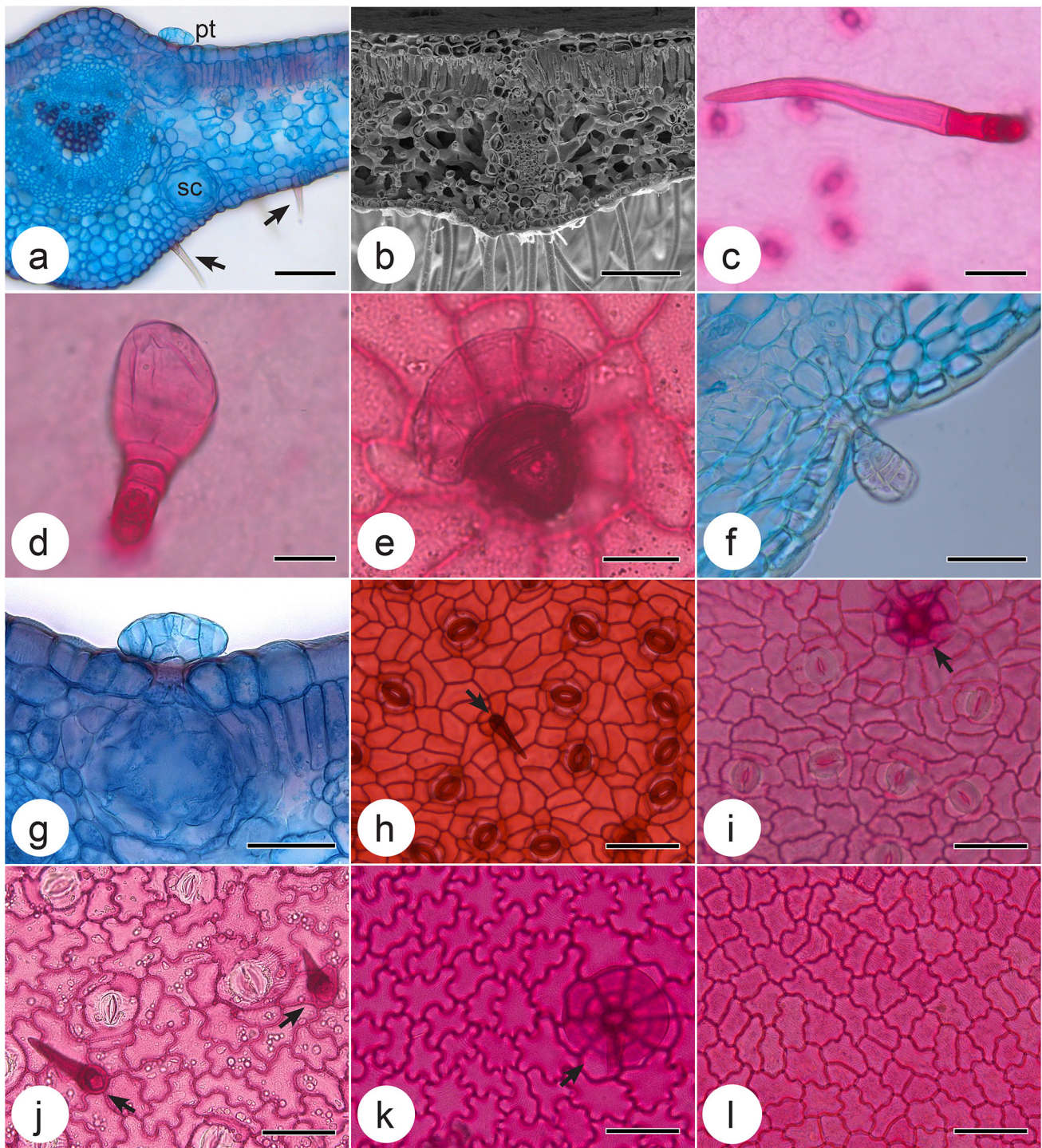
Fig. 2 Leaf blade. **a** *Metrodorea mollis*. Cross section. Uniseriate epidermis, unicellular non-glandular trichomes (*arrows*), and peltate glandular trichome. Secretory cavity in the midrib region. **b** *Esenbeckia irwiniana*. SEM of cross section. Higher density of trichomes on abaxial side when compared with adaxial side. **c** *Conchocarpus heterophyllus*. Multicellular non-glandular trichome. **d** *C. heterophyllus*. Capitate glandular trichome with multicellular peduncle and unicellular head. **e–f** Capitate glandular trichomes with multicellular peduncle and head; **e** *E. pilocarpoides*; **f** *E. irwiniana*; **g** *M. mollis*. Cross section. Peltate trichome associated with secretory cavity in the midrib region. **h–l** Epidermal cells in front view; **h** *E. grandiflora*, abaxial surface with anomocytic stomata, unicellular non-glandular trichome (*arrow*), and straight anticlinal cell walls; **i** *M. stipularis*, abaxial surface with peltate trichome (*arrow*) and sinuous anticlinal cell walls; **j** *M. mollis*, abaxial surface with sinuous anticlinal cell walls and trichomes (*arrow*); **k** *M. maracasana*, adaxial surface with sinuous anticlinal cell walls and peltate trichome (*arrowed*); **l** *M. stipularis*, adaxial surface with sinuous anticlinal cell walls. *Bars* **a**, **b**, **h** 100 μm; **c**, **f**, **g**, **i–l** 50 μm; **d**, **e** 20 μm; *pt* peltate trichomes; *sc* secretory cavities

Anatomy of the leaf base (characters 10–18)

The dermal system of the petiole shows in some regions a periderm with a subepidermal phellogen (Fig. 1a, b) and, sometimes, lenticels (Fig. 1b). The exceptions are *Metrodorea mollis* and *Esenbeckia leiocarpa*, which bear papillose epidermis with trichomes (Fig. 1c, d) that is not replaced by a periderm. In *Metrodorea* and *Raulinoa,* the proximal and adaxial part of the petiole exhibits a region with long intertwined multicellular trichomes. However, only *Metrodorea* species present capitate glandular trichomes (Fig. 1e) in the proximal region of the petiole.

Along the axis of the petiole, the vascular system consists of three bundles at the proximal region (Fig. 1c) and a closed cylinder at the distal region (Fig. 1a), except in *Metrodorea stipularis*, that does not present a region with a closed cylinder in its leaf base, neither a free petiole (its leaflets are sessile, the blades directly attached to the stipule). Most species present perivascular fibers forming an interrupted cylinder (Fig. 1a, b), except *Metrodorea mollis*, *Esenbeckia leiocarpa*, *Galipea trifoliata*, *Helietta apiculata*, and *Balfourodendron riedelianum*, which lack such fibers. The intrapetiolar stipules in *Metrodorea* species are vascularized, except in *M. nigra*. Adhesion between the margins of the stipules is provided by interlocking epidermal cells in *M. mollis* (Fig. 1d), *M. flavida*, and *M. concinna*, while in other species the stipule margins are glabrous (Fig. 1e).

Dermal system of the leaf blade (characters 19–24)

Unicellular non-glandular trichomes occur on both adaxial and abaxial surface of most species, but do not occur in *Conchocarpus heterophyllus*, *Esenbeckia pilocarpoides*, and most of *Metrodorea* spp. In *Metrodorea,* this type of trichomes is present only in *M. mollis* (Fig. 2a). *Esenbeckia irwiniana* has these trichomes distributed more densely on the abaxial surface of the leaves (Fig. 2b), while other species does not exhibit a visible difference of distribution between the two surfaces. Multicellular non-glandular trichomes on the abaxial surface occur only in *Conchocarpus heterophyllus* (Fig. 2c). Glandular trichomes are present in all studied species with the exception of *Esenbeckia grandiflora* and *Zanthoxylum rhoifolium*. Capitate trichomes with a multicellular peduncle occur in *Conchocarpus heterophyllus*, *E. irwiniana,* and *E. pilocarpoides* (Fig. 2d–f), whereas peltate trichomes are present in *Metrodorea* spp. (Fig. 2g), *Raulinoa echinata*, and *Helietta* spp.

In most of the studied species, anticlinal walls of epidermal cells are straight in frontal view (Fig. 2h), while in *Metrodorea* spp. they are sinuous (Fig. 2i–l), as well as in

Fig. 3 Cross sections of the leaf blade. **a** *Conchocarpus heterophyllus*. Druse (*arrow*), uniseriate palisade parenchyma, and spongy parenchyma containing small intercellular spaces. **b** *Metrodorea mollis*. Uniseriate palisade parenchyma and spongy parenchyma with loosely arranged arm cells. **c** *M. nigra*. Bundle sheath (*arrow*). **d** *Esenbeckia grandiflora*. Detail of bundle sheath (*arrow*). **e** *E. irwiniana*. Extensions (*arrows*) of bundle sheath oriented toward adaxial and abaxial surfaces. **f** *M. maracasana*. Vascular cylinder in the midrib region formed by two arches (*arrows*). Secretory cavities can be observed close to the abaxial surface. **g** *E. irwiniana*. Midrib region with medullary arch (*arrow*); **h** *M. maracasana*. Detail of midrib with secretory cells (*arrows*). **i** *M. nigra*. Crystals of palisade parenchyma in compartments separated by cell wall (*arrow*). Secretory cavity surrounded by epithelial cells under polarized light. **j** *E. pilocarpoides*. Raphides bundle under polarized light. **k** *M. stipularis*. Palisade parenchyma with prismatic crystals in compartments separated by walls (*arrows*) transversal to the length of the cell. **l** *M. nigra*. Prismatic crystals under polarized light, some of them concentrated in the hypodermis of the midrib region. *Bars* **a–d**, **h** 50 μm; **e** 100 μm; **f**, **g**, **l** 200 μm; **j–k** 20 μm; *pp* palisade parenchyma; *sp* spongy parenchyma; *sc* secretory cavities

Esenbeckia pilocarpoides and *Galipea trifoliata*. Anomocytic stomata (Fig. 2h–j) occur only on the abaxial surface, except in *E. irwiniana*. This species has this type of stomata on both surfaces, but they are rare on the adaxial side.

Mesophyll and midrib region (characters 25–31)

All species have dorsiventral mesophyll with uniseriate palisade parenchyma (Fig. 3a–c), except *Esenbeckia pumila* and *Helietta* spp., which present multiseriate palisade parenchyma. Vascular bundles are collateral and involved by a sheath (Fig. 3c, d). Extensions of the bundle sheath occur in *E. pumila* and *E. irwiniana* oriented toward the adaxial and abaxial surface (Fig. 3e).

In the midrib region, the vascular system consists of two vascular arches, one in the adaxial side and the other on the abaxial side, forming a vascular cylinder (Fig. 3f). In *Esenbeckia irwiniana*, a central vascular arch occurs in the medullary parenchyma of this cylinder (Fig. 3g). Secretory cells (Fig. 3h) and secretory cavities with epithelial cells (Fig. 3i) are present in the mesophyll and in the region of the midrib.

Different types of crystals occur in the midrib region, mesophyll and vascular tissues. They can be raphides (Fig. 3j), druses (Fig. 3a), or prismatic crystals that may appear in a cubic or in a rod-like form (Fig. 3k). Prismatic crystals (when they occur) and *Pilocarpus* druses are found in compartments in the palisade parenchyma created by walls transversal to the length of the cell. However, in *Esenbeckia pilocarpoides* and *Metrodorea mollis,* such crystals are less frequent in all tissues and were not observed in the palisade parenchyma. In *M. concinna*, *M. nigra*, *M. maracasana*, and *M. flavida*, some crystals are concentrated in the hypodermis of the midrib region (Fig. 3l).
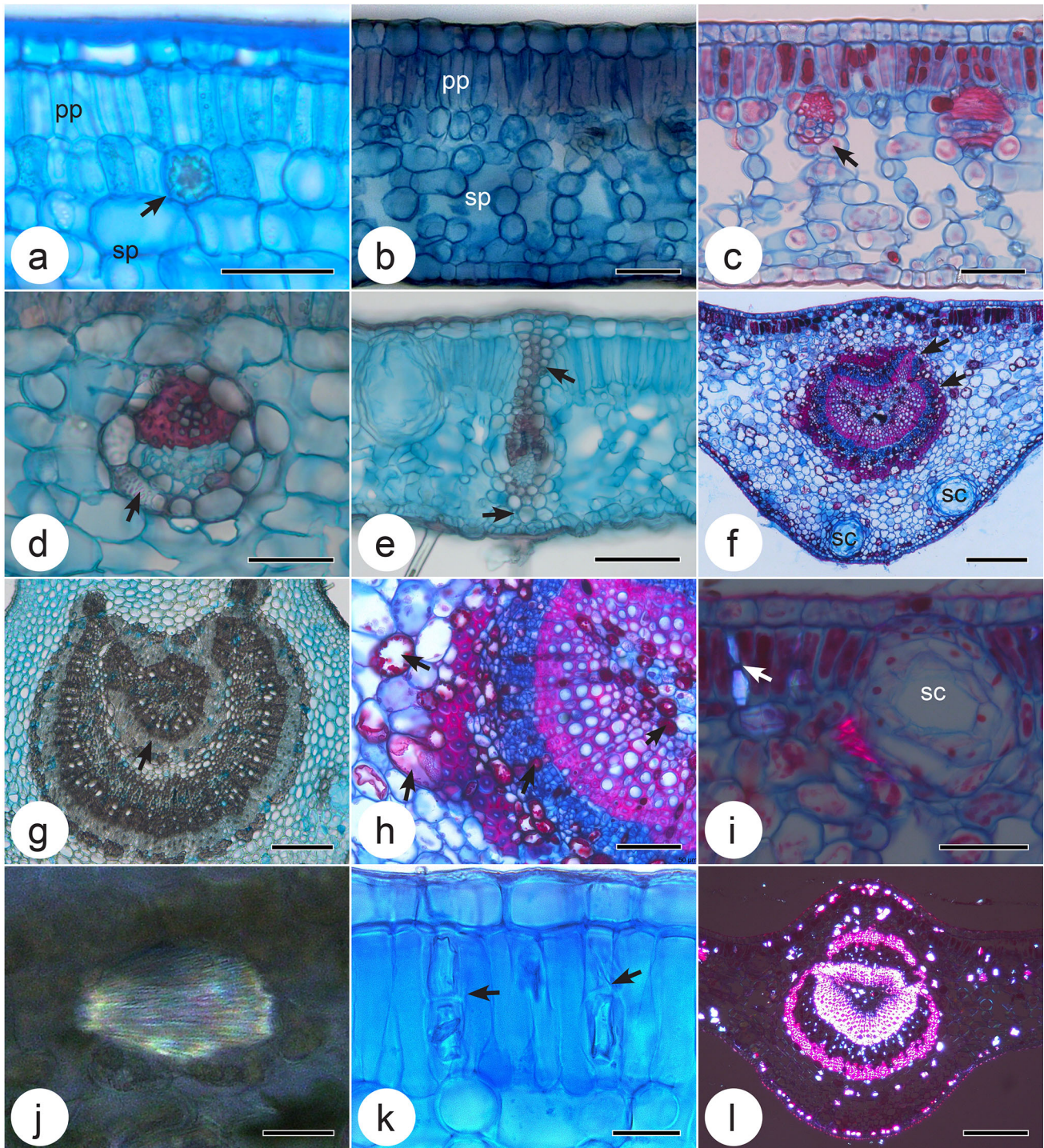
Venation (characters 32–36)

The venation pattern of the studied species is pinnate and simple brochidodromous, as in *Metrodorea mollis* (Fig. 4a). Connections of the secondary veins with the midrib are excurrent in *Balfourodendron riedelianum*,

Esenbeckia grandiflora (Fig. 4b), *E. pumila*, *Galipea trifoliata*, and *Helietta puberula*; decurrent only in the proximal secondary veins in *Raulinoa echinata*, *Metrodorea mollis* (Fig. 4a), *M. nigra*, *M. concinna*, and *M. stipularis*; and decurrent in the other studied species, as in *Conchocarpus heterophyllus* (Fig. 4c). Intersecondary

veins are absent only in *E. leiocarpa*. Tertiary veins are all mixed percurrent (Fig. 4d), whereas quaternary veins are irregular reticulate in *E. irwiniana*, *E. pilocarpoides* (Fig. 4e), *E. pumila*, *Pilocarpus jaborandi,* and *Zanthoxylum rhoifolium* and freely ramifying in the other species, as in *Metrodorea maracasana* (Fig. 4f). The veins of all studied species have branched ends (Fig. 4e–g) and exhibit areolation (Fig. 4g–i). The areolas show poor development (i.e., irregular sizes and shapes with more than seven sides) in *B. riedelianum*, *Helietta* spp. (Fig. 4g), *R. echinata*, *Pilocarpus* spp., and *Zanthoxylum rhoifolium* and good development (i.e., regular sizes and shapes with three to six sides) in the other species (Fig. 4h, i). The marginal ultimate venation is incomplete in *B. riedelianum*, *E. grandiflora* (Fig. 4j), *E. leiocarpa*, *E. pumila*, *Helietta* spp., and *Pilocarpus* spp., and it is looped in all other species (Fig. 4k).

Reproductive structures (characters 37–45)

Although the literature used to compile these results presents different details for different species in some cases (e.g., types of imbricative estivation of the corolla and endocarp anatomy) or may lack data for some characters (e.g., polyembryony), no inconsistency was found in reproductive structures descriptions.

The flowers of *Balfourodendron riedelianum* and *Raulinoa echinata* are tetramerous, while those of the other species studied are pentamerous. Some species of *Helietta* other than *H. puberula* present some tetramerous flowers among the prevailing pentamerous ones, though. Corollas are red to purple in *Raulinoa echinata* and *Metrodorea nigra*, and they are white to cream in all other species. The estivation of the corolla is valvate in *Metrodorea* and imbricate in the other species. Complete connation of the carpels occurs in all studied species except for *Pilocarpus microphyllus* and *Esenbeckia pilocarpoides*, in which the carpels are connected through the base, and *Conchocarpus heterophyllus*, in which they are connected at the central axis and at the base.

All *Metrodorea* species, *Esenbeckia pilocarpoides*, *E. pumila*, *E. irwiniana*, and *Zanthoxylum rhoifolium* present muricate fruits; *E. grandiflora* bears echinate fruits; and other species present smooth fruits. Dorsal expansions are present and may be dorsal apophyses in *Esenbeckia*, *Metrodorea*, *Pilocarpus*, and *Raulinoa*; or winged structures in *Balfourodendron* and *Helietta*. All of the studied species have been described as having biovulate locules, although they may differ regarding the number of seeds per locule. In *Metrodorea nigra*, for example, there are normally two seeds per locule, although variations on this pattern may occur, having also been described by Souza

et al. (2008). The occurrence of polyembryony is reported in *E. grandiflora* and *E. pilocarpoides* (Kaastra 1982).

Phylogenetic inferences

The result of combined BA (Fig. 5), compared with the Bayesian tree made exclusively with molecular data by Dias et al. (2015), shows a similar topology for *Metrodorea* species, with a high posterior probability support value (PPS = 100%) corroborating the monophyly of the genus. Otherwise, the addition of morphological data reduced the support of the clade *M. maracasana* + *M. concinna* + *M. flavida* + *M. mollis* (PPS from 94 to 80%).

Raulinoa is the sister group of *Metrodorea* in our tree (PPS = 86%). The clade formed by *Metrodorea* + *Helietta* + *Balfourodendron* + *Raulinoa echinata* in Dias et al. (2015) has the posterior probability of 64%, lower than the 84% value obtained by us with combined data.

Relationships of other studied species, most of them devoid of molecular information, showed a lower resolution (polytomies or lower PPS value). For example, *Esenbeckia grandiflora*, *E. leiocarpa*, and *E. pilocarpoides* species devoid of molecular data appeared in a polytomy together with *Galipea trifoliata* and a clade formed by other 13 species. However, the larger data set (with morphological and molecular data) allowed us to retrieve *Raulinoa* and the "*Balfourodendron* alliance" as close relatives of *Metrodorea* with a better support.

ML analysis (Fig. 6) with the molecular data set of Dias et al. (2015) recovered the same topology of their Parsimony bootstrap tree. For *Metrodorea*, we have found similar bootstrap values. Notwithstanding, the relations between *Metrodorea*, *Raulinoa*, and the "*Balfourodendron* alliance" presented bootstrap values under 50, so it is not possible to point the sister group of *Metrodorea* in this analysis since these values do not represent accurate clades (Hillis and Bull 1993).

Concerning the ML optimizations of morphological characters within the ML tree generated with molecular data, possible synapomorphies of the genus *Metrodorea* are: leaflet abortion, vascularized intrapetiolar stipule (with loss of vascularization in *M. nigra*), sinuous cell walls contour in surface view of the epidermis, glandular trichomes on the adaxial and proximal region of the petiole, good development of areoles, muricate fruits, and valvate estivation of the corolla (Fig. 7). Also, the presence long non-glandular trichomes concentrated in the adaxial-proximal region (character 12) and the presence of looped veins (character 36) would be synapomorphic for *Metrodorea* with 0.98 and 1.00 proportional likelihood (PL), respectively. These features are found in *Raulinoa echinata* and appear with 0.38 PL (character 12) and 0.2 PL (character
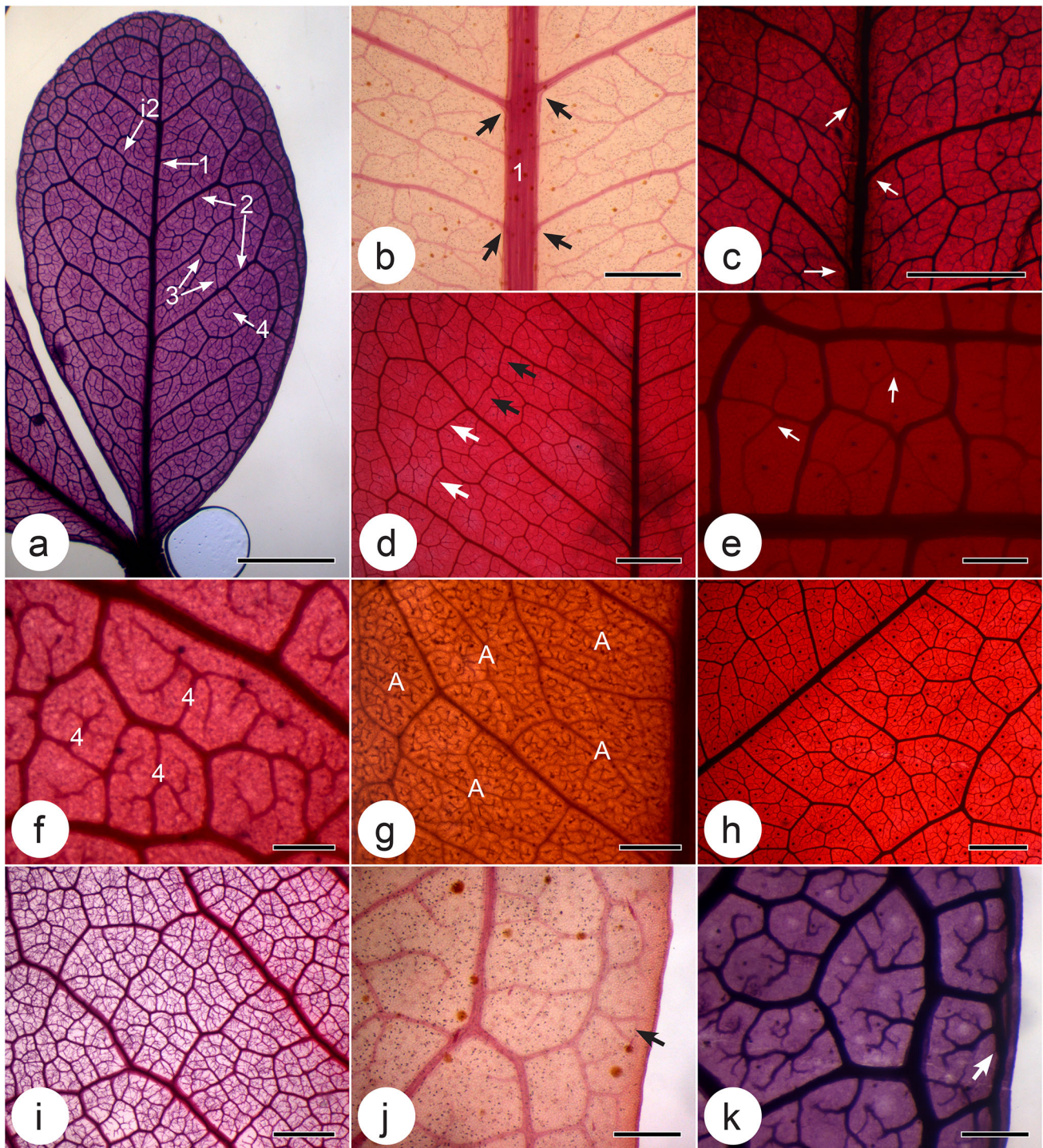
Fig. 4 Leaf venation. **a** *Metrodorea mollis*. Primary vein forms a pinnate pattern together with secondary veins. Connections are decurrent at the base of the leaf and excurrent at the apex. Intersecondary veins, tertiary veins, and quaternary veins are shown. **b** *Esenbeckia grandiflora*. Excurrent connections between primary and secondary veins (*arrows*). **c** *Conchocarpus heterophyllus*. At the leaf apex, connections between primary vein and secondary veins are decurrent (*arrows*), like those of the leaf base. **d** *M. nigra*. Tertiary veins with opposed (*black arrows*) or alternate (*white arrows*) patterns forming mixed percurrent arrangement. **e** *E. pilocarpoides*.

Irregular quaternary reticular veins with branched ends. Meeting points between quaternary veins pointed by *arrows*. **f** *M. maracasana*. Quaternary veins freely branched. **g** *Helietta apiculata*. Poor development of areoles. **h** *M. stipularis*. Good development of areoles. **i** *E. irwiniana*. Good development of areoles. **j** *E. grandiflora*. Incomplete terminal venation. **k** *M. nigra*. Looped terminal venation. *Bars* **a**, **c**, **d**, **h** 3 mm; **b** 1 mm; **e**, **f**, **i**, **k** 400 µm; **g** 900 µm; **j** 500 µm; *1* primary veins; *2* secondary veins; *i2* intersecondary veins; *3* tertiary veins; *4* quaternary veins; *A* areoles

Fig. 5 Majority-rule consensus Bayesian tree of *Metrodorea* and related species. *Numbers* above branches are posterior probability percentages
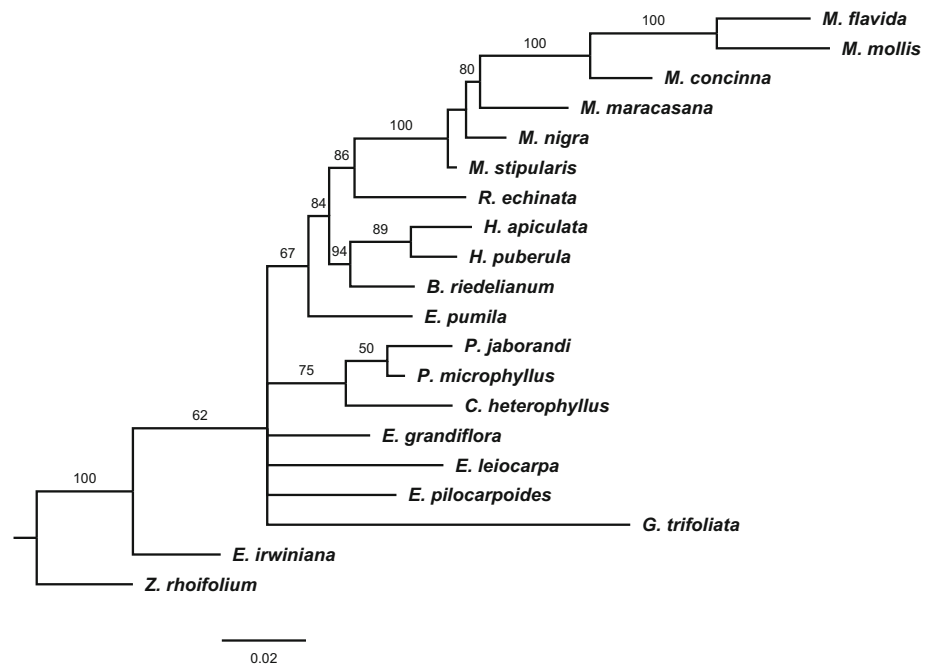
36) in the common ancestor of *Raulinoa* and *Metrodorea* that is shared with *Balfourodendron* and *Helietta*. Since the relationship between *Metrodorea* and these genera is not solved with a good support in our phylogeny, it is not possible to confirm that this feature appeared in the ancestral of *Metrodorea*.

Our topology shows an early divergence of *Metrodorea stipularis,* with the remaining species forming a clade supported by the concentration of crystals in the hypodermis, but this feature was not observed in *M. mollis.* Nevertheless, it can be just a result of the rarity of crystals in all tissues of this species leaves rather than a reversion. Within this clade, *M. nigra* emerged as sister group of a clade formed by the other four species, and *M. maracasana* as the sister group of the clade formed by *M. concinna, M. flavida,* and *M. mollis*, but no morphological synapomorphies could be identified that support these relationships. Otherwise, the crown group, composed by *M. concinna, M. flavida,* and *M. mollis*, is corroborated by the presence of margins of the stipules containing short intertwined trichomes and papillary cells that enhance adhesion.
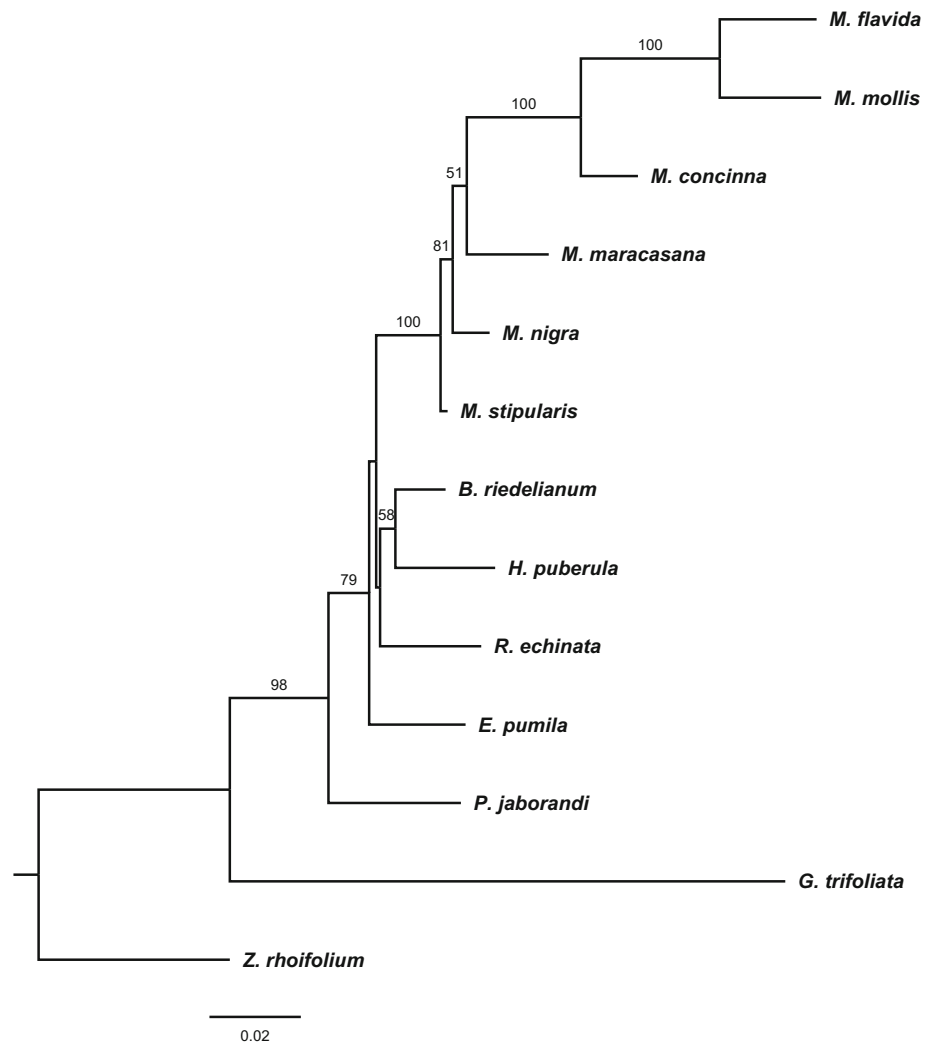
Discussion

"Unifoliolate leaves" are a condition usually applied to several Rutaceae-bearing leaves with a joint or articulation at the base of the blade (e.g., Kaastra 1982; Kallunki and Pirani 1998; Pirani 1999; Kubitzki et al. 2011). This is mostly upon the reasoning that in a phylogenetic sense these plants would have reduced the number of leaflets

during evolution to one. Any trying to find an anatomical or ontogenetic correlation between articulations and the division of the leaf blade has been unsuccessful since there is no evidence that these structures represent some residual feature from ancestral extra leaflets, as previously shown (Muntoreanu et al. 2011; Cruz et al. 2015). Thus, unifoliolate leaves reported for the Rutaceae members studied herein are identical to simple leaves with respect to development, except for some *Metrodorea* species leaves (in all described species, except *M. maracasana*) whose leaf primordia bear three leaflet primordia each (two of them may be aborted during development), so we classify them as early trifoliolate leaves (as in Cruz et al. 2015).

It is remarkable that some species like *Esenbeckia leiocarpa*, even though presenting a small swelling at the petiole, are classified as simple-leaved (Kaastra 1982). *E. grandiflora* also bears a swelling but associated with a constriction and is classified as unifoliolate by the same author. On the other hand, Kaastra (1982) classified as unifoliolate the leaves of *E. pilocarpoides*, which bear no conspicuous swelling but instead a distinct articulation at the petiole apex. Here, we conclude that it is more accurate to distinguish the possession of articulations and characterize associated constrictions or swellings, instead of mingling such character with the states of simple blade versus divided in leaflets. Anyway, we recovered the abortion of leaflet primordia as a possible synapomorphy of *Metrodorea* (Fig. 7), while the evolution of swellings and constrictions at the petiole apex of several among the sampled taxa have not shown a clear pattern. A similar condition of abortion of leaflet primordia may occur also

Fig. 6 Maximum likelihood
tree of *Metrodorea* and related
species. *Numbers* above
branches are bootstrap values
based on 1000 replicates (values
below 50% are not shown)

Fig. 6 Maximum likelihood tree showing:
M. flavida, M. mollis (100), M. concinna (100), M. maracasana (51), M. nigra (81), M. stipularis (100), B. riedelianum, H. puberula (58), R. echinata (79), E. pumila, P. jaborandi (98), G. trifoliata, Z. rhoifolium. Scale bar 0.02

in *E. pilocarpoides* subsp. *maurioides* Kaastra (not sampled here), since it presents in the same specimen simple leaves and leaves with two or three distinct leaflets (the lateral ones are smaller and sometimes partially connate to the terminal one). This diversity of leaf development and of leaf base structures is certainly promising to be investigated in further more extensive studies in the family.

Regarding other correlations with the division of the leaf, Duval (1903) associated the number of palisade parenchyma layers to compound and simple leaves in *Pilocarpus*. According to this author, species with simple leaves do not have palisade parenchyma with one single layer of cells. The simple-leaved species *Raulinoa echinata* and *Esenbeckia leiocarpa* (according to Kaastra 1982) have one layer of palisade parenchyma, so it was not possible to expand the generalization of Duval (1903) to other genera. Also, Muntoreanu et al. (2011) described one layer of palisade parenchyma in simple leaves of *P. spicatus* and *P. pauciflorus*, contradicting Duval (1903).

Metcalfe and Chalk (1950) reported that hairs may be infrequent in many members of Rutaceae. This may apply for *Metrodorea*, for example, that lacks non-glandular hairs in the leaf blades of most of its species. Kaastra (1982) and Pirani (1998) observed that the occurrence or absence of these trichomes can vary within the same species, but we did not observe such variation within the analyzed material. It is possible that the reported variation can be observed in younger or older leaves than the ones that we analyzed and it may be related to dehiscence process of these trichomes. More development studies are necessary to investigate this possibility.

Secretory cavities are the most characteristic feature of the Rutaceae and are described as schizogenous or lysigenous (Metcalfe and Chalk 1950; Kaastra 1982). However, cell lysis is often an artifact of the fixation procedure employed during the preparation of plant material (Turner et al. 1998), and our anatomical data with the applied technics do not bring any further evidence to
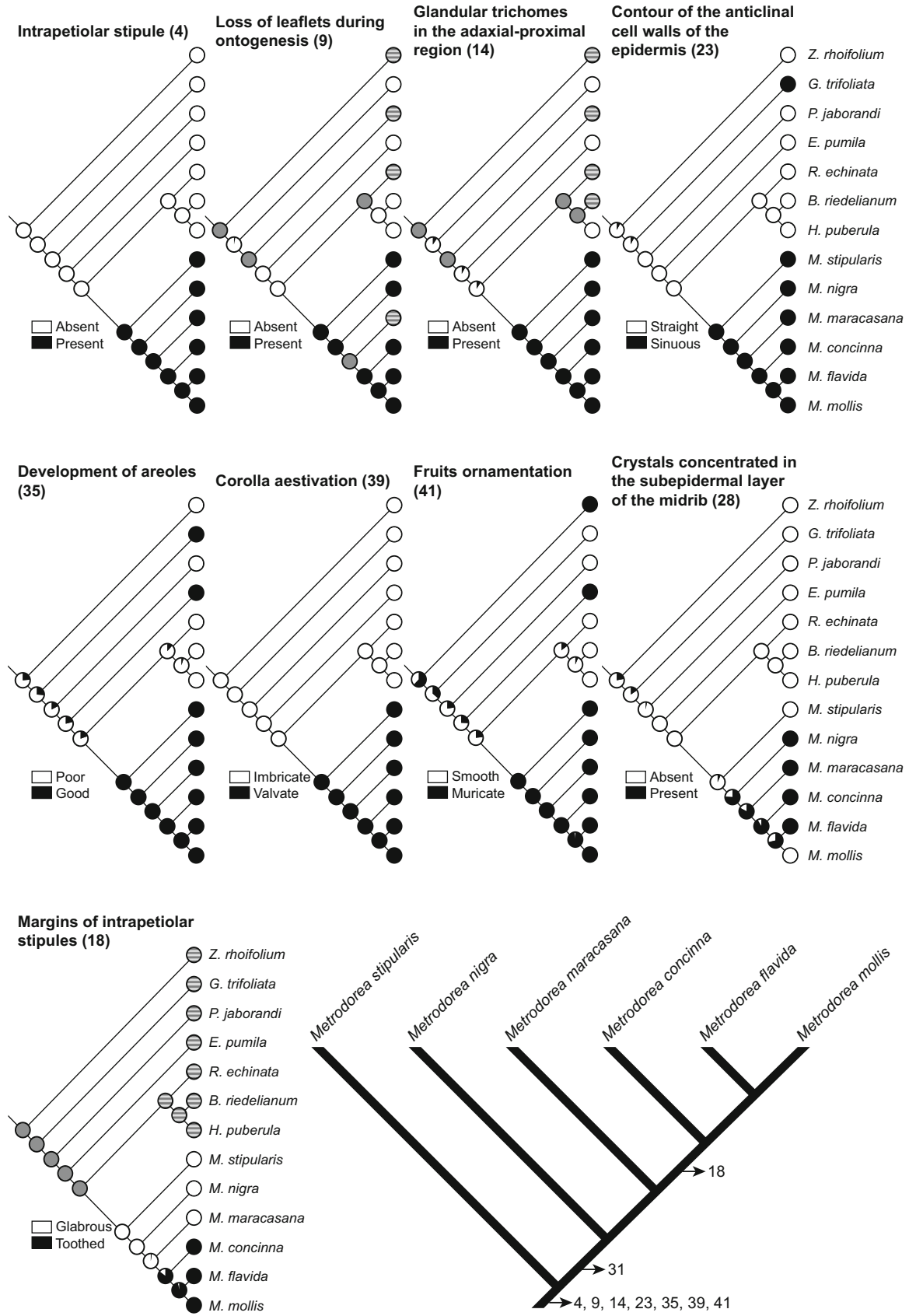
Fig. 7 Optimizations of characters that states changes represent possible synapomorphies in *Metrodorea*. *Black* and *white balls* in the nodes are probabilities graphs of which state these ancestral present. *Striped balls* represent non-applicable characters related to equivocal nodes (*plain gray balls*). At the *right bottom corner*, a compilation of all detected possible synapomorphies in the *Metrodorea* phylogeny

contribute to this discussion. Machado et al. (2016) present a detailed study of the histochemistry, ultrastructure, and ontogeny with careful fixation procedures of the oil cavities in *Metrodorea nigra*, describing a schizo lysigenous and seasonal development for the species.

The central vascular arch observed in the midrib region of *Esenbeckia irwiniana* is a feature that had been described in some plants as a medullary plate (Radford et al. 1974), and it is the first report for this structure in the vascular system of Rutaceae.

The types of crystals observed in the present study have already been described in some Rutaceae, and here, we register their occurrence for more species. Prismatic crystals found in compartments created by walls transversal to the length of the cell in palisade parenchyma are similar to *Pilocarpus* druses that also are organized in this way in the species studied by us and by other authors (Metcalfe and Chalk 1950; Muntoreanu et al. 2011).

The venation pattern typically pinnate and simple brochidodromous observed in all studied species of the present paper was also cited by Muntoreanu et al. (2011) in other species of the family. This pattern seems to be very old, as it was described in fossilized specimens of the Rutaceae (*Vepris* and *Clausena*) dating from the late Oligocene period (Pan 2010). It may be the only venation pattern of Rutaceae species for primary and secondary veins, or at least the most common. Dede (1962) proposed a classification of veins patterns in Rutaceae based on the position of secretory cavities in 80 genera. Despite he presented an extensive study, this classification does not allow a comparison with other families and it is hard to be made with regular clearing technics, and with herbarium or fossilized material, as they do not properly preserve secretory cavities. So, we recommend for new Rutaceae venation studies a detailed description according to Ellis et al. (2009), as we did. More details about the venation, like areolation and terminal veins, may be of big importance in new studies with other species and may provide potential phylogenetically informative characters, as we state below.

Although reuniting morphological data is essential to systematics studies, when used to make phylogenetic analyses, it usually generates matrixes much smaller than matrixes made with molecular data. So, when we prepared the combined matrix, morphological data were clearly underrepresented, except when molecular data were not available. This does not necessarily imply in a smaller
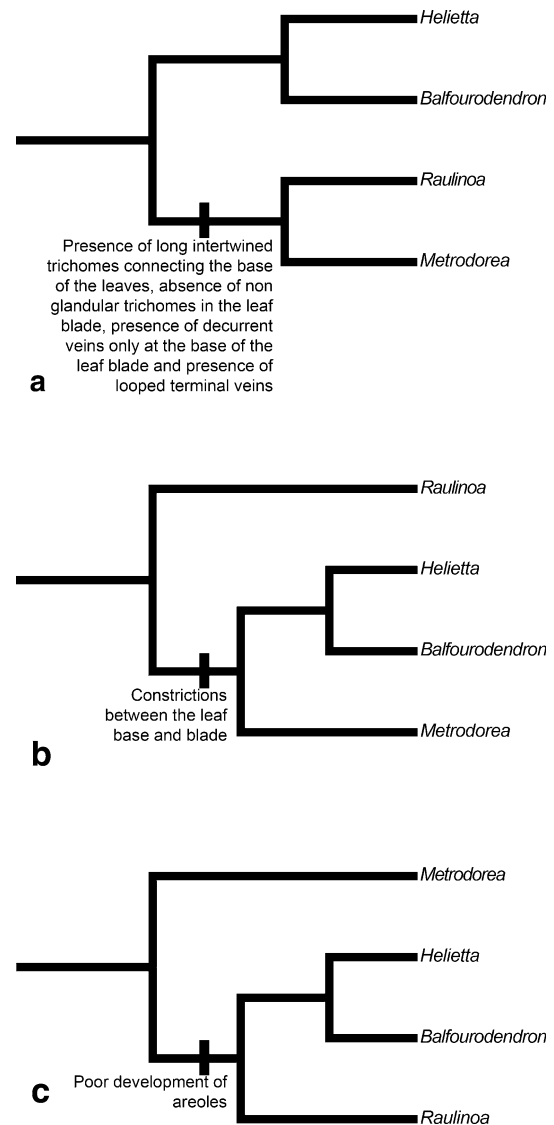
Fig. 8 Possible relationships between *Metrodorea*, *Helietta*, *Balfourodendron* and *Raulinoa*, and potential synapomorphies. **a** *Metrodorea* and *Raulinoa* are sister groups. This hypothesis is supported by a larger number of synapomorphies. **b** "*Balfourodendron* alliance" is the sister group of *Metrodorea*. **c** *Metrodorea* is the sister group of a clade formed by the three other genera

accuracy, even with a lot of missing data, as Jenner (2004) stated in his good discussion about the use of combined morphological and molecular data in phylogenetic reconstructions.

The adhesion promoted by intertwined trichomes in the stipules of *Metrodorea* species was already explored by us in our previous study (Cruz et al. 2015), and here, we detected that when present in the margin of stipules, these trichomes represent a good synapomorphy for the crown clade, composed by *M. concinna*, *M. flavida*, and *M. mollis*.

There are discrepancies between the relationships of *Raulinoa* with the *Helietta* + *Balfourodendron* clade and

with *Metrodorea* in the topologies recovered by us and by Dias et al. (2015). Considering a polytomy containing these three groups, there are three possible rooted trees as resolutions (Fig. 8). The clade *Raulinoa + Metrodorea* would find support based on the following structural characters: the presence of long intertwined trichomes connecting the base of the leaves, the absence of glandular trichomes on the leaf blade, the presence of decurrent veins only at the base of the leaf blade, and presence of looped terminal veins. In the second proposition, *Helietta + Balfourodendron* is considered sister group of *Metrodorea* based on the existence of constrictions between the leaf base and blade. The third possibility, in which *Raulinoa* is sister group of *Helietta + Balfourodendron,* would find support based on the synapomorphy of poor development of areoles. The acceptance of any one of these solutions implies the presence of homoplasies in character states that sustains the other two proposals. Clearly, the first assumption minimizes the number of necessary homoplasies to explain the relationship.

The findings presented herein allowed some robust hypotheses to be drawn about the evolution of some anatomical aspects of the studied species. Thus, the relationship of *Metrodorea* and *Raulinoa* postulated previously by Cowan (1960) and Kaastra (1982) is now additionally supported by a large number of possible structural synapomorphies. However, it is important to emphasize that the relationship between the stipules of *Metrodorea* and the thorns of *Raulinoa* (which are modified branches) postulated by Cowan (1960) has no grounds of homology and so cannot be taken as an appropriate evidence of the close relationship of the two genera, a fact previously pointed out by Kaastra (1982).

In summary, our study shows morphological features which are phylogenetically informative characters in *Metrodorea* and related Rutoideae, by using new leaf anatomy and venation data, including areolation and terminal veins, and from available data for reproductive structures. Further investigation on their structure certainly will bring additional evidence to keep on improving the knowledge about the systematics and evolution of the group.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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