

Comparative systematic study of colleter and stipule of Rhizophoraceae with implications for adaptation to challenging environments

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Colleters are multicellular secretory structures found on various organs in flowering plants. Colleters on the adaxial sides of stipules have been hypothesized to play a role in protecting the developing shoot. Rhizophoraceae is a stipulate family with a broad distribution from mangrove to montane environments, which makes the family well suited for the examination of this hypothesis, but the colleters of Rhizophoraceae are not well known. We compared species from all three tribes of Rhizophoraceae, including five inland genera and all four mangrove genera. In all species, several to hundreds of colleters, sessile or stalked, arranged in rows aggregated in genus-specific shapes, are found at the adaxial bases of open and closed stipules. *Pellacalyx* uniquely has additional colleters at the stipule margins. Colleters are all of the standard type, comprising a central axis of core parenchyma with large vacuoles and tannins, and an outer palisade-like epidermis with organelles involved in secretory activity. An exception is *Pellacalyx axillaris*, in which colleters appear as extremely small epidermal protrusions. *Kandelia obovata* has a tracheary element in some colleters. *Pellacalyx* uniquely has an unusual fleshy outgrowth on the adaxial stipule base. We propose an evolutionary sequence in which *Macarisia* has plesiomorphic stipule and colleter traits and the mangrove *Kandelia obovata* with colleter vascular traces is most derived. Colleter and stipule structures are largely concordant with habitat and phylogeny, and show taxonomic value. The strong alignment of colleter and stipule patterns with habitat is suggestive that colleters have a protective function, although some components of these patterns may be phylogenetically determined. © 2013 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2013, **172**, 449–464.

ADDITIONAL KEYWORDS: *Cassipourea* – fleshy outgrowth – *Gynotroches* – *Kandelia* – *Macarisia* – mangroves – *Pellacalyx* – standard type – ultrastructure.

INTRODUCTION

Rhizophoraceae (Malpighiales) is diversified both ecologically and morphologically. In addition to four exclusively mangrove genera (*Bruguiera* Sav., *Ceriops* Arn., *Kandelia* Wight & Arn. and *Rhizophora* L.), there are

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15 terrestrial genera with 135 species occurring in inland forests (Juncosa & Tomlinson, 1988a). All taxa of Rhizophoraceae s.s. (excluding *Anisophyllea* R.Br. ex Sabine) have large and conspicuous interpetiolar, glabrous and caducous stipules, strongly sheathing the young leaves and inflorescences (Hou, 1958). At the adaxial bases of stipules in the mangrove Rhizophoraceae, several to hundreds of finger-like glandular structures, called colleters (Lersten & Curtis, 1974; Fahn, 1990), can be observed with the naked eye, mixed with milky mucilage (Tomlinson, 1986). As the stipules enclose the developing shoot, it has been hypothesized that colleter secretions have a shoot protective function (Kronstedt-Robards & Robards, 1991; Paiva, 2009, 2012). In addition, in a comprehensive study of mangrove Rhizophoraceae (Sheue, Chen & Yang, 2012), it has been suggested that stipules and colleters provide a system for mechanical protection and physiological immersion, aiding the development of the young shoots of these mangrove plants coping with a challenging environment. Under this hypothesis, challenges in different environments should be reflected by differences in stipule and colleter morphology, colleter number and secretory function. However, suitable comparative studies to test this idea have not been conducted previously.

According to Hou (1958), colleters of Rhizophoraceae were first described by Alston in African *Cassipourea* Aubl. (inland species) and later by Metcalfe & Chalk (1957) in *Carallia* Roxb., but appear to occur in all Malaysian genera, both mangrove and inland species. The colleters of a mangrove species of this family were first described in *Rhizophora mangle* L. (Gill & Tomlinson, 1969). In an anatomical study of *R. mangle*, Lersten & Curtis (1974) reported that colleters of this taxon have a finger-like structure with a central axis of slender elongated cells surrounded by a palisade-like epidermis. This structure is similar to that found in several other families, and defines the 'standard type' (Lersten & Curtis, 1974). Recently, the taxonomic value of colleters of the mangrove Rhizophoraceae (tribe Rhizophoreae) has been demonstrated (Sheue, Liu & Yang, 2003; Sheue, Liu & Yong, 2003; Sheue, Yong & Yang, 2005; Sheue *et al.*, 2010, 2012).

Although many studies of colleters have been carried out in Rubiaceae (Lersten, 1974a, b, 1975; Miller, Scott & Gardner, 1983; Mangalan *et al.*, 1990; Klein *et al.*, 2004), Apocynaceae (Thomas & Dave, 1989, 1991; Appezzato-da-Glória & Estelita, 2000; Schwarz & Furlan, 2002) and Myrtaceae (da Silva *et al.*, 2012), colleters of Rhizophoraceae have been less comprehensively studied beyond mangrove taxa (Lersten & Curtis, 1974; Sheue *et al.*, 2012). However, Rhizophoraceae is a family well suited to the examination of the idea that colleters have an

important role in protecting the developing shoot, because members of this family are distributed over a broad range of environments, including mangrove habitats, lowland forests and montane forests. Here, we provide comparative data for nine genera of Rhizophoraceae from all three major habitat types to reveal associations between environment and colleter features. These include all three tribes (Macarisieae, Gynotrocheae and Rhizophoreae) of Rhizophoraceae (Schwarzbach & Ricklefs, 2000), five inland genera (*Carallia*, *Cassipourea*, *Gynotroches* Blume, *Macarisia* Thou., *Pellacalyx* Korth.) and all four mangrove genera.

MATERIAL AND METHODS

Plant materials of Rhizophoraceae containing stipules with colleters were collected from four mangrove genera from Asia and Australia and five inland genera from Asia and Madagascar during the period 2005 to 2010 (Table 1). Voucher specimens were deposited in the Herbarium of National Chung Hsing University (TCB), Taichung, Taiwan.

Three individual plants were sampled from each population studied. From each individual, three stipules were carefully detached from the shoot apices at the incipiency of extension of the young leaves. In addition, three younger stipules of *Kandelia obovata* from three individuals were collected for the study of ultrastructure. Stipules were stored in 70% ethanol in the field, washed with an ultrasound cleaner and observed with a Leica S8AP0 stereoscope (Wetzlar, Germany) equipped with an Olympus digital camera (Tokyo, Japan) to determine stipule morphology, the numbers of colleters and their form of aggregation in the stipule. Photographs of colleters in a stipule taken from different focal planes (*c.* five to eight images) were used to make three-dimensional still and video images, using Helicon Soft (Kharkov, Ukraine). For shape and size determination, a portion of the materials was observed and photographed with a table-top microscope (TM 3000, Hitachi, Tokyo, Japan).

For field fixation for anatomical study, the base of each stipule with colleters was cut into several small pieces and placed into 1.25–1.50% glutaraldehyde in 0.1 M phosphate buffer with 5% sucrose. Materials were stored in this fixative for 5–7 days whilst collecting overseas (Sheue, 2003). For the second fixation, materials were subsequently transferred to 1% OsO₄ in 0.1 M phosphate buffer for about 4 h. After dehydration through an ethanol series, materials were infiltrated for 3 days and embedded in Spurr's resin. The embedded materials were polymerized in an oven at 70 °C for 12 h. Semithin sections (1 µm)

were made by a MTX Microtome (RMC, Tucson, AZ, USA), stained with 0.1% toluidine blue for around 1 min, and then observed and photographed with a light microscope (Olympus BX-51, Tokyo, Japan). Ultrathin sections (70 nm) were cut and stained with 5% uranyl acetate in 50% methanol and 1% lead citrate in water before examination with a transmission electron microscope (JEOL JEM-1400, Tokyo, Japan). In addition, three stipules with colleters from each of three individuals of *K. obovata* were cleared (Sheue, Liu & Yang, 2003) for further investigation of vascular tissue after tracheid-like cells were observed from a semithin section.

RESULTS

STIPULE MORPHOLOGY AND EXUDATES

Although the mangrove genera of Rhizophoraceae have large stipules in pairs enclosing young leaves at the shoot apex, there is substantial variation between inland members in stipule size and nature (Fig. 1). Two types of stipule were distinguished on the basis of stipule vernation. In all mangrove species (Fig. 1A, B) and two inland genera of Gynotrocheae (*Carallia*, *Gynotroches*) (Fig. 1C, D), stipule vernation is clasping with one margin free (imbricate). We designated these stipules as the 'closed' type (Fig. 1A–D; Table 1).

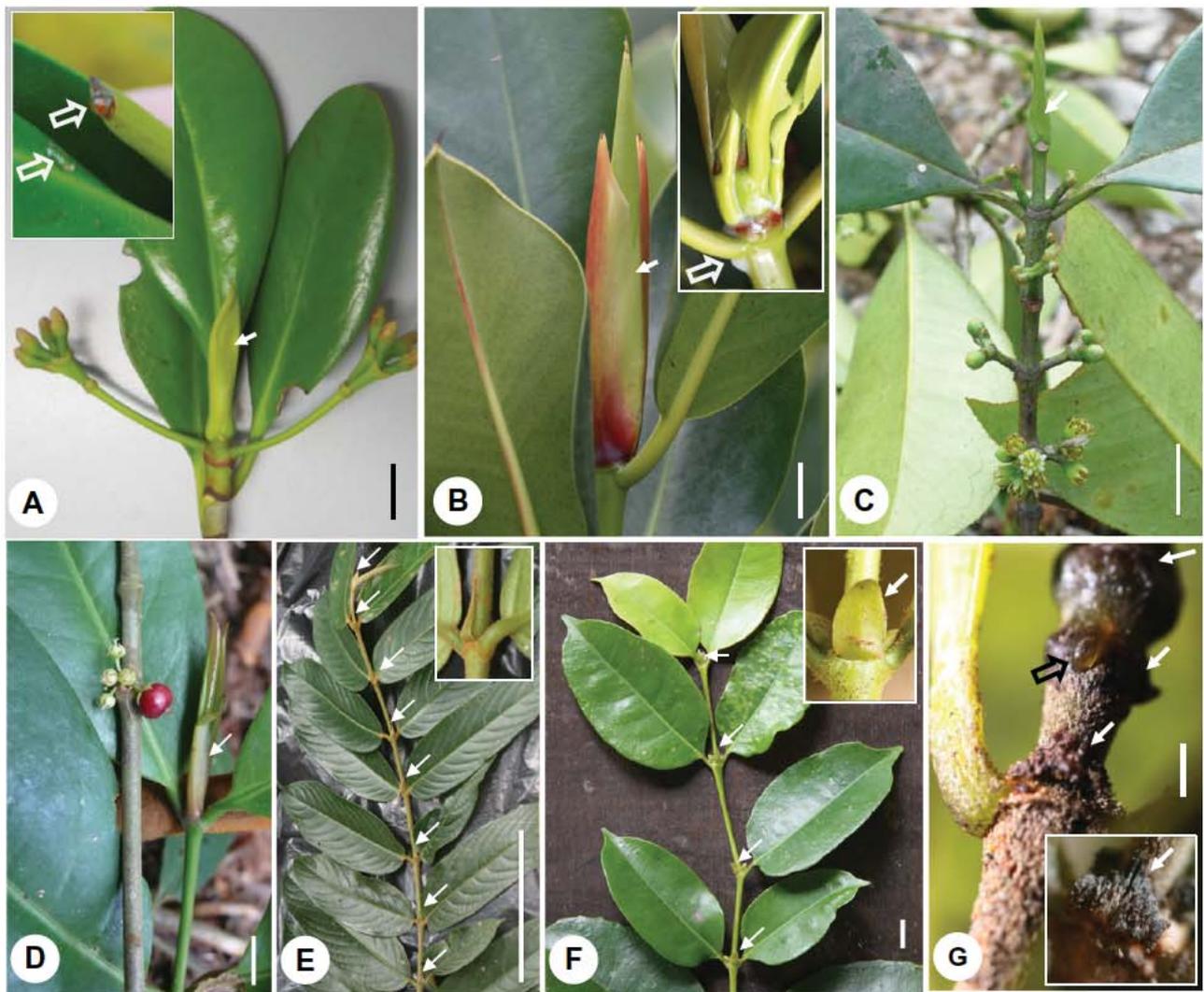


Figure 1. Shoot morphology and types of stipule of Rhizophoraceae. The closed-type stipule (A–D) is conspicuous (15–80 mm in length) and caducous, and is found in both inland and mangrove species; the open-type stipule (E–G) is small (1–15 mm) without overlapping margins, more persistent and found only in inland species. A, *Kandelia obovata*. B, *Rhizophora stylosa*. C, *Carallia brachiata*. D, *Gynotroches axillaris*. E, *Pellacalyx axillaris*. F, *Cassipourea* sp. G, *Macarisia humbertiana*. Open arrow, exudates; arrow, stipule. Scale bars: A–D, F, 1 cm; E, 10 cm; G, 5 mm.

Figure 2. Adaxial view of stipule and the aggregation form of colleter of Rhizophoraceae. A, B, *Rhizophora stylosa*. A, Fresh stipules with large yellowish collectors (arrows) aggregated as a band. B, Close-up view of fresh collectors. C–E, *Carallia brachiata*. C, A fresh stipule showing small and brown collectors. D, Close-up view of small collectors. E, All collectors aggregate as a band. F, *Carallia suffruticosa* often with collectors of uneven size. G, H, *Pellacalyx axillaris*. G, A fresh stipule showing a novel fleshy outgrowth (open arrow), with extremely small colleter-like protrusions (arrows) above this outgrowth (top inset). Note that the lateral view of the stipule shows the striking bend at its base (bottom inset). H, The top of the outgrowth showing the free upper margin with lobes (arrows). I, *Pellacalyx saccadianus* with a less evident fleshy outgrowth (open arrow) below the colleter region. Inset is a close-up view of collectors. Note that additional collectors occur on stipule margins (arrows). J, K, *Cassipourea* sp. J, A fresh stipule with several collectors arranged in a single row. Occasionally, collectors can be found attached to the corresponding side of the stipule scar on a stem (inset, arrows). K, The collectors at senescence are dark brown. L, M, *Macarisia humbertiana* with very small stipules, but abundant collectors uniquely filling entire stipules. B, E, F are reconstructed three-dimensional images. Scale bars: A, 10 mm; B–F, H–K, 1 mm; G, 5 mm; L, M, 0.5 mm.

Closed stipules are conspicuous (10–55 mm), but caducous, only seen at the terminal shoot apices. In contrast, the other type of stipule is small (1–15 mm), but more persistent, and the margins of the stipule pair do not overlap (valvate). We designated this stipule type as ‘open’. Although the leaves below the second node are fully mature in these species, stipules can be seen persistently from the shoot apex to the fourth or even sixth node in the inland genera *Cassipourea* (Fig. 1F) and *Macarisia* (Fig. 1G), or to the tenth or twelfth node in the inland genus *Pellacalyx* (Fig. 1E). Open-type stipules are further distinguished by being hairy, whereas closed-type stipules are glabrous. Most of these stipules, whether open or closed, are green to yellow–green (video S1), although, in some taxa (e.g. *Rhizophora*, *Bruguiera* and *Gynotroches*), they may also be red–purple or deep brown (*Macarisia*).

Pellacalyx axillaris is unusual in having a significant fleshy outgrowth (1.2–3.5 mm long) at the adaxial base of the stipule, free at the upper margin, forming a shallow pocket (Fig. 2G, H; video S2). The top of this outgrowth is lobed and covered by dense trichomes (Fig. 3E). A similar structure is also found in *Pellacalyx saccadianus*, but is less developed (Fig. 2I).

Field observation revealed that stipule exudates are always abundant and visible in the mangrove species. These exudates are milky or resinous and slightly sticky (Fig. 1A, B). For the inland species, only *Macarisia humbertiana* Arènes showed abundant resinous exudates (Fig. 1G; Table 1).

AGGREGATION FORM AND SIZE OF COLLETTERS

Collecters can be observed in the adaxial bases of stipules of all species of Rhizophoraceae studied (Fig. 2), regardless of the morphology and size of the stipules. These collectors are aggregated in genus-specific forms: rectangles, trapezoids, triangles, semi-

circles, narrow or thick bands, a single row or scattered (Table 1). Collecters are commonly milky-white initially (Fig. 2A, B, J), gradually turning black with age (Fig. 2K).

For convenience of description, colleter size can be divided into three categories on the basis of colleter length: large (> 601 μm), medium (301–600 μm) and small (101–300 μm). Compared with the inland species, all four mangrove genera have more abundant collectors (95–300 per stipule), aggregated in 4–26 rows. They are all medium or large in size and aggregated as semicircles or trapezoids (*Bruguiera exaristata* Ding Hou), thick bands (*Rhizophora stylosa* Griff., Fig. 2A; video S1) or triangles (*Ceriops* and *Kandelia*). Among the species of Rhizophoraceae studied, *R. stylosa* has the largest collectors, up to 1500 μm in length.

Colleter aggregation forms of the inland species are more diverse than those of the mangrove species. Single rows, scattered forms (*P. axillaris* Korth.) (Fig. 2H) and irregular triangles (*P. saccadianus* Scort.) (Fig. 2I) were only found in inland species. Small numbers of collectors occur in *Gynotroches* (two to ten collectors) and *Cassipourea* (< 20 collectors, Fig. 2J), usually arranged as a single row. In addition, in *Cassipourea* sp., a few collectors were found attached to the corresponding side of the stipule scar on a stem (Fig. 2J, inset).

The inland species often have a wide range of colleter size within a stipule. This phenomenon is particularly noticeable in *Carallia suffruticosa* Ridl. (Fig. 2F) and *P. saccadianus* (Fig. 2I). Inland species have medium or small collectors. *Macarisia humbertiana* stands out in having collectors that completely fill the very small stipule (Fig. 2L, M). The genus *Pellacalyx* also has unusual features: *P. axillaris* has extremely small colleter-like structures (82 μm) scattered above the fleshy outgrowth (Fig. 2G, H; video S1) and *P. saccadianus* has collectors along the margins of stipules, in addition to colleter aggregations in the stipule base (Fig. 2I).

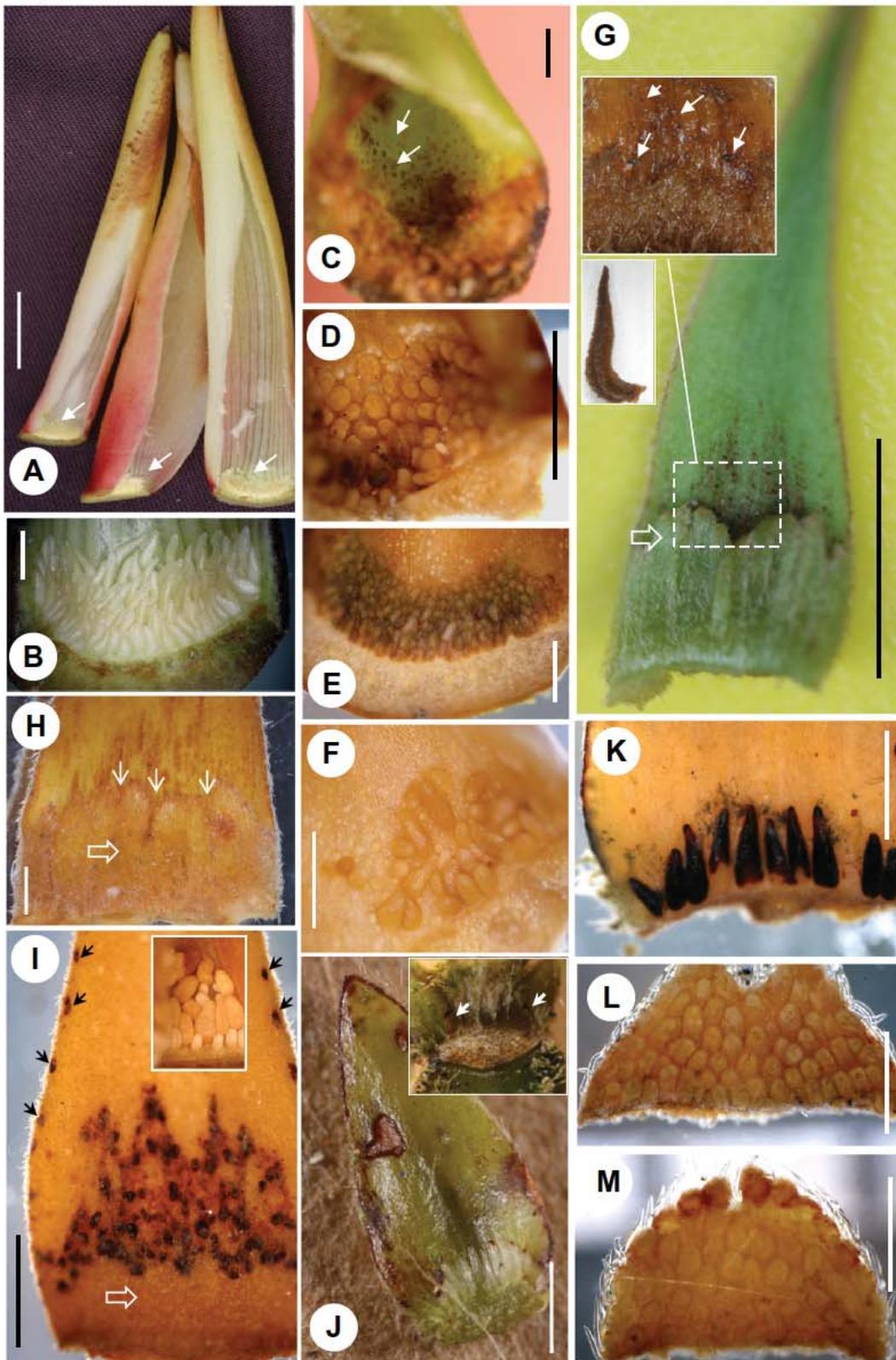


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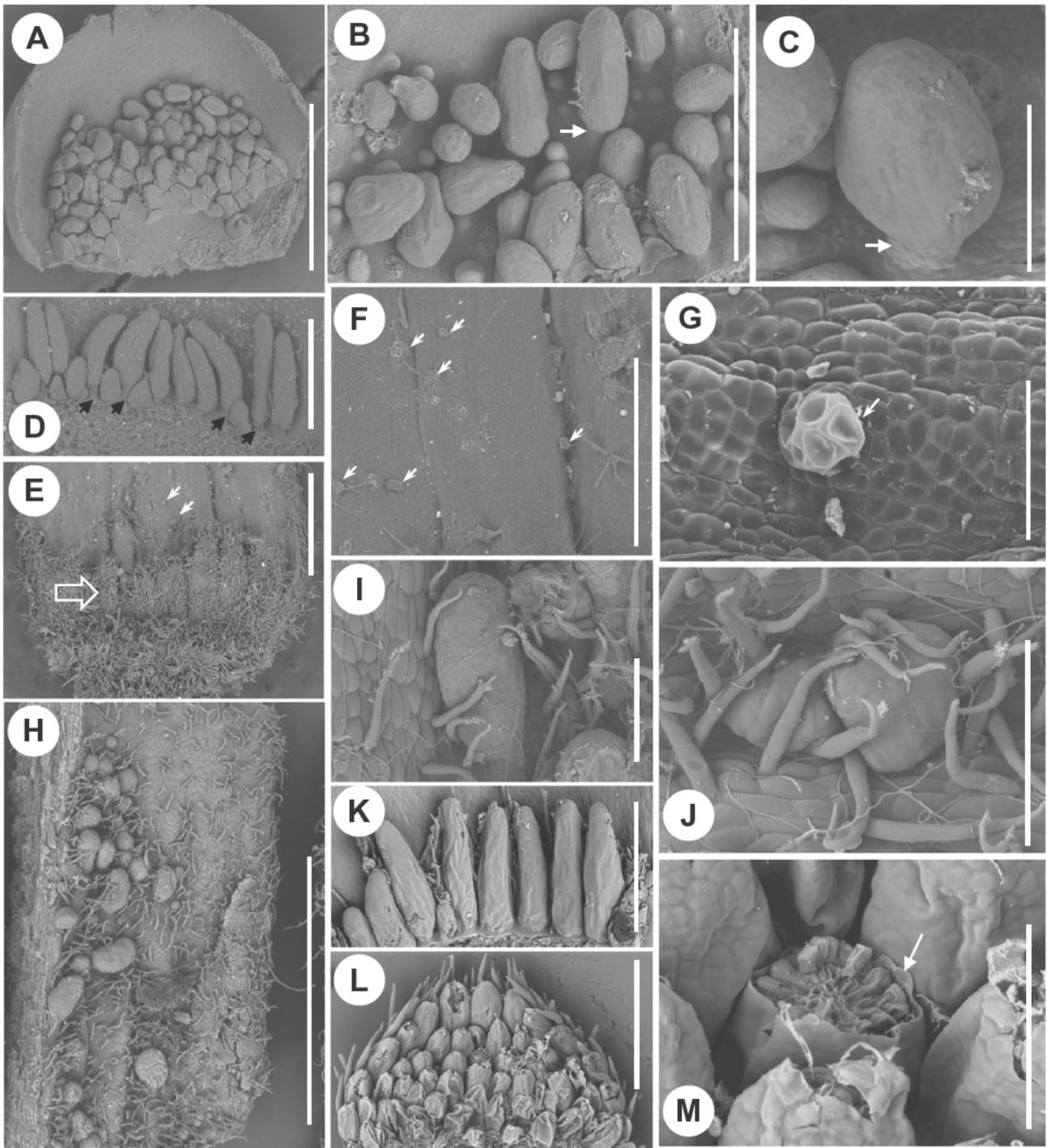


Figure 3. Scanning electron micrographs of collectors of the inland species of Rhizophoraceae. A, *Carallia brachiata* with sessile rod collectors. B, C, *Carallia suffruticosa* with sessile rod and short-stalked rod (arrows) collectors. D, *Gynotroches axillaris* with stalked rod collectors (arrows indicate stalks). E–G, The extremely small colleter-like structures (arrows) above a fleshy outgrowth (open arrow) of *Pellacalyx axillaris*. G is a close-up view. H–J, *Pellacalyx saccadianus* with sessile rod collectors intermingled with trichomes. K, *Cassipourea* sp. with sessile rod collectors. L, M, *Macarisia humbertiana*. L, Sessile rod collectors filling the entire stipule. M, A transverse view of a colleter showing a central axis surrounded by epidermal cells covered by a cuticular layer (arrow). Scale bars: A, E, H, 1 mm; B, D, F, K, L, 500 μm ; C, I, J, M, 100 μm ; G, 50 μm .

Figure 4. Anatomical structure of colleters of Rhizophoraceae. A, B, C, J and K are transverse sections; D, E, F, H and I are longitudinal sections; G is cleared. A, D, *Kandelia obovata*. B, E, *Carallia brachiata*. Note the detached cuticle (arrows) outside the palisade epidermal cells forming a space, which can accumulate exudates. C, F, *Cassipourea* sp. with smaller palisade epidermal cells. G–I, The vascular trace (tracheary element, arrow and circles in red) of *K. obovata*, which is found in some colleters. J, K, The extremely small colleter-like structures on the stipule of *Pellacalyx axillaris* is shown here to be epidermal protrusions (open arrows). CP, core parenchyma; Cut, cuticular layer; Mu, mucilage cell; PP, palisade epidermal cell; S, stalk. St, stipule. Tr, trichome. Scale bars: A–F, H, I, K, 100 μm ; G, 20 μm ; J, 200 μm .

COLLETER MORPHOLOGY

The individual morphology of colleters of Rhizophoraceae varies between genera (Table 1). The colleter morphology of the inland taxa is provided in Figure 3. [For the mangrove taxa, see fig. 4 in Sheue *et al.* (2012)]. Five types of colleter were discriminated in this study: short-stalked rods (*Bruguiera*, *Carallia*) (Fig. 3B, C), long-stalked rods (*Ceriops*, *Kandelia*, *Gynotroches*) (Fig. 3D), acuminate sessile rods (*Rhizophora*), sessile rods (*Carallia*, *Cassipourea*, *Macarisia*, *Pellacalyx*) (Fig. 3A, H–M) and small epidermal protrusions (*P. axillaris*) (Fig. 3E–G). Except in *Carallia* and *Pellacalyx*, colleter type does not vary within a genus.

Stipule pubescence is associated with trichomes mingled with colleters: all four mangrove genera and two inland genera (*Carallia* and *Gynotroches*) have glabrous stipules and no trichomes mingled with colleters. Three inland genera (*Pellacalyx*, *Cassipourea* and *Macarisia*) have pubescent stipules and trichomes mingled with colleters. These trichomes are unbranched in *Cassipourea* and *Macarisia* (tribe Macariseae), whereas both unbranched and branched trichomes occur in *Pellacalyx* (Gynorocheae).

COLLETER STRUCTURE

Although the colleters reported for this family vary in shape and size, they all have similar anatomical features. They have two types of cell: a core of elongated parenchymatous cells (*c.* five to ten rows) sheathed by palisade-like epidermal cells arranged in one layer perpendicular to the axis of the core parenchyma (Fig. 4). The stalk of the colleter is a basal constriction composed of a single epidermal cell layer surrounding core parenchyma cells (Fig. 4D, G, I) attached to the stipule tissue. A cuticular layer covers the entire colleter, including the palisade-like epidermis and the stalk when present. A space between the cuticular layer and the epidermal cells may be greatly increased and filled with a mucilaginous substance exuded by the palisade-like epidermis at the secretory stage (Fig. 4B, E).

With the exception of *Kandelia*, no vascular tissue was found in the colleters of the species of Rhizophoraceae studied. Tracheary elements of *K. obovata* Sheue, H.Y.Liu & J.W.H.Yong, identified by the thick-

ened secondary cell walls and elongated cell shape, were occasionally found in the core parenchyma of stalks or the basal parts of colleters (Fig. 4G–I). Extensive investigation of samples by the clearing method showed that, even in the same stipule, only a few colleters have this vascular trace. Each of these vascular traces is just a single tracheary element, with no visible connection to the main vascular tissue of the stipule (Fig. 4G).

Colleters of mangrove genera appear to be distinguished by larger epidermal cells. For example, colleters of *K. obovata* and *R. stylosa* have palisade-like epidermal cells of *c.* 50 and 60 μm in length, respectively, whereas the colleters of the inland species *Carallia brachiata* (Lour.) Merr., *M. humberiana* and *Cassipourea* sp. have smaller epidermal cells (*c.* 45, 35 and 25 μm , respectively). Tannins are commonly seen in the core parenchyma of colleters of the mangrove species. Druse crystals are abundant in the stipule tissue of both mangrove and inland species, but rarely in the core parenchyma of the colleter, and not at all in the outer colleter epidermal cells.

The extremely small colleter-like protrusions of *P. axillaris* have no typical colleter structure (Figs. 3F, G, 4J–K). They consist of only 8–15 epidermal cells, slightly protruding from the stipule to form a small peak.

COLLETER ULTRASTRUCTURE

Colleter ultrastructural information is based here mostly on both young and mature stipules from *K. obovata* and *R. stylosa*, and on some mature stipules of *Rhizophora apiculata* Blume described in a previous report (Sheue *et al.*, 2012). We were unsuccessful in obtaining adequate ultrathin sections and images for inland species, although materials were fixed in the field and treated following the same general electron microscopy protocols.

Colleter cells have different ultrastructural features at different stages (Fig. 5). In the young stipule, the epidermal cell has a distinct nucleus, dense cytoplasm, many small vesicles, oil drops, abundant endoplasmic reticula (ER), mitochondria and some Golgi apparatus (Fig. 5A, B). The small vesicles appear lighter in the images and are scattered in the cyto-

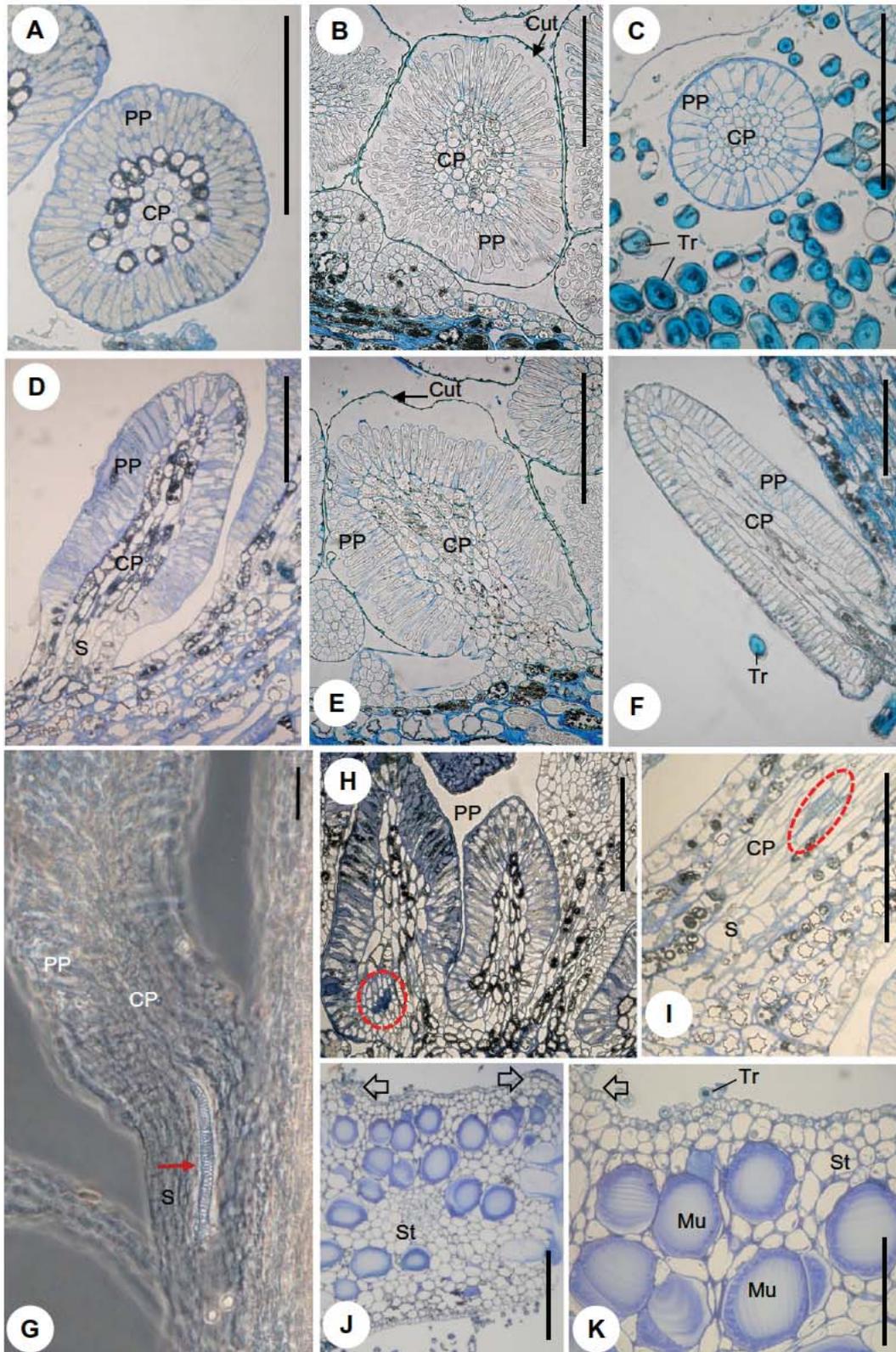


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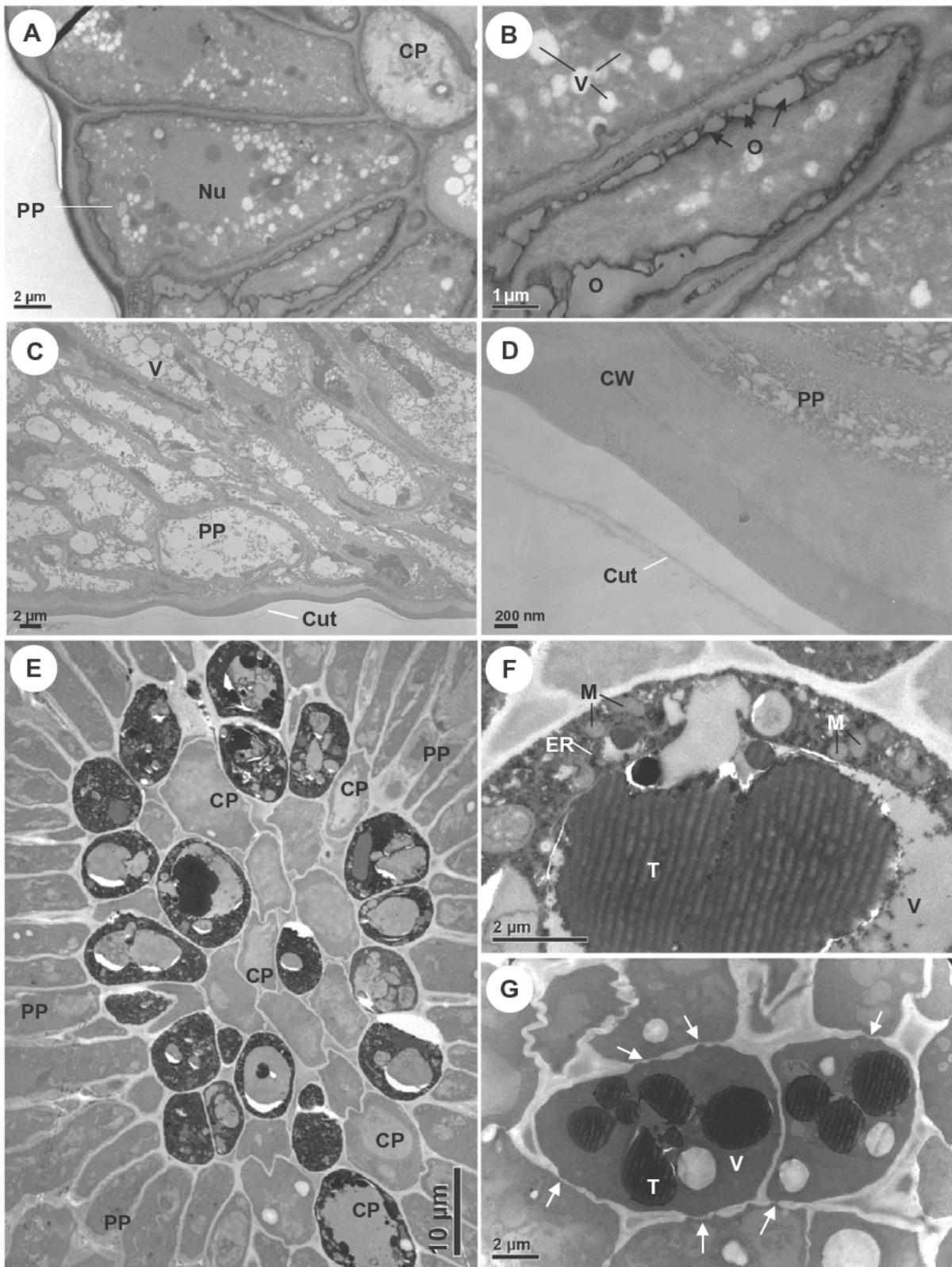


Figure 5. See caption on next page.

Figure 5. Transmission electron micrographs of colleters of *Kandelia obovata* (A, B, E–G) and *Rhizophora apiculata* (C, D). A–D, Palisade epidermal cell. E–G, Core parenchyma cell. A, B, During the secretory phase, dense cytoplasm with large nuclei, endoplasmic reticula, Golgi apparatus and mitochondria were found in outer epidermal cells. Note the many vesicles and oil drops of various sizes along the cell walls in (B). C, D, At senescence, these epidermal cells are disorganized with many vacuoles. Note the space between the detached cuticular layer and the outer cell wall in which the exudates from the palisade epidermal cells may accumulate (D). E–G, Core parenchyma during the secretory phase, with large vacuoles containing tannins, endoplasmic reticula and mitochondria scattered along the cell walls. Plasmodesmata are commonly found between core parenchyma cells (arrows in G), but not in palisade epidermal cells. CP, core parenchyma cell; Cut, cuticular layer; CW, cell wall; ER, endoplasmic reticulum; M, mitochondrion; O, lipid drop; PP, palisade epidermal cell; Nu, nucleus; T, tannins; V, vacuole.

plasm. Oil drops are grey, being more electron dense, and are mostly restricted to the borders of the cell walls (Fig. 5B). ER parallel to the axis of the epidermal cells, associated with vesicles and oil drops, are common. All of these ultrastructural features are indicative of a secretory function for the outer epidermal cells. An evident cuticular layer surrounds these secretory cells (Fig. 5D). The space between the outer epidermal cells and the cuticular layer is normally small and invisible, but flexible. It may be filled with secretory substances from the outer epidermal cells during the colleter secretory stage. This cuticular layer ruptures and collapses gradually during colleter senescence.

The core parenchyma often contains darker more electron-dense materials than the outer epidermal cells (Fig. 5E). Mitochondria and ER can be found in these cells only at younger stages (Fig. 5F). Tannins, large vacuoles and plasmodesmata are observed in this tissue (Fig. 5G).

Colleters from expanded stipules collected in this study showed disorganized organelles within cells of both the epidermis and the core parenchyma. Only irregular shaped vesicles were evident in the outer epidermal cells (Fig. 5C), but these were numerous. Usually, only a large vacuole containing tannins could be observed in the core parenchyma cells.

DISCUSSION

The role of colleters in the stipules of plant groups is under-reported and poorly understood. Here, colleters were found in the adaxial bases of stipules of all members of Rhizophoraceae studied. Thus, this feature is a family-level taxonomic character. With the exception of *P. axillaris*, the colleters of all species studied are finger-like. In *P. axillaris*, the colleters are rudimentary, reduced to colleter-like protrusions. A surprise is the presence of additional colleters on stipule margins of *P. saccadianus*, not previously reported to our knowledge in this or any other plant group, although colleters on leaf margins are known from *Cariniana estrellensis* (Raddi) Kuntze (Lecythidaceae) (Paiva, 2012). Except for *Bruguiera*, colleters,

although few in number, are also present in the bracteoles of mangrove Rhizophoraceae inflorescences, as noted previously (Sheue, 2003; Sheue, Liu & Yong, 2003). Reports of colleters in various locations, although most commonly in stipules, have been described in other families of Malpighiales, including Caryocaraceae (Paiva & Machado, 2006b), Erythroxylaceae (Thiebaut & Hoffmann, 2005), Euphorbiaceae (Thomas, 1991), Passifloraceae (Thomas, 1991), Salicaceae (Thomas, 1991) and Turneraceae (Thomas, 1991; González, 1998). In addition, colleter-like structure was reported in Ochnaceae (Matthews, Amaral & Endress, 2012) and Putranjivaceae (Matthews & Endress, 2013).

In this study, we provide information on the concordance of colleter and stipule structure with habit and phylogeny. Our results, summarized in Figure 6, show definitively that the mangrove taxa (taxa 1–4) of Rhizophoraceae have larger and more abundant colleters than inland taxa. There is a stark difference between the species *R. stylosa* (a mangrove) with the largest colleters, up to 1490 μm , and the inland species *C. brachiata* with the smallest finger-like colleters (taxon 7), about 140 μm in length. In *P. axillaris* (taxon 6), the colleter-like protrusions are only 83 μm in length. Colleters of the inland taxa, although generally smaller than those of the mangrove taxa, vary greatly in size between species. In the mangrove tribe Rhizophoreae, *Rhizophora*, usually occurring at the front of mangroves facing the sea, has the largest colleters (Sheue, Chen & Yang, 2012). Indeed, the colleters in *Rhizophora* are amongst the largest observed in any species. A few genera of Apocynaceae (*Nerium* L. and *Alstonia* R.Br.) also have large colleters, up to 1100 μm in length (Thomas & Dave, 1991), but are less abundant (10–35 colleters) than in the mangrove Rhizophoraceae, and do not occur in stipules, but on petioles. However, a complication in providing a clear summary is that some species have broad elevational ranges. For example, the inland species of Gynotrocheae are constituents of the lowland mixed Malaysian rainforest rarely found on mountains above 1000 m, but several species range to higher elevations. For example, *P. axillaris* has been found up to 1300 m,

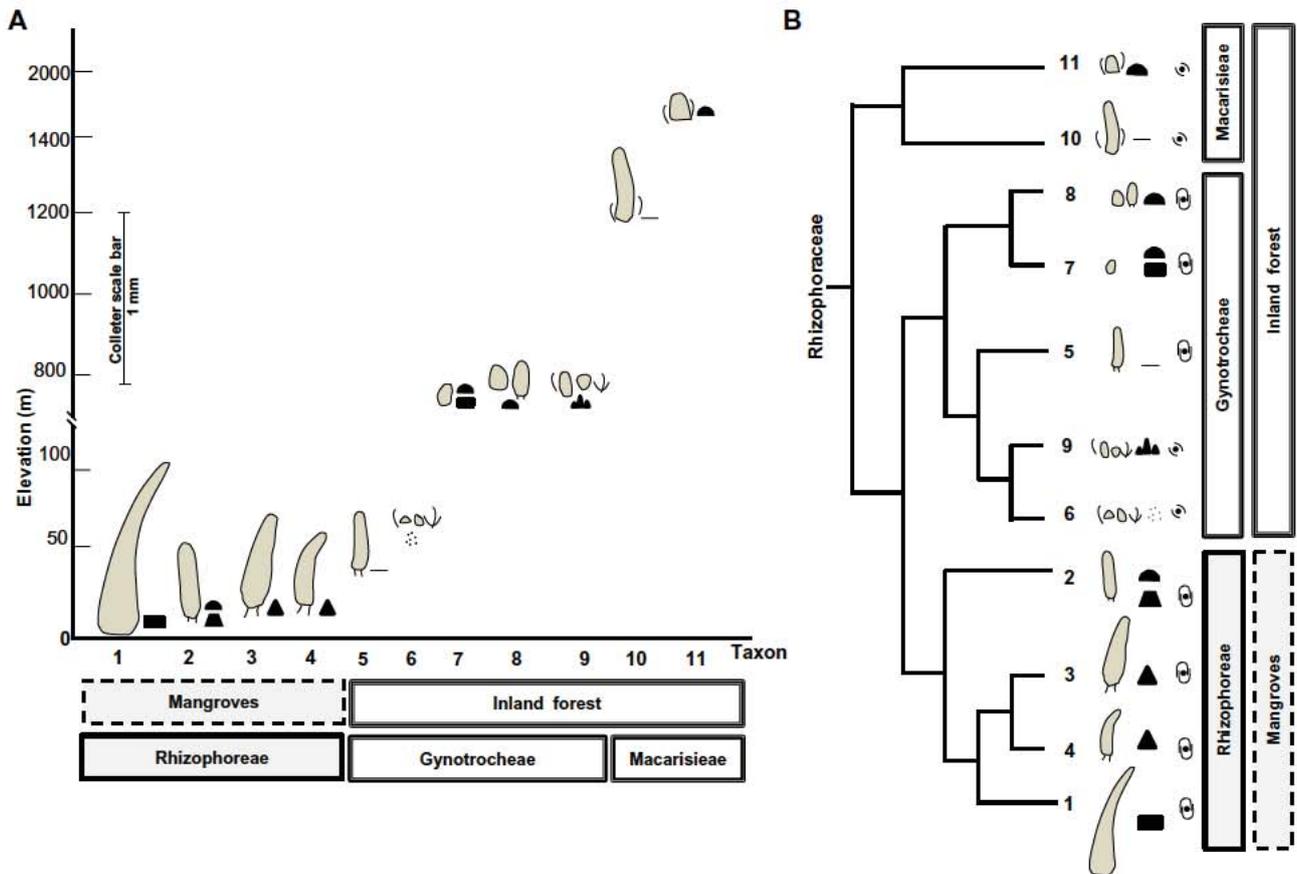


Figure 6. The characters of colleters of Rhizophoraceae arranged in accordance with habitat (A) and phylogeny (B). The cladogram in (B) is based on Schwarzbach and Ricklefs (2000). A, The mangrove taxa have significantly larger and more abundant colleters than inland taxa, and the inland taxa at lower elevation tend to have fewer colleters, when compared within tribes. B, Gynotrocheae has more diverse characters of stipules and colleters than Macarisieae and Rhizophoreae, spanning almost the full range of the entire family. Stipule type: \ominus , closed; \odot , open. Aggregation form of colleters: \blacksquare , band; \blacktriangle , trapezoid; \blacktriangle , semicircle; \blacktriangle , triangle; $-$, a single row; \circ , scattered; \blacktriangle , irregular triangles. Taxa: 1, *Rhizophora stylosa*; 2, *Bruguiera exaristata*; 3, *Ceriops tagal*; 4, *Kandelia obovata*; 5, *Gynotroches axillaris*; 6, *Pellacalyx axillaris*; 7, *Carallia brachiata*; 8, *Carallia suffruticosa*; 9, *Pellacalyx sacardianus*; 10, *Cassipourea* sp.; 11, *Macarisia humbertiana*.

C. brachiata up to 1800 m and *Gynotroches axillaris* up to 2250 m (Hou, 1958).

Colleters presumably work in conjunction with stipules in protecting the shoot and, as stipules house the colleters, they necessarily constrain their morphology and functioning. In this study, two types of stipule were observed: closed and open. The open type of stipule is small, but is more persistent. It is found only in inland species (taxa 6, 9, 10 and 11 in Fig. 6). Among them, *Pellacalyx* (taxa 6 and 9) has a novel stipulate feature not previously reported, namely a fleshy outgrowth. This outgrowth makes the stipule base thick, and appears to support the insertion of the stipule at a large angle to the stem, on the order of 40–90°. The stipule subsequently bends back towards the axis of the shoot, forming a spring-like arrangement, potentially providing superior mechanical

support to the shoot apex. The total number of colleters per stipule and their aggregation form in the stipule are not directly associated with stipule type. *Macarisia*, with the smallest stipules in this family, was found uniquely to have numerous colleters covering the entire adaxial surface of the stipule. However, *Gynotroches*, an inland species with the largest stipule of the closed type, has only a few colleters arranged in a single row.

The various colleter aggregation forms are consistent in a genus, but variable between genera, and therefore of taxonomic value. More diverse aggregation forms were observed in inland taxa than in mangrove taxa (Sheue, Chen & Yang, 2012). Irregular triangles, single rows and scattered forms were found only in the inland genera. These colleter aggregation forms were observed in open and closed stipules.

Gynotroches and *Cassipourea* are the only two genera with few colleters arranged as a single row (taxa 5 and 10 in Fig. 6). However, these two genera have different habitats and stipule characteristics. *Gynotroches*, with closed-type large stipules, is a monotypic genus occurring in marshy places and swamps, particularly along creeks in tall rainforest or in secondary forest in Asia (Hou, 1958). In contrast, *Cassipourea*, with small open stipules, is a species-rich genus mostly growing in drier places (Schwarzbach & Ricklefs, 2000). Thiebaut & Hoffmann (2005) reported that *Cassipourea malosana* Alston, a tree commonly found in moist montane forest or in upland dry forest in continental Africa (AgroForestryTree Database; <http://www.worldagroforestrycentre.org/sea/products/afdbases/af/asp/SpeciesInfo.asp?SpID=18008>), has colleters forming one row. This is the same colleter aggregation form as observed in the *Cassipourea* sp. found growing as an understorey shrub of rainforest at mid-elevation in Marojejy National Park, Madagascar.

In this study, colleters of most inland species appear as sessile rods. Mangrove genera of Rhizophoraceae have been reported previously with three types of colleter: short-stalked rods, long-stalked rods and acuminate rods (Sheue, Chen & Yang, 2012). *Gynotroches* (taxon 5 in Fig. 6) is the only inland genus with evident stalked colleters similar to those of mangrove taxa. Generally, each species has one type of colleter morphology. However, the colleters of *C. sufruticosa* (taxon 8 in Fig. 6) may appear with both short-stalked rods or sessile rods. Based on the morphology of stipule and colleter, *Carallia* and *Gynotroches* seem to be more closely related to the mangrove Rhizophoraceae than to other inland species. This result may support a hypothesis proposed previously. Members of Macariseae share capsular fruits, those of Gynotrocheae (except *Crossostylis* Forst.) have berries and those of Rhizophoreae have indehiscent fruits with seeds germinating on the mother plant (viviparous). According to Juncosa & Tomlinson (1988b), the most parsimonious interpretation is that the capsular fruit constitutes the plesiomorphic character state in the family: a common ancestor of *Carallia*, *Gynotroches*, *Pellacalyx* and the mangrove clade evolved berry-like fruits, followed by a shift to viviparous, indehiscent fruits in the mangrove group. Based on the combined analysis of molecular and morphological data, Schwarzbach & Ricklefs (2000) also supported a plesiomorphic capsular fruit of Macariseae. Our results demonstrate that members of Gynotrocheae show more diverse characters of stipules and colleters than do members of Macariseae and Rhizophoreae. Indeed, colleter and stipule diversity in Gynotrocheae spans almost the full range found in the entire family, perhaps representing evolutionary diversification from the ancestral state found in Macariseae.

According to previous reports (Lersten & Curtis, 1974; Sheue, Chen & Yang, 2012) and this study, colleters of both mangrove and inland species of Rhizophoraceae are anatomically of the standard type (a central axis of slender, elongate cells surrounded by an outer palisade-like epidermis). An exception is the inland species *P. axillaris*, which has extremely small and irregularly shaped epidermal protrusions scattered above the fleshy stipule outgrowth. As no visible exudates can be observed, and the standard internal structure is absent, we conjecture that these protrusions are nonfunctional vestigial colleters. The standard-type colleter is the most common type (Robbrecht, 1988), which is also found in Apocynaceae (Apezato-da-Glória & Estelita, 2000), Rubiaceae (Thomas & Dave, 1990; Klein *et al.*, 2004) and Turnaceae (González, 1998). It is noteworthy that the mangrove species not only have larger colleters, but also have larger outer palisade-like epidermal cells in their colleters.

A unique feature was found in *K. obovata* (mangrove, Rhizophoreae), in which some colleters showed limited vascular traces (tracheary elements). However, we found no visible connection to the vascular tissue of a stipule, even though we carefully examined many colleters with the clearing method. This finding differs from a previous report indicating that vasculature in the colleter is always connected to the organ to which it is attached (Thomas, 1991). This feature, together with other unique attributes of *Kandelia*, such as cold tolerance, slender calyx lobes and an indefinite number of stamens (polyandry) with long filaments, suggest a more derived status (Juncosa & Tomlinson, 1988b; Sheue, Liu & Yong, 2003). Combined with molecular data, *Kandelia* has also been suggested to be the most derived mangrove clade (Schwarzbach & Ricklefs, 2000).

Based on the results of this study and the proposed phylogeny (Juncosa & Tomlinson, 1988b; Schwarzbach & Ricklefs, 2000), we suggest a possible evolutionary sequence for stipule and colleter traits in Rhizophoraceae (Fig. 7). Members of Macariseae (inland) have only open stipules and sessile colleters mingled with unbranched trichomes. We hypothesize that Macariseae represents the most plesiomorphic state. Gynotrocheae, with both types of stipules, stalked and sessile colleters sometimes mingled with branched and unbranched trichomes, shows evolutionary diversification. The ancestor of Rhizophoreae (mangroves) was derived from Gynotrocheae. Rhizophoreae, adapted to the challenging mangrove environment, has developed conspicuous closed stipules with large colleters. In this family, *K. obovata* is the most derived species, with vascular traces developed in some colleters.

The colleter ultrastructure of Rhizophoraceae is similar to that reported from Rubiaceae and

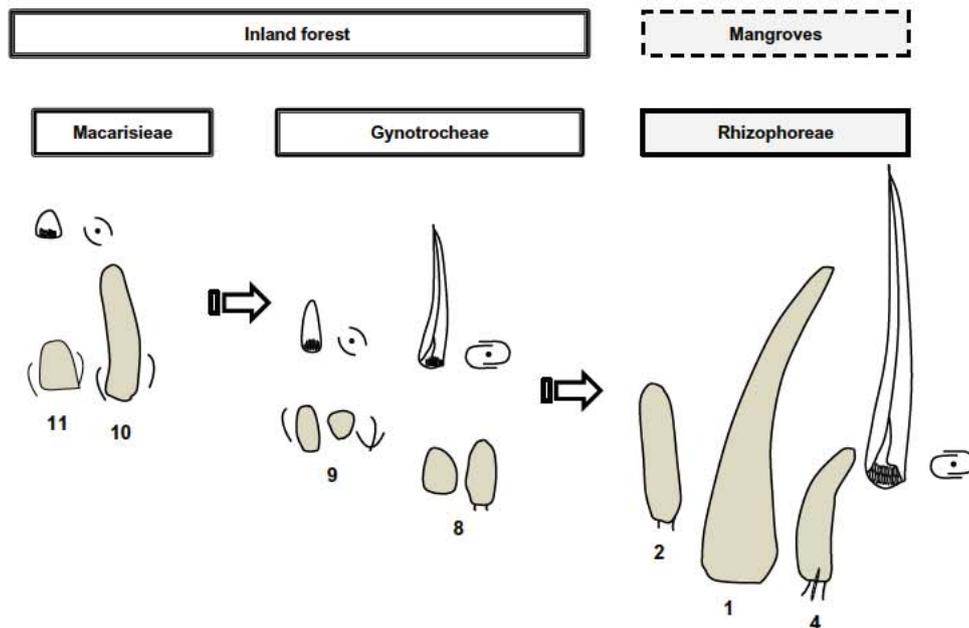


Figure 7. Proposed evolutionary sequence for stipule and colleter traits in Rhizophoraceae. The taxon number is the same as in Figure 6. The inland tribe Macariseae, with only small open stipules and sessile colleters mingled with unbranched trichomes, represents the most plesiomorphic state. Gynotrocheae, with both types of stipule, stalked and sessile colleters, sometimes mingled with branched and unbranched trichomes, shows evolutionary diversification. The ancestor of Rhizophoreae derived from Gynotrocheae and adapted to the mangrove environment develops conspicuous closed stipules and large stalked or acuminate rod colleters. In this family, the colleters of *Kandelia obovata* (4), possessing a vascular trace, are the most derived state.

Apocynaceae (Klein *et al.*, 2004; Paiva & Machado, 2006a, b). Secretory and senescent phases can be recognized by ultrastructural features. Our studies show that the senescent phase of colleters is initiated before the young leaves have expanded from the outer sheathing stipules. During senescence, colleter epidermal cells and core parenchyma show a disorganized subcellular structure, filled with many vesicles, but without intact organelles, as reported by Miguel *et al.* (2010). Tannins and calcium oxalate crystals in the druse form are commonly found in core parenchyma cells and stipular tissue, but not in the outer epidermal cells. These subcellular features reveal significant cellular differentiation and functioning between the core parenchyma and outer epidermal cells.

The colleter secretions have been termed 'blastocolla' by Hanstein (González, 1998) or 'bud-glue'. They contain carbohydrates, mucilages and proteins or lipophilic substances (Kronstedt-Robards & Robards, 1991). In *R. stylosa*, colleter secretions contain galactose (Primack & Tomlinson, 1978). Although the key functional aspects of colleters are unknown, many authors have reported that colleter secretions cover and protect the developing shoot apex (Williams, Metcalf & Gust, 1982; Thomas & Dave, 1989, 1990; Paiva, 2009, 2012). In addition, the colleter secretions

are surmised to play a vital role in the nutrition of symbiotic bacteria in nodulated rubiaceae species (van Hove & Kagoyre, 1974; Lersten, 1975). It has also been suggested that they have evolved as a defence against pathogens and insects (Farrell, Dussourd & Mitter, 1991; Zalucki, Brower & Alonso, 2001; Cruz *et al.*, 2002). Colleters may inhibit the growth of axillary buds in *Nerium* and govern the apical dominance in vegetative shoots (Williams *et al.*, 1982). According to Dell (1977) and Paiva (2009, 2012), the resinous coating from colleters may help to reduce water loss from cuticular transpiration in warm tropical climates. In view of the various untested hypotheses for colleter functioning, further critical experimentation is needed for a better understanding beyond speculation.

Macarisia humbertiana is the only species of Rhizophoraceae with colleters covering the entire adaxial surface of the stipule. Shoots significantly bathed in resin-like exudates were found on this canopy shrub, which, in this study, was collected near a high mountain summit (1250–1400 m). These exudates are reminiscent of the rich exudates observed from mangrove taxa (Sheue, Chen & Yang, 2012). In contrast, the inland species growing at lower elevations (especially taxa 5, 6, 8–10 in Fig. 6) often have less exu-

dates, notably the genera *Pellacalyx* and *Cassipourea*, in which no exudates were observed. These differences suggest environmental associations of colleter form and functioning. Thiebaut & Hoffmann (2005) used herbarium specimens to observe collectors of *Cassipourea malosana* and *Rhizophora mucronata* Lam. Their observation of obvious secretions gluing the collectors together in *R. mucronata*, but not in *Ca. malosana*, is similar to ours. However, at the present time, the biology of collectors is poorly understood. Here, we have demonstrated various patterns with elevation and tribe, but more data are needed for a definitive association between collectors and habitats. We hope that the results of this comparative study of Rhizophoraceae will stimulate such research.

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Video S1. Three-dimensional video of the basal part of a stipule showing the colleters of *Rhizophora stylosa*.

Video S2. Three-dimensional video of a stipule of *Pellacalyx axillaris* showing the basal outgrowth and rudimentary colleters.