Morphological and ultrastructural studies of floral nectaries in Rhamnaceae

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Morphological and ultrastructural studies of floral nectaries in Rhamnaceae[a,b]

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Abstract. Morphology, anatomy, and ultrastructure of nectaries of 12 species belonging to five tribes of Rhamnaceae (Pomaderreae, Colletieae, Paliureae, and Gouanieae of the Ziziphoids clade and Rhamneae of the Rhamnoids clade) were studied by light and electron microscopy. Four types of nectaries were observed in this study. Species of the tribes Rhamneae, Gouanieae, Paliureae, and *Cryptandra tomentosa* Lindl. of the Pomaderreae tribe have annular nectaries. *Siegfriedia darwinioides* C.A. Gardner and *Stenanthemum humile* Benth. (Pomaderreae) have revolute nectaries, as do *Colletia paradoxa* (Spreng.) Escalante and *Colletia spinosissima* J.F. Gmel. (Colletieae). *Retanilla patagonica* (Speg.) Tortosa and *Kentrothamnus weddellianus* (Miers) M.C. Johnst. (Colletieae) have indistinct and adpressed nectaries, respectively. Nectar secretion occurs through modified nectary stomata in all species. Differences in the ultrastructure, mode of nectar secretion, and position of the nectaries are discussed. According to the organelles found in the nectary cells of all the species studied, granulocrine secretion is most likely to occur. Further research on these structures in additional species of this family could help to establish nectary homologies.

Key words: electron microscopy, nectary, Rhamnoids clade, Ziziphoids clade

Nectaries are specialized tissues that secrete a sugary solution and may be situated in the flower or other parts of the plant (Nicolson *et al.* 2007). Vogel (1997, 1998) provided an exhaustive description of the types and structures of nectaries in many angiosperm families. Several topographic nectary classifications, the major criteria for distinguishing among nectaries, have been published (*e.g.*, Bonnier 1879; Ewert 1932; Brown 1938; Fahn 1953, 1979, 1982; Daumann 1970; Smets 1986; Smets and Cresens 1988) and, according to Bernardello (2007), many more can undoubtedly be formulated. Three components are present in nectaries: an epidermis, a specialized parenchyma, and a vascular bundle (Fahn 2000; Pacini *et al.* 2003). Nectaries vary in ontogeny, morphology, and structure among species (Fahn 1979, 1988; Durkee 1983; Nepi *et al.* 1996; Küchmeister *et al.* 1997; Smets *et al.* 2000; Fahn

and Shimony 2001; Pacini *et al.* 2003; Nepi 2007). According to Bernardello (2007), differences in histological components in structural nectaries have a systematic value.

Several authors have demonstrated the taxonomic and evolutionary value of floral nectaries for understanding the classifications of many angiosperm groups (*e.g.*, Bernardello 2007; Chesselet *et al.* 2002). Floral nectaries are either homogeneous or have changed dramatically within groups. Therefore, the floral nectaries show evolutionary trends in plant groups. In addition, within a lineage, the floral nectaries may be easily lost or acquired, which helps us to understand the evolution of the group and its mating systems (Bernardello 2007).

Rhamnaceae is a family of about 55 genera and 900 species and is cosmopolitan in distribution, but it mostly occurs in warm, temperate regions (Medan and Schirarend 2004, Perveen and Qaiser 2005). Three strongly supported Rhamnaceae clades (Ziziphoids, Rhamnoids, and Ampelozizi-phoids) have been identified in a phylogenetic analysis using *rbcl* and *trnl-f* plastid DNA sequences (Richardson *et al.* 2000). Medan and Aagesen (1995) provided a general description of nectary morphology and position in some members of the family. However, there are many species of Rhamnaceae whose nectary morphology is still unknown, and there are no known anatomical and/or ultrastructural studies of nectaries in any species of this family.

Table 1. Materials studied and nectary characters in Rhamnaceae.

Clade	Tribe	Species	Material provenance[a]	Nectary		
				Type	Trichomes	Cuticle ornamentation
Ziziphoids	Colletieae	*Retanilla patagonica* (Speg.) Tortosa	2, Cuesta de Toquero (Medan) (X, 1995)	Indistinct	Present	Striate
		Kentrothamus weddellianus (Miers) Johnst.	2, Tilly (XII, 1992)	Adpressed	Absent	Striate
		Colletia paradoxa (Spreng.) Escal	1	Revolute	Absent	Loosely striate
		C. spinosissima Gmel.	1	Revolute	Absent	Striate
	Pomaderreae	*Stenanthemum humile* Benth.	2, Kellermann (III, 2002)	Revolute	Absent	Rugulate
		Siegfriedia darwinioides C. Gardner	2, Walsh (VII, 2002)	Revolute	Absent	Smooth
		Cryptandra tomentosa Lindl.	2, Kellermann (2002)	Annular	Present	Smooth
	Paliureae	*Ziziphus mucronata* Willd.	1	Annular	Absent	Striate
		Z. mistol Griseb.	1	Annular	Present	Striate
		Z. jujuba Mill	1	Annular	Absent	Striate
	Gouanieae	*Gouania ulmifolia* Triana and Planch.	1	Annular	Absent	Striate
Rhamnoids	Rhamneae	*Scutia buxifolia* Hutch. and Moss	1	Annular	Absent	Striate

[a] 1 = Flowers were collected from cultivated plants in the Jardin Botánico Lucien Hauman, Facultad de Agronomía, Universidad de Buenos Aires (Buenos Aires, Argentina) (IX, 2012); 2 = Flowers were taken from fixed specimens in formalin-alcohol-acetic acid (FAA).

The aim of this research was to provide a detailed description of nectaries, based on their position in the flower, ultrastructure, and mode of nectar secretion to contribute to the general knowledge of the nectaries in Rhamnaceae. Therefore, representative species of the tribes belonging to the Ziziphoids clade (Pomaderreae, Colletieae, Paliureae, and Gouanieae) and to the only tribe of the Rhamnoids clade (Rhamneae) were studied.

Material and Methods. In total, 12 species belonging to 9 genera of Rhamnaceae were investigated. Flowers of seven species were collected from cultivated plants in the Jardin Botánico Lucien Hauman, Facultad de Agronomía, Universidad de Buenos Aires (Buenos Aires, Argentina) (Table 1). Flowers of the other species were taken from fixed specimens in formalin-alcohol-acetic acid (FAA) (Table 1). Voucher specimens were deposited in the Herbarium Gaspar Xuarez (BAA).

For light microscopy, flowers were fixed in FAA and embedded in paraffin. Sections (5–10 μm) were cut and stained with safranin combined with fast green, following the Zarlavsky (2014) technique, and mounted in synthetic resin.

For scanning electron microscopy studies, the material was prepared in accordance to procedures described by Zarlavsky (2014). Scanning micrographs were taken with a Philips XL 30 microscope (Philips, Amsterdam, The Netherlands).

Four representative species of the Ziziphoids clade (*Colletia spinosissima* J.F. Gmel. [Colletieae]; *Ziziphus jujuba* Mill., *Ziziphus mistol* Griseb., and *Ziziphus mucronata* Willd. [Paliureae]) and one of the Rhamnoids clade (*Gouania ulmifolia* Hook. & Arn. [Gouanieae]) were studied with transmission electron microscopy (TEM). Flowers at anthesis were prefixed overnight in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) and then postfixed in osmium tetroxide (OsO$_4$) at 2 °C in the same buffer for 3 h. After dehydration in an acetone series, the material was embedded in

Spurr's resin (Spurr 1969). Sections (1 μm) were stained with toluidine blue and photographed with a digital camera incorporated to a Motic light microscope (Ted Pella, Redding, CA). Ultrathin sections (75–90 nm) were made on a Reichert-Jung ultramicrotome (Reichert, Vienna, Austria), stained with uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963), and observed and photographed with a JEOL 1200 EX II transmission electron microscope (JEOL, Akishima, Tokyo, Japan) at 85.0 kV.

To analyze character evolution using parsimony, five states for the type of nectary were mapped in cladistics trees, using the traditional search with 1,000 replicates, with TNT (Tree analysis using New Technology) software (version 1.1, Fundación Miguel Lillo, San Miguel de Tucumán, Tucumán, Argentina) (Goloboff *et al*. 2000). *Dirachma socotrana* Schweinf. ex Balf.f (Dirachmaceae) was used as the outgroup. Bootstrap support and jackknife were calculated using 1,000 replicates. Presence of trichomes was hand mapped.

Results. MORPHOLOGY. Four types of nectaries were distinguished in the species studied according to their position in the flower (Table 1; Fig. 1a–d). In **annular nectaries**, an hypanthium encloses the ovary, and the nectary forms an annular structure surrounding the base of the style (*Scutia buxifolia* Reissek, *G. ulmifolia*, *Z. mucronata*, *Z. mistol*, *Z. jujuba*, and *C. tomentosa*) (Fig. 1a, b, 2a–h). **Revolute nectaries** were laminar projections of the hypanthium, above the ovary level near the revolute rim (*Siegfriedia darwinioides*, *Stenanthemum humile*, *C. paradoxa*, and *C. spinosissima*) (Fig. 1c, 3a, b). **Adpressed nectaries** are delimited distally by a slightly elevated rim (*K. weddellianus*) (Fig. 1d, 3c). **Indistinct nectaries** appear at the inner surfaces of the hypanthium (*R. patagonica*) (Fig. 1e).

ANATOMY. In all types, the nectary is composed of an epidermis and a secretory parenchyma supplied by vascular bundles, principally composed by phloem (Fig. 2h, 3b).

The nectary disc of *G. ulmifolia*, *Z. mistol*, and *C. tomentosa* presents trichomes (Fig. 4a), which are unicellular, with smooth cuticles that do not show secretory anatomical characteristics (Fig. 4a). In all other species studied, no trichomes were observed (Fig. 4b).

Modified nectary stomata are present in all species (Fig. 2b, d, f, 3d, 4c–f). In revolute nectaries, the stomata are rare, small, and positioned in a row through the rim curve. The guard cells of these stomata do not have a visible cuticular ridge (Fig. 3d). In indistinct and adpressed nectaries, the stomata are bigger and slightly sunken (Fig. 5f, g). In annular nectaries, the stomata are abundant through the entire nectariferous surface (Fig. 4a).

The nectary cuticle is smooth in *Siegfriedia darwinioides* and rugulate in *Stenanthemum humile* (Fig. 5a, b, respectively). *Colletia paradoxa*, *C. spinosissima*, and *G. ulmifolia* have a loosely striated cuticle (Fig. 5c–e), whereas the rest of the species studied have a striate cuticle (Fig. 5f–h). Cuticular pores were observed in *R. patagonica* (Fig. 5i).

ULTRASTRUCTURE. Epidermal cells of *G. ulmifolia* present a large, central vacuole, with the cytoplasm restricted to the periphery. Mitochondria, dictyosomes, and ribosomes can be observed (Fig. 6a). Subepidermal cells show mitochondria, plastids with plastoglobuli, rough endoplasmic reticulum, and large vacuoles with fibrillar and electron-dense content in *G. ulmifolia* and *Z. mucronata* (Fig. 6b, c). The guard cells of the stoma in this *Z. mucronata* have plastids with starch grains and many mitochondria (Fig. 6d).

An electron-dense and fibrillar secretion accumulates between the walls of the epidermal cells and its cuticle in *Z. mistol* (Fig. 6e–f). Mitochondria, ribosomes, and some plastids can be observed in the cytoplasm, and some vacuoles are filled with electron-dense content (Fig. 6e–g). Guard cells of the stomata present with many mitochondria, plastids with starch grains, ribosomes, and dictyosomes (Fig. 6g). Secretion can be observed between these two cells (Fig. 6g).

In *Z. jujuba*, secretion can be observed outside the cuticle of the epidermal cells, which are vacuolated cells with mitochondria and free ribosomes in the cytoplasm (Fig. 7a). The vacuoles show membranous vesicular bodies inside. The guard cells of the stomata have many mitochondria, some dictyosomes, and plastids with starch grains. Secretion accumulates in the substomatal chamber and between the two guard cells (Fig. 7b, c).

Epidermal cells of *Discaria americana* Gillies & Hook. present with a large, central vacuole, and the cytoplasm is restricted to the periphery. Some mitochondria, endoplasmic reticulum, and plastids
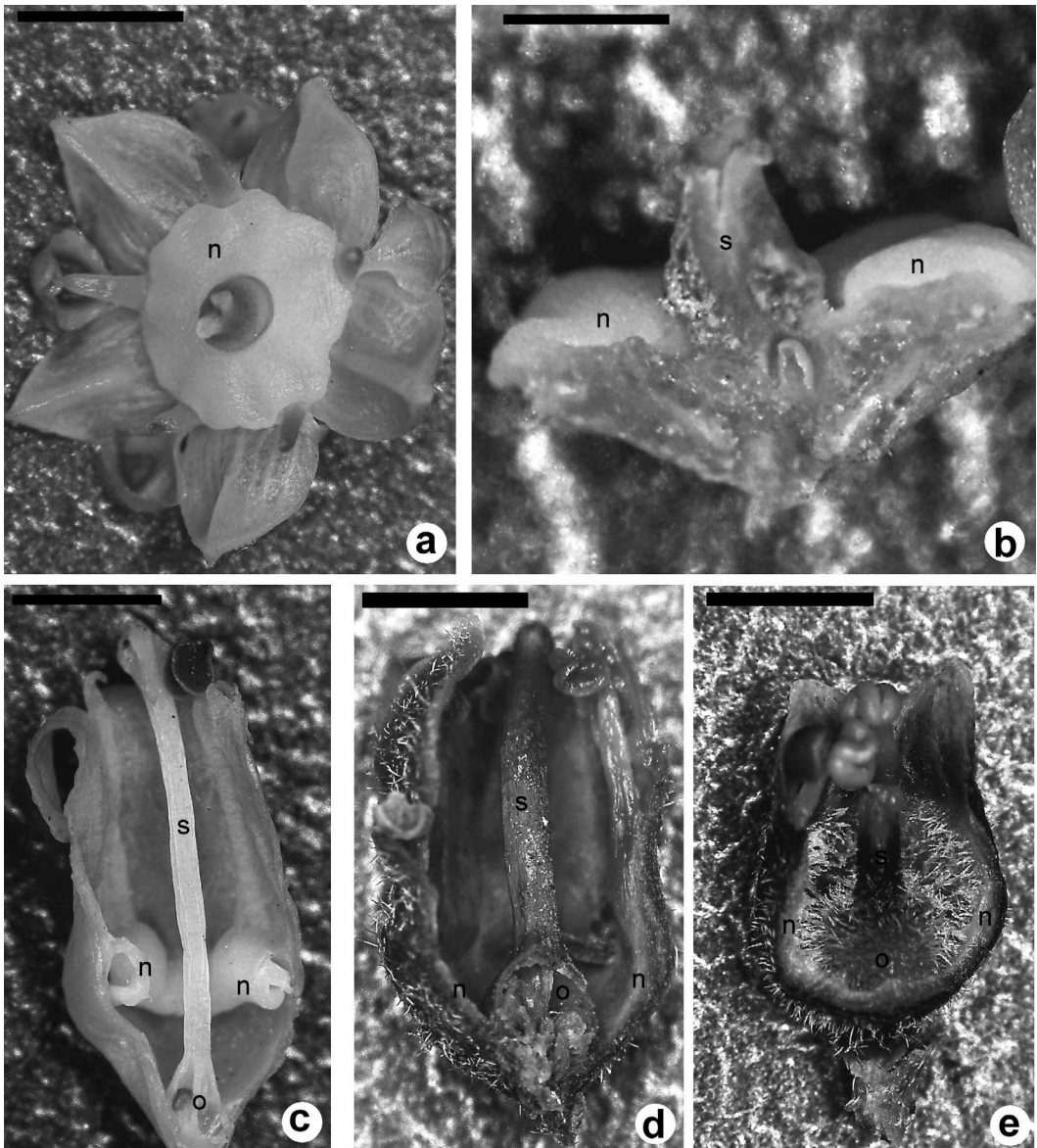
FIG. 1.   Light microscopy micrographs. (a, b) Annular type of nectary (*Ziziphus jujuba*). (c) Revolute type of nectary (*Colletia paradoxa*). (d) Adpressed type of nectary (*Kentrothamus weddellianus*). (e) Indistinct type of nectary (*Retanilla patagonica*). Scale bars = 2 mm (a, c, d, e); 1 mm (b). Abbreviations: n = nectary; s = style; o = ovary.

with starch grains can be observed. Vacuoles have membranous vesicular bodies. The cuticle is thick, and some electron-dense droplets can be seen in it (Fig. 7d). There are large, intercellular spaces between the subepidermal cells of the secretory parenchyma. Ribosomes, numerous mitochondria, dictyosomes, abundant plastids with starch grains, and plastoglobuli and endoplasmic reticulum of a

rough type can be found in the cytoplasm of these cells (Fig. 7e, f).

Discussion. Medan and Aagesen (1995) described four types of hypanthial nectaries in species of the tribe Colletieae. In our research, we extended the study of nectaries to other tribes of the family as well, and the same four types of
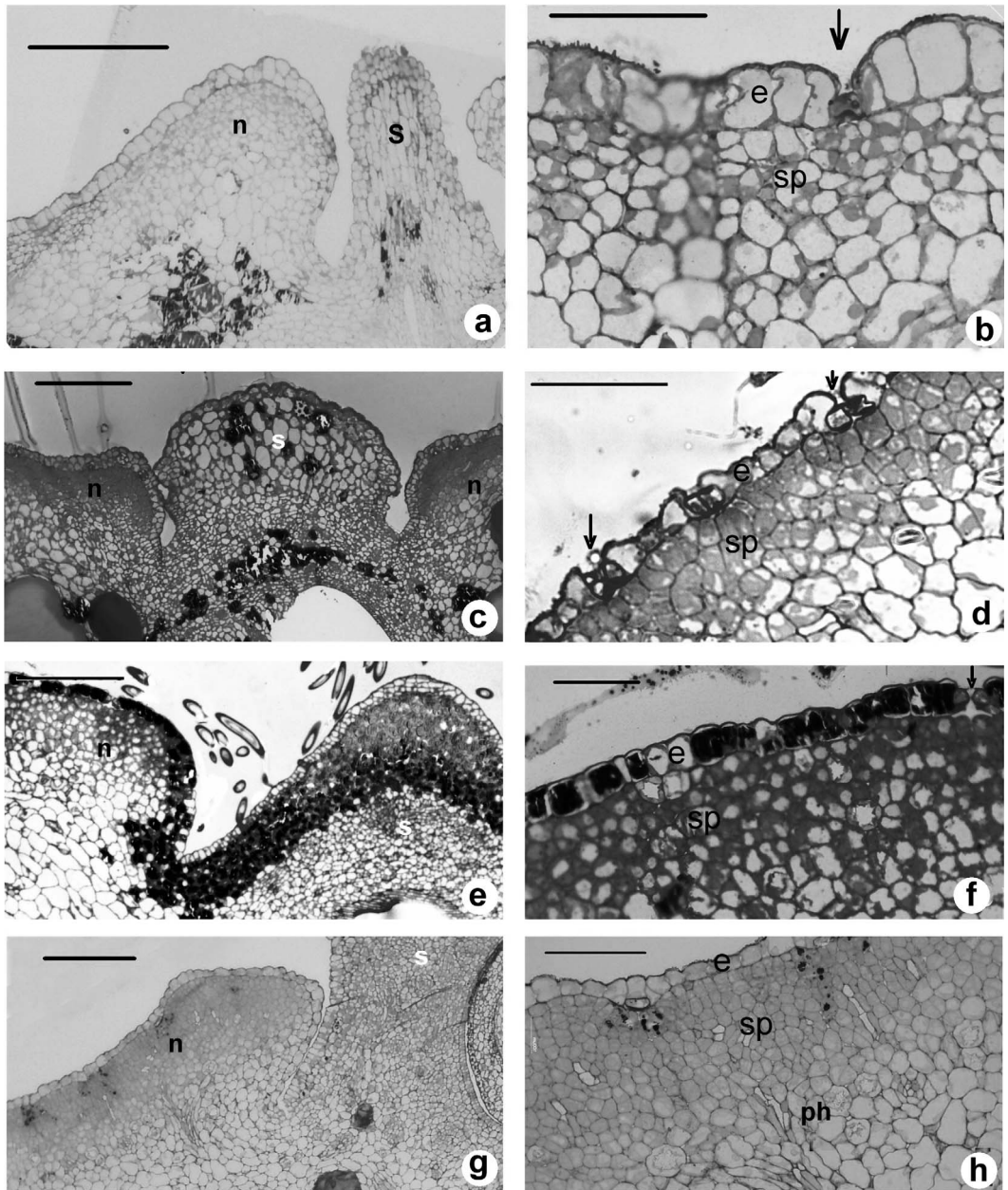
FIG. 2. Light microscopy micrographs showing annular nectaries. (a, b) *Gouania ulmifolia* (Gounaieae). (a) General aspect. (b) Nectary tissue with modified stoma (arrow). (c, d) *Ziziphus jujuba* (Paliureae). (c) General aspect. (d) Nectary tissue with modified stomata (arrows). (e, f) *Ziziphus mistol* (Paliureae). (e) General aspect. (f) Nectary tissue with modified stoma (arrow). (g, h) *Ziziphus mucronata* (Paliureae). (g) General aspect. (h) Nectary tissue with phloem. Scale bars = 200 μm (a); 70 μm (b); 150 μm (c, e); 35 μm (d); 40 μm (f); 150 μm (g); 20 μm (h). Abbreviations: n = nectary; ph = phloem; s = style; e = epidermis; sp = secretory parenchyma.

nectaries were recognized. *Colletia paradoxa* and *C. spinosissima* (Colletieae) and *Siegfriedia darwinioides* and *Stenanthemun humile* (Pomaderreae) have the revolute type of nectary. *Retanilla*

patagonica and *K. weddellianus*, both belonging to Colletieae, present with indistinct and adpressed types of nectary, respectively. All species studied of the tribes Paliureae, Gouanieae, and Rhamneae
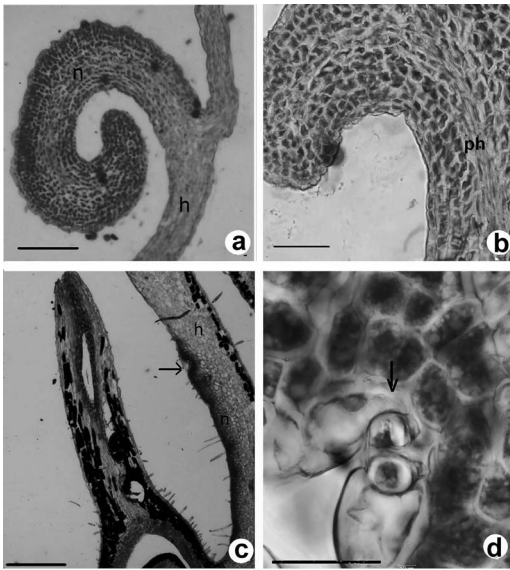
FIG. 3. Light microscopy micrographs showing anatomical structure of the nectaries in the studied species. (a) Pomaderreae. (b–f) Colletieae. (a) Stoma of *Cryptandra tomentosa*. (b–d) *Colletia paradoxa*. (b) General aspect of the nectary as a laminar projection of the hypanthium. (c) Detail of the nectary with phloem (ph). (d) Detail of the nectary surface showing a stoma (arrow). (e) General aspect of the inner secretory surface of the hypanthium in *Kentrothamus weddellianus* (n: nectary, arrow: rim). (f) Detail of the indistinct nectary of *Retanilla patagonica* showing stoma (arrow). Scale bars = 25 μm (a, d, f); 150 μm (b); 50 μm (c); 350 μm (e). Abbreviations: H = hypanthium; n = nectary.
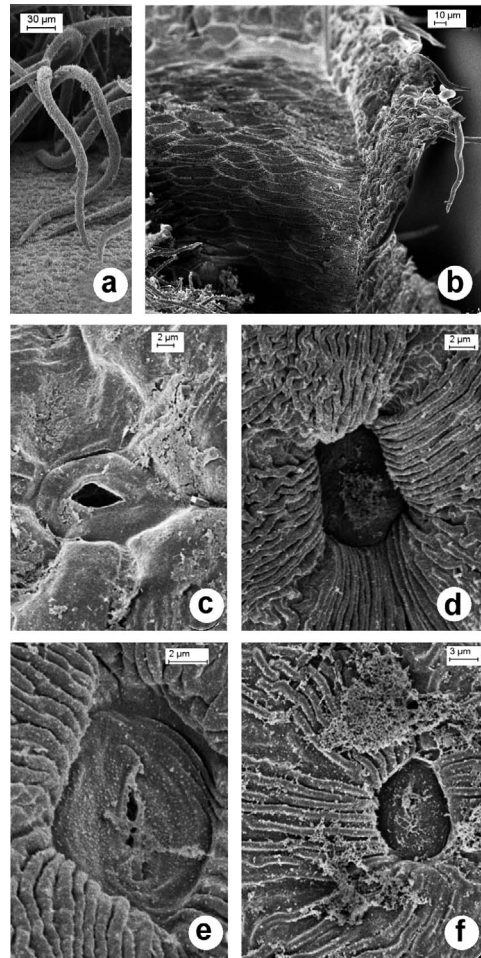

FIG. 4. Scanning electron microscopy micrographs showing the nectary epidermis. (a) Detail of trichomes of *Ziziphus mistol*. (b) Detail of secretory surface of *Retanilla patagonica*. (c) Detail of a modified stomata in *Scutia buxifolia*. (d) Detail of a modified stomata in *Gouania ulmifolia*. (e) Striate cuticle and a modified stoma in *Ziziphus mucronata*. (f) Striate cuticle and a modified stoma in *Ziziphus jujuba*.

and one species of Pomaderreae (*C. tomentosa*) have annular nectaries.

Nectar secretion occurs through modified nectary stomata in all species. According to Nepi (2007) when nectar secretion does not occur via stomata, the epidermis gets involved in the secretion process through epidermal secreting cells or secreting trichomes. Although some of the species studied have trichomes on their nectaries, those trichomes do not have any secretory function. The presence of secretion between the walls and the cuticle in *Z. jujuba*, the presence of electron-dense droplets in the cuticle of the epidermal cells in *D. americana*, and the pores observed in *R. patagonica* suggest that secretion is released through other epidermal cells as well. In *Z. jujuba* and *D. americana*, the cuticle may be permeable to the secretion.

The ultrastructure of the epidermal cells of the species studied coincides with previous general descriptions (Nepi 2007). According to Razem and Davis (1999), plastids do not store starch unless high levels of nectar are produced, as in *Passiflora* L. sp. (Durkee 1983), *Rosmarinus officinalis* L. (Zer and Fahn 1992), and *Cucurbita pepo* L. (Nepi *et al.* 1996). Fahn and Shimony (2001) suggested that the prenectar originating in the phloem is stored in the plastids as starch grains, which are
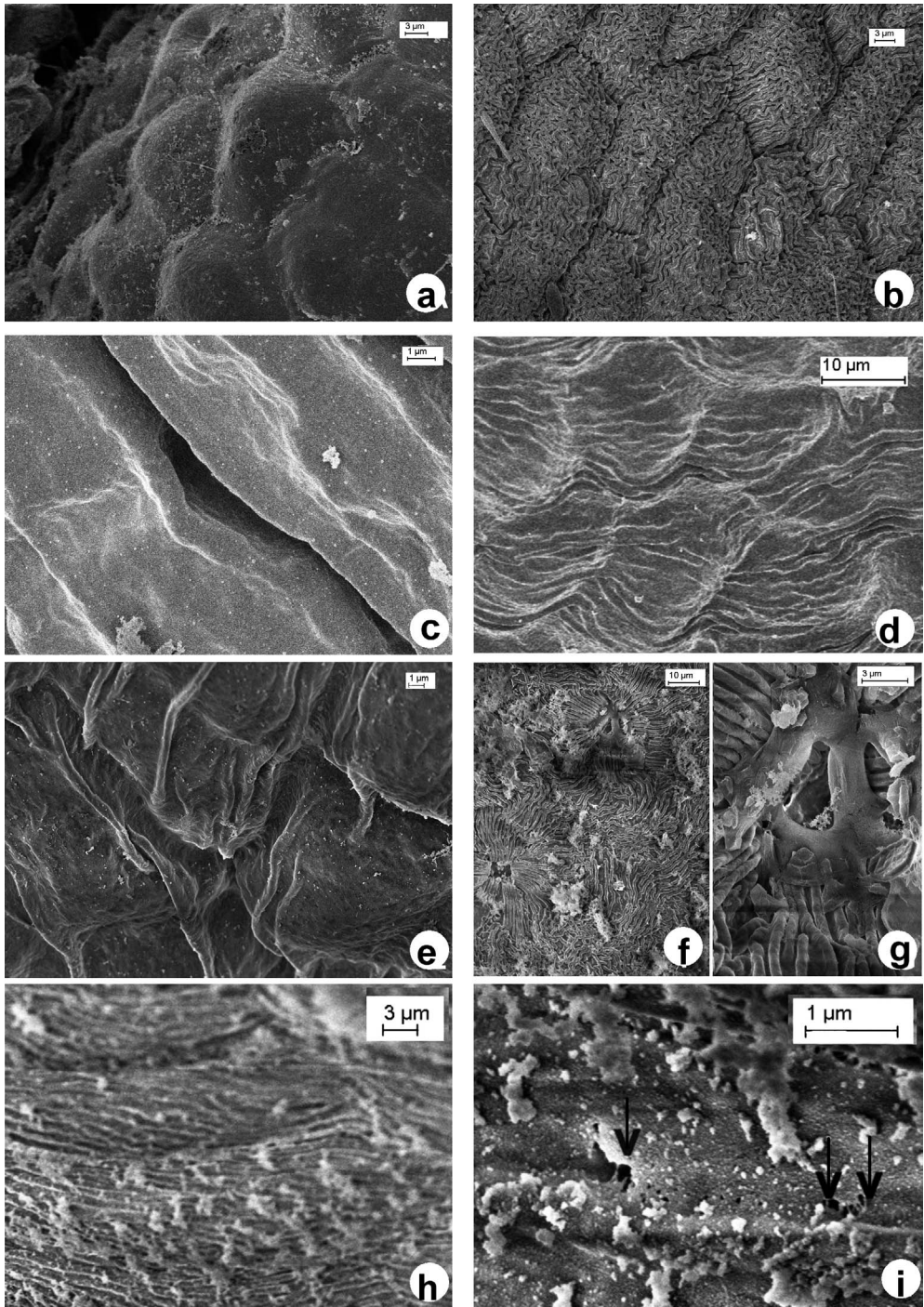
Fɪɢ. 5.   Scanning electron microscopy. Detail of the nectary epidermis. (a) *Siegfriedia darwinioides* with a smooth cuticle. (b) *Stenanthemun humile* with a regulate cuticle. (c) *Colletia paradoxa* with a loosely striate cuticle. (d) *Colletia spinosissima* with a striate cuticle. (e) *Discaria americana* with a loosely striate cuticle. (f, g) *Kentrothamus weddellianus*. (f) Striate cuticle with stomata. (g) Detail of a stoma. (h, i) *Retanilla patagonica*. (h) Striate cuticle. (i) Detail of the cuticle with pores (arrows).
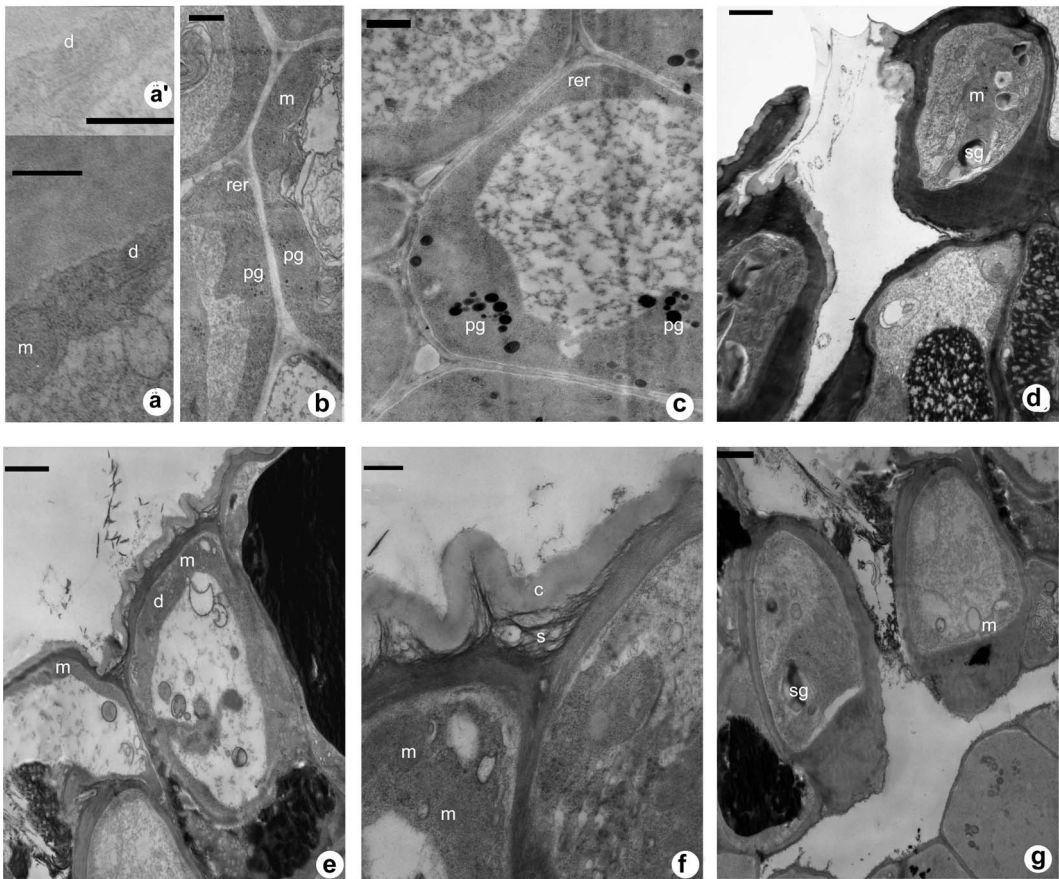
Fig. 6. Transmission electron microscopy micrographs showing the nectary ultrastructure. (a, b) *Gouania ulmifolia*. (c, d) *Ziziphus mucronata*. (e–g) *Ziziphus mistol*. (a) Epidermal cell of the nectary with mitochondria and dictyosome (d). (b) Subepidermal cell with plastids with plastoglobuli, mitochondria, and endoplasmic reticulum. (c) Subepidermal cells with plastids with plastoglobuli, mitochondria and endoplasmic reticulum. (d) Guard cells of the stoma showing plastids with starch grains, mitochondria. (e) Epidermal cell with striate cuticle, vacuole with tannin content. (f) Details of epidermal cells with secretion between the wall and the cuticle. (g) Stomata. Scale bars: 500 nm (a, f); 2 μm (b, d, e, g); 1 μm (c). Abbreviations: d = dictyosome; er = endoplasmic reticulum; m = mitochondrion; pg = oil bodies in plastids; sg = starch grains.

then hydrolyzed to sugar before the nectar secretion stage. Cell of the epidermis in *D. americana* and the guard cells of the stomata in the three species of *Ziziphus* species studied have plastids with starch grains, which suggest sugar-rich nectar. In addition, the nectary is principally innervated by phloem. All species of Colletieae studied show xenogamy, self-incompatibility, and pollen transfer mediated by insects (Medan and Torretta 2014), and insect pollination is common in Rhamnaceae (Medan and Schirarend 2004).

The abundance of mitochondria in the cells suggests a high-energy demand for the transport of nectar (Paiva 2009). According to Taiz and Zeiger

(1998) in the apoplastic pathway, sucrose uptake requires metabolic energy. These features, along with the absence of plasmodesmata between cells, suggest an apoplastic transport route for the nectar precursors toward the secretory cells in Rhamnaceae family. An apoplastic route for the prenectar was also suggested by Genc (1996) for nectaries of *Euphorbia* L.

Nectary parenchyma ultrastructure depends on the mechanism of prenectar transport and the source of nectar carbohydrates (Nepi 2007). Parenchyma cells rich in endoplasmic reticulum cisternae, dictyosomes, and vesicles are often associated with granulocrine secretion (Rachmilevitz and Fahn
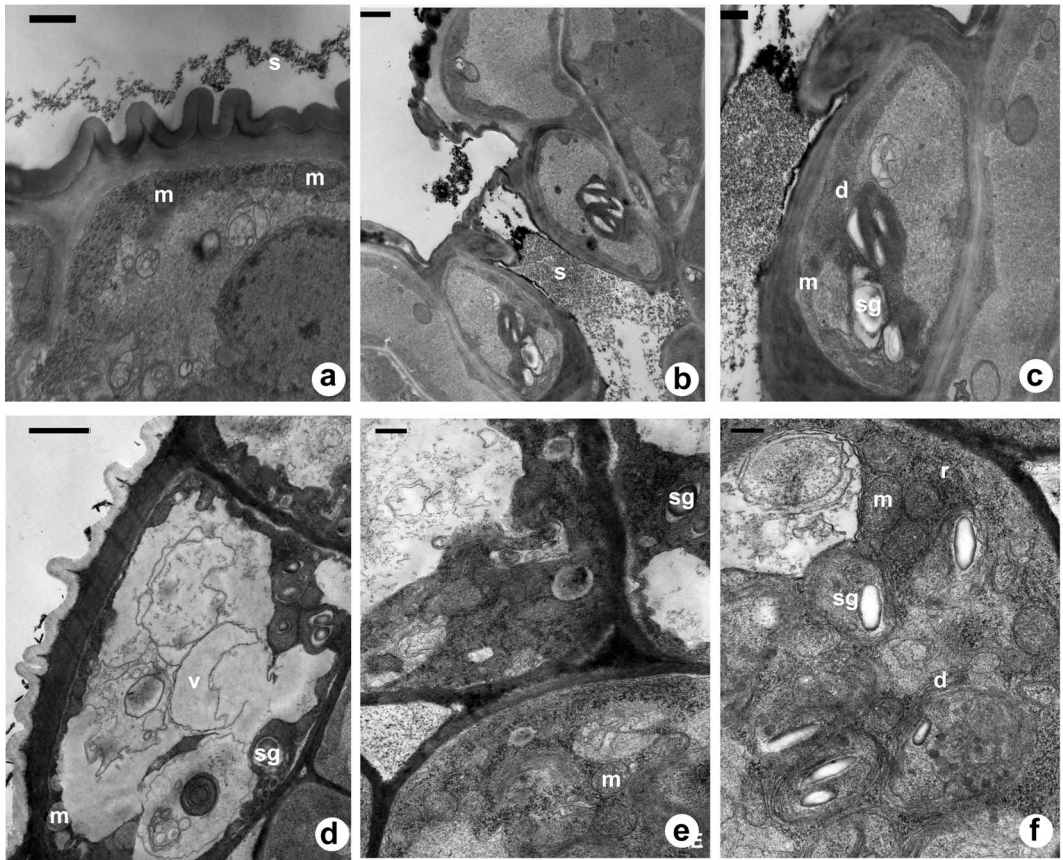
Fɪɢ. 7.   Transmission electron microscopy micrographs showing the nectary ultrastructure. (a–c) *Ziziphus jujuba*. (d–f) *Discaria americana*. (a) Detail of an epidermal cell, secretion, mitochondria (m). (b) Stomata with secretion in sub-stomatal chamber. (c) Detail of a guard cell of the stomata with mitochondria and starch plastids. (d) Epidermal cell with a large vacuole, mitochondria, and starch plastids. (e) Subepidermal cells. (f) Detail of a subepidermal cell with mitochondria ribosomes = Golgi bodies (d) starch plastids with starch grains and endoplasmic reticulum. Scale bars: 1 μm (a, c); 2 μm (b, d); 500 nm (e, f). Abbreviations: d = dictyosome; er = endoplasmic reticulum; m = mitochondrion; p = oil bodies in plastids; s = secretion; sc = substomatal chamber; sg = starch grains; v = vacuole.

1973; Fahn 1979; Durkee 1983; Fahn 1988, 2000; Arumugasamy *et al.* 1990). When eccrine secretion occurs, dictyosomes and endoplasmic reticulum are rare (Elias *et al.* 1975; Eriksson 1977; Nepi *et al.* 1996; Razem and Davis 1999; Stpiczyńska 2003). According to the organelles found in nectary cells of all the species studied, granulocrine secretion is the most likely to occur.

Nectaries in Dirachmaceae, a family closely related to Rhamnaceae, arise on a protuberance at the base of the petals (de Craene and Miller 2004). In Rhamnaceae. nectaries are intrastaminal, sometimes separated from the gynoecium by intercalary growth (Medan and Aagesen 1995). According to de Craene and Miller (2004), the association of

nectaries with the petal bases may represent a further sequence in the centrifugal displacement of the nectary. Medan and Aagesen (1995) proposed that all nectary forms are derived from a nectarial primordium located around the gynoecium primordium. That hypothesis is supported by our observations.

Aagesen (1999) estimated the phylogenetic relationships within the Colletieae tribe, and Richardson *et al.* (2000) made a phylogenetic analysis of the family Rhamnaceae using plastid DNA sequences. Several authors claim that determining nectary homologies is essential to understanding their evolution (*e.g.*, Smets 1986; Smets and Cresens 1988; Smets *et al.* 2000).
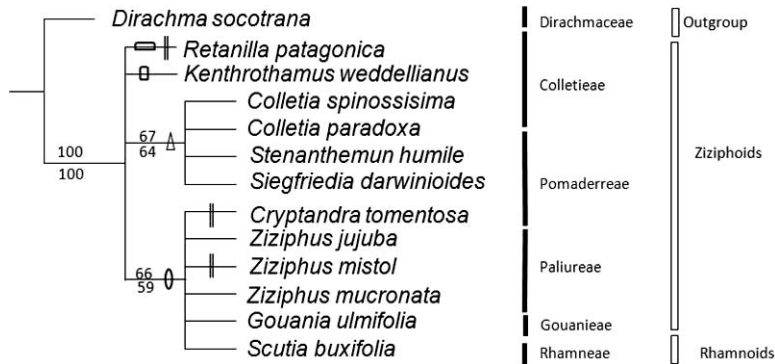
Fig. 8.   Optimal tree from the type of nectary analysis with standard bootstrap values (above the branches) and Jackknifing ($P = 36$) (below the branches). Group frequencies, 1,000 replicates, cut = 50. (0) Annular nectary. (Δ) Revolute nectary. (□) Adpressed nectary. (□) Indistinct nectary. (||) Presence of trichomes (hand mapped).

Therefore, we analyzed the morphological characters and considered the maximum parsimony principle, with *D. socotrana* (Dirachmaceae; de Craene and Miller 2004) used as an outgroup.

According to the observations described in this article, the characters summarized in Table 1, and the characters mapped on the tree in Fig. 8 (showing the type of nectary and presence of trichomes), we conclude that the presence of stomata could be a plesiomorphic character in the family. The annular nectary suggest synapomorphy between Rhamneae, Gouanieae, and Paliureae; the revolute type of nectary could be a synapomorphy between Colletieae and Pomaderreae (excluding *C. tomentosa*). The adpressed nectary of *K. weddellianus* and the indistinct nectary in *R. patagonica* appear to be apomorphies. The presence of trichomes in *Z. mistol*, *C. tomentosa*, and *R. patagonica* could also be considered apomorphies (Fig. 8). Considering that the Rhamnaceae family comprises about 900 species, further studies on nectary morphology and ultrastructure in additional species of this family could help to establish homologies. However, the morphology of nectaries studied here seems to support the phylogenetic analysis using plastid DNA (Richardson *et al.* 2000).

Conclusion. Characters described here substantiates future research on the structures in other species of the Rhamnaceae family.

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