Flora 292 (2022) 152078

Contents lists available at ScienceDirect

Flora

journal homepage: www.elsevier.com/locate/flora

Anther and ovule development in *Pittosporopsis* and its implications for the systematics of Metteniusaceae (Metteniusales)

Dong-Rui Kong [a,*], Melanie Schori [b], Lu Li [c], Yan Luo [d], De-Chang Hu [a]

[a] School of Life Science, Ludong University, Yantai 264025, Shandong, P.R. China
[b] National Germplasm Research Laboratory, Beltsville Agricultural Research Center, United States Department of Agriculture, Beltsville, Maryland 20705, United States
[c] Yunnan Academy of Biodiversity, Southwest Forestry University, Kunming 650224, Yunnan, P.R. China
[d] Gardening and Horticulture Department, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Menglun Mengla 666303, Yunnan, P.R. China

ARTICLE INFO

Editor: Edited by Favio Gonzalez

Keywords:
Icacinaceae s.l.
Megagametogenesis
Megasporogenesis
Microgametogenesis
Microsporogenesis

ABSTRACT

Pittosporopsis Craib, previously considered a member of the Icacinaceae *sensu lato* (s.l.), was transferred recently to the expanded Metteniusaceae, a family of 11 genera that needs morphological reevaluation to assess possible synapomorphies given its new circumscription. We investigated the anther and ovule developmental characters of *Pittosporopsis* and compared them with those of other members of Metteniusaceae as well as Icacinaceae s.l. to the extent possible. These characters are important to establish morphological synapomorphies of Metteniusaceae and to provide insights into embryology of the early diverging clades of core asterids. Within the family, *Pittosporopsis* shares several uncommon embryological characters with both *Metteniusa* H. Karst. and *Emmotum* Ham., such as a connective with numerous tanniferous cells and two superposed ovules within one locule. The ovule of *Pittosporopsis* is bitegmic, the third report of this condition (after *Emmotum* and *Quintinia* Baker f.) in the recently recircumscribed campanulids. Characters not shared with other members of Metteniusaceae include an unusual outward protuberance in the anther wall derived from the division and enlargement of endothecial cells, and a hypostase connecting the embryo sac and the ovular vascular bundle. Interestingly, a hypostase is known from Bruniaceae, which is sister to the core campanulids. Although further studies are needed to fully characterize the embryology and floral development of *Pittosporopsis* and the other genera now placed in Metteniusaceae, our study provides new insights into the embryology of the first diverging campanulid clades.

1. Introduction

Pittosporopsis is a monotypic genus of evergreen shrubs or small trees, distributed in Laos, Myanmar, Thailand, northern Vietnam and Yunnan Province of China (Peng and Howard, 2008). In traditional classifications, *Pittosporopsis* was treated as a member of the Icacinaceae *sensu lato* (s.l.) (e.g. Sleumer, 1971; Cronquist, 1981; Peng and Howard, 2008), which were shown to be polyphyletic in phylogenetic analyses based on molecular and morphological data and were provisionally split into four families in three orders: Cardiopteridaceae and Stemonuraceae (Aquifoliales, campanulids), Pennantiaceae (Apiales, campanulids), and the Icacinaceae (Garryales, lamiids) (Kårehed, 2001). The reduced Icacinaceae were still non-monophyletic and were tentatively arranged into three groups within the family: the *Apodytes* group, the *Emmotum* group, and the *Icacina* group, with the genus *Cassinopsis* Sond. in none of the three groups. *Pittosporopsis* was placed in the *Icacina* group based on morphological data because molecular data were lacking (Kårehed, 2001).

Recently, in a phylogenetic analysis based on 50 plastid genes, Stull et al. (2015) found that the *Apodytes* group, the *Emmotum* group, and *Pittosporopsis* of the *Icacina* group are more closely related to *Metteniusa* and moved these groups into Metteniusaceae in its own order, Metteniusales. The expanded Metteniusaceae comprise *Metteniusa* and 10 genera from Icacinaceae sensu Kårehed (2001) and were classified into three subfamilies: Plateoideae (*Calatola* Standl. and *Platea* Blume), Apodytoideae (*Apodytes* E. Meyer ex Arn., *Dendrobangia* Rusby, and *Rhaphiostylis* Planch. ex Benth.), and Metteniusoideae (*Emmotum, Metteniusa, Oecopetalum* Greenm. & C. H. Thomps., *Ottoschulzia* Urb., *Poraqueiba* Aubl., and *Pittosporopsis*). *Cassinopsis* has since been proposed as another member of Metteniusaceae (Stevens, 2001 onwards; Stull et al., 2020; Zhang et al., 2020). Though the phylogenetic placement of the family is still tentative, expansion of Metteniusaceae has been accepted

* Corresponding author: School of Life Science, Ludong University, Yantai 264025, Shandong, P.R. China.
E-mail address: drkongcong@foxmail.com (D.-R. Kong).

https://doi.org/10.1016/j.flora.2022.152078
Received 10 January 2022; Received in revised form 10 May 2022; Accepted 12 May 2022
Available online 14 May 2022

(APG, 2016; Stevens, 2001 onwards) and is supported by analysis of asterid nuclear genomes (Stull et al., 2020) and a variety of analyses on the genomes/transcriptomes of 365 species of asterids (Zhang et al., 2020). The latter study places the family as one of the clades sister to the core campanulids, more closely related to Bruniaceae and Oncothecaceae (possibly sister to all other core asterids) and Stemonuraceae and Cardiopteridaceae (now the first divergent clade of lamiids) than to Icacinaceae s.s. (sister to core lamiids). An alternate classification based on plastomes (Li et al., 2021) placed Metteniusaceae in an early diverging clade of lamiids, but with minimal support values; assessments in the current study follow the classification presented in Zhang et al. (2020). Morphological synapomorphies of the expanded Metteniusaceae have not been identified, nor have potential synapomorphies been evaluated in this new concept of asterid relationships. A close relationship between *Metteniusa* and *Oncotheca* Baill. was proposed by González et al. (2007), who recognized each genus as belonging to its own family. Metteniusaceae were treated as comprising these two genera, with the possible inclusion of *Dendrobangia*, in the most recent comprehensive treatment of morphological characteristics (Dickison and Bittrich, 2016), which was prepared before Stull et al. (2015) was published. Androecium and gynoecium characters are useful and important in the study of plant phylogeny and macroevolution (Endress 2011a). However, Metteniusaceae are poorly understood embryologically; González and Rudall (2010) and Endress and Rapini (2014) reported critical embryological characters of *Metteniusa* and *Emmotum* when they investigated the floral structure/development of those genera. *Pittosporopsis* has not been studied embryologically. In this paper, we focus on developmental and embryological characters of the anther and ovule in *Pittosporopsis*. Although it is difficult to anticipate embryological characteristics or establish synapomorphies in a family when data are scant or absent for most genera, we expected to find features shared with *Metteniusa* and *Emmotum*, which have been investigated.

2. Material and methods

2.1. Material

Material of *Pittosporopsis kerrii* Craib was obtained from cultivated plants in the Xishuangbanna Tropical Botanical Garden, Chinese Academy of Science (introduction number 0,020,012,100; voucher HITBC159258).

2.2. Methods

Floral buds at different developmental stages were fixed and

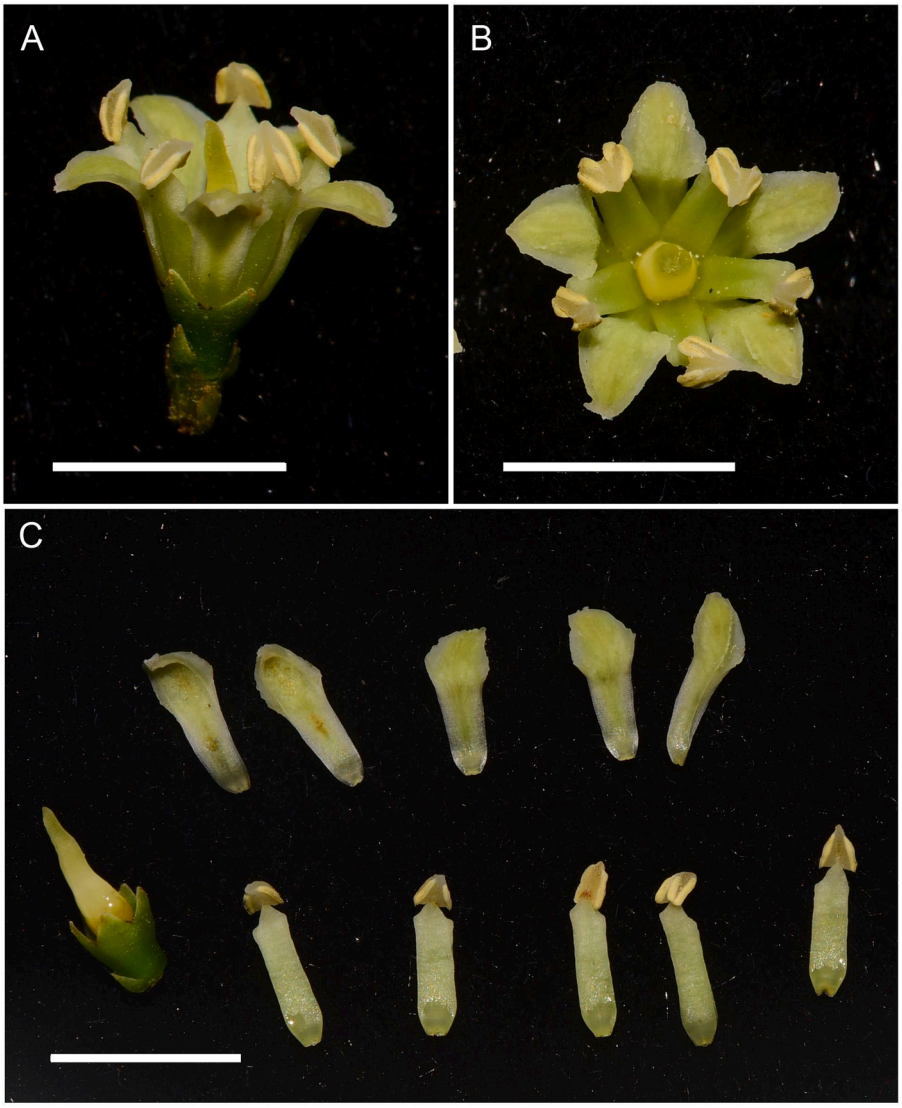

Fig. 1. A-C. Flowers of *Pittosporopsis kerrii* in oblique, lateral (A) and top (B) view, and dissected floral organs (C). Scale bars = 5 mm.

preserved in FAA (formaldehyde: acetic acid: 50% ethanol = 4: 6: 90). The preserved material was washed with distilled water twice, immersed in the Ehrlich's Hematoxylin solution (ratio of material to dye solution was approximately 1:5), and stained at 40 °C for 15 days. The stained material was washed in a plastic bottle with many minute holes that was placed under a slow-running tap water faucet for 15 days. Then the material was dehydrated in an ethanol series, infiltrated with xylenes, and embedded in paraffin wax. The embedded material was sectioned at 8 μm thickness. Several slides were stained with IKI and examined with polarized light to check for the presence of starch grains. The slides were examined under an Olympus BX50 microscope and micrographs were taken with an Olympus DP70 camera. Photos of developing seeds were taken with Nikon D810 camera.

3. Results

3.1. Floral structure

Flowers of *Pittosporopsis kerrii* are bisexual, pentamerous, and glabrous except for a few hairs on the sepals (Fig. 1A-C). The sepals are fused at the base, with a deltoid, acute apex (Fig. 1A). The clavate petals are free, with a slightly keeled midrib adaxially, an erose margin, and the upper third more or less reflexed at anthesis (Fig. 1A-C). The petal apex varies from plane to slightly cucullate (Fig. 1C). The alternipetalous stamens are free. The flattened, fleshy filaments are abruptly contracted shortly below the point of attachment with the connective and the anthers are deflexed towards the center of the flower. Anther dehiscence is extrorse (Fig. 1B). The connective protrudes past the anther sacs (Fig. 1C). The ovoid-ellipsoid ovary is slightly asymmetrical and tapers into the slightly geniculate style. The stigma is not distinct from the style (Fig. 1C).

3.2. Microsporangium, microsporogenesis, and microgametogenesis

The anther of *Pittosporopsis kerrii* is tetrasporangiate (Fig. 2A). Developing anthers have three to five hypodermal archesporial cells that differentiate at the four corners and divide periclinally, producing the primary peripheral cell layer and the primary sporogenous cells (Fig. 2B). The primary parietal cells then divide both anticlinally and periclinally, giving rise to an endothecium, two middle layers, and the

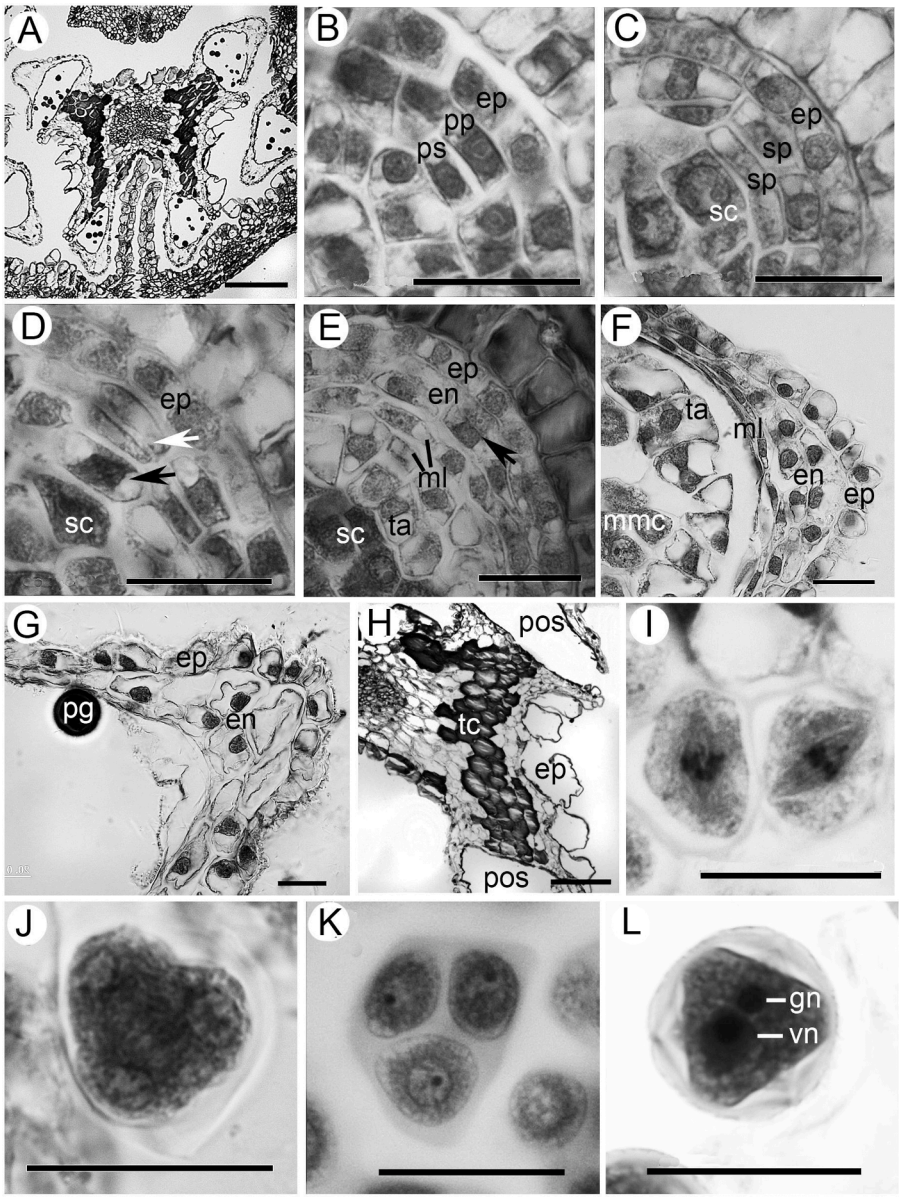

Fig. 2. Microsporogenesis and microgametogenesis of *Pittosporopsis kerrii*. A-H, transverse sections through the anther showing anther development. **A,** Anther with four pollen sacs, each with a protuberance perpendicular to the dehiscence slit; note tannins in the connective, and bulliform epidermal cells between the dehiscence slits. **B,** Primary peripheral cell and primary sporogenous cell resulting from the division of the archesporial cell. **C,** Two secondary peripheral layers. **D,** Outer and inner secondary peripheral layers (pointed to by the white and black arrow, respectively). **E,** Primary 5–6 layered anther wall; note the epidermis, one or two layers of the endothecium, two middle layers, and the tapetum; the arrow points to the two-layered endothecium. **F,** Anther wall during maturation; note middle layers starting degeneration, glandular tapetum with one or two nuclei per cell, endothecium becoming 2–3-layered, and with large cells. **G,** Mature anther wall; note epidermis and endothecium with enlarged cells forming a protrusion perpendicular to the dehiscence slit. **H,** Connective between the two pollen sacs of one theca; note tanniferous cells and bulliform epidermal cells. **I,** Microspore mother cells at metaphase I. **J,** Microspore mother cells at telophase II. **K,** Tetrahedral tetrads. **L,** Two-celled mature pollen grain. Abbreviations: *en* endothecium; *ep* epidermis; *gn* generative nucleus; *ml* middle layer; *mmc* microspore mother cell; *pg* pollen grain; *pp* primary peripheral cell; *pos* pollen sac; *ps* primary sporogenous cell; *sc* sporogenous cell; *sp* secondary peripheral cell; *ta* tapetum; *tc* tanniferous cell; *vn* vegetative nucleus. Scale bars = 100 μm in **A**; = 20 μm in **B–F, I–L**; = 50 μm in **G, H**.

tapetum (Figs. 2C, D). The middle layers have a common origin both with the endothecium and the tapetum. According to Davis (1966), this indicates the Basic development type of the anther wall. The mature anther wall comprises 5–6 layers (Fig. 2E). The tapetum is glandular and its cells are one- or two-nucleate and become vacuolated prior to meiosis of the microspore mother cell (Fig. 2F). During maturation of the anther walls, the middle layer and tapetum degenerate and the epidermal cells partially collapse (Figs. 2F, G). The endothecial cells do not develop fibrous thickenings but divide and grow into 2 layers of large parenchyma cells in the median position of the anther wall, forming an outward protuberance (Fig. 2G). The two pollen sacs of each theca are distant from each other, and each has an independent longitudinal slit (Fig. 2A). The connective has patches of tanniferous cells and bulliform epidermal cells between the two pollen sacs of a theca (Figs. 2A, H).

The microspore mother cell undergoes meiosis, accompanied by simultaneous cytokinesis, forming the tetrahedral microspore tetrads (Figs. 2I-K). Pollen grains are two-celled at the dispersal stage (Fig. 2L).

3.3. Ovular arrangement, development, and structure

There are two pendent, syntropous, anatropous ovules in the single locule. During development, the funiculus of one ovule elongates substantially, resulting in a superposed arrangement of a proximal and distal ovule in relation to the locule apex (Figs. 3A, B). The proximal ovule is larger than the distal one, and the funiculus of the proximal ovule is thicker but shorter than that of the distal one (Figs. 3A, B).

The two ovules are initiated from the upper part of the ovary wall (Fig. 4A). The funiculus of the distal ovule is as wide as the ovule before the nucellus starts to differentiate, whereas the funiculus of the proximal ovule is only a few cells wide at this stage (Fig. 4A, B). When the ovule begins bending, the inner and outer integuments arise from the epidermal and hypodermal cells, respectively (Fig. 4C). The inner integument grows faster than the outer one. When the apex of the ovule is nearly perpendicular to the locule wall, the inner integument encloses the nucellus and forms the micropyle while the outer integument remains shorter (Figs. 4D, 5A). At maturity, the ovule becomes anatropous, and the two integuments are tightly appressed (Fig. 5B). The outer and inner integuments have approximately 15 cell layers in total (Figs. 5B, F). The hypostase becomes evident at the chalazal region (Figs. 3A-B, 5B, D) as an aggregation of small, parenchymatous cells deeply stained by hematoxylin, indicating dense cytoplasm and conspicuous nuclei (Fig. 5C). Approximately nine vascular bundles enter the proximal ovule, with several extending through the raphe, and at least one reaches the chalaza and connects with the hypostase (Figs. 5D-F). In contrast, only one vascular bundle enters the distal ovule and reaches the chalaza (Figs. 3B, 5E).

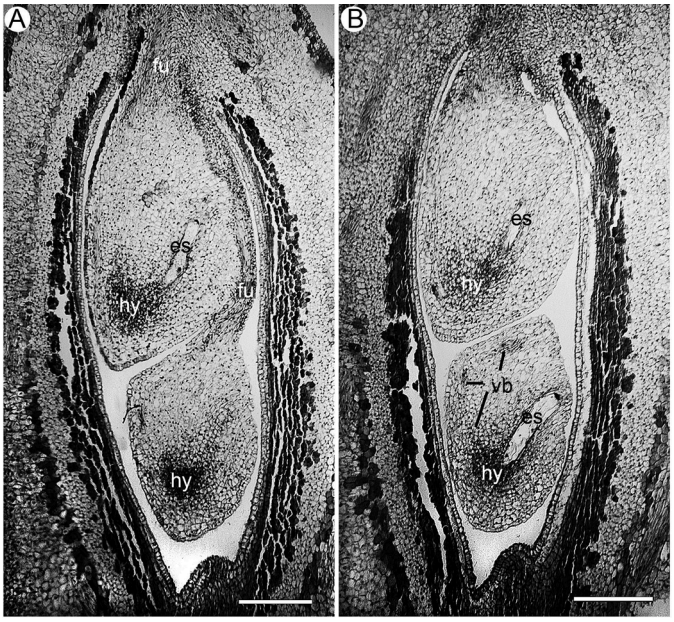

Fig. 3. Two superposed ovules in *Pittosporopsis kerrii*. The ovary apex is at the top, and the ventral side of the ovary is on the right in Figs. 2-6. A, B, Two successive longitudinal sections (LS) through syntropous, anatropous, superposed ovules; note funiculus of proximal ovule shorter and thicker than that of the distal ovule. Abbreviations: *es* embryo sac; *fu* funiculus; *hy* hypostase; *vb*, vascular bundle. Scale bars = 200 μm.

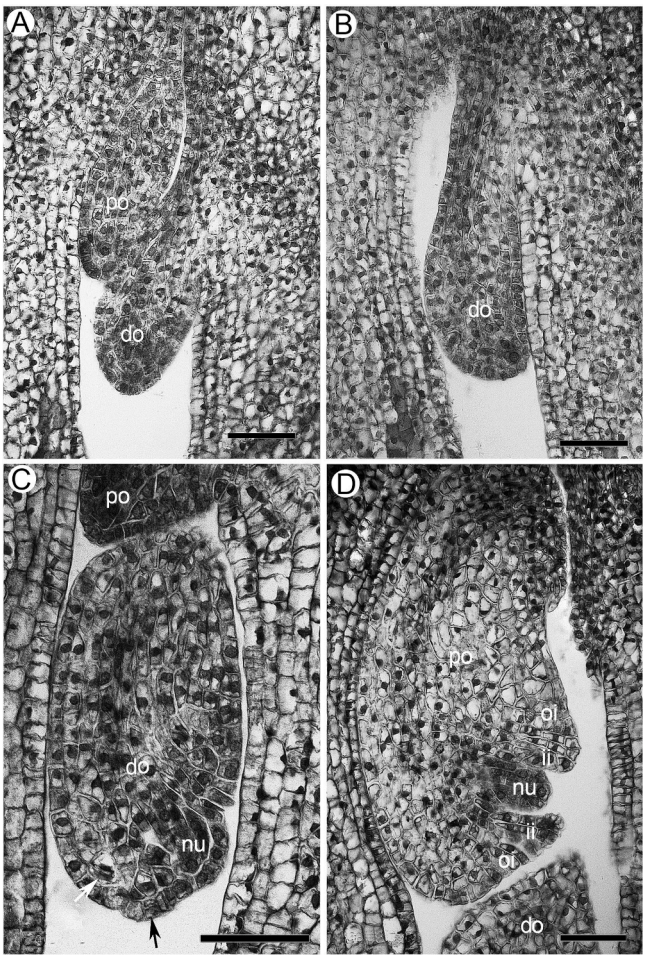

Fig. 4. Young ovules in *Pittosporopsis kerrii*. A, LS through the ovary showing the two ovules arising at the apex of the ventral ovary wall, one above the other. **B,** LS through the ovary showing elongation of the funiculus of the distal ovule. **C,** LS through the ovary showing the ovule beginning to curve and integument initiation; the black arrow points to the epidermal initiation of the inner integument; the white arrow points to the hypodermal initiation of the outer integument. **D,** LS through the ovary showing ovule at a hemianatropous stage with its inner integument longer than outer integument. Abbreviations: *do* distal ovule; *ii* inner integument; *nu* nucellus; *oi*, outer integument; *po*, proximal ovule. Scale bars = 50 μm.

3.4. Megasporogenesis, megagametogenesis, and nucellus

A single hypodermal nucellar cell differentiates as an archesporial cell that divides periclinally into a peripheral cell and a primary sporogenous cell (Figs. 6A, B). The peripheral cell further divides into three cell layers, with the megaspore mother cell positioned deeper in the nucellus; thus, the ovule is crassinucellar (Fig. 6C).

The primary sporogenous cell functions as the megaspore mother cell that undergoes meiosis, resulting in a linear megaspore tetrad (Fig. 6D).

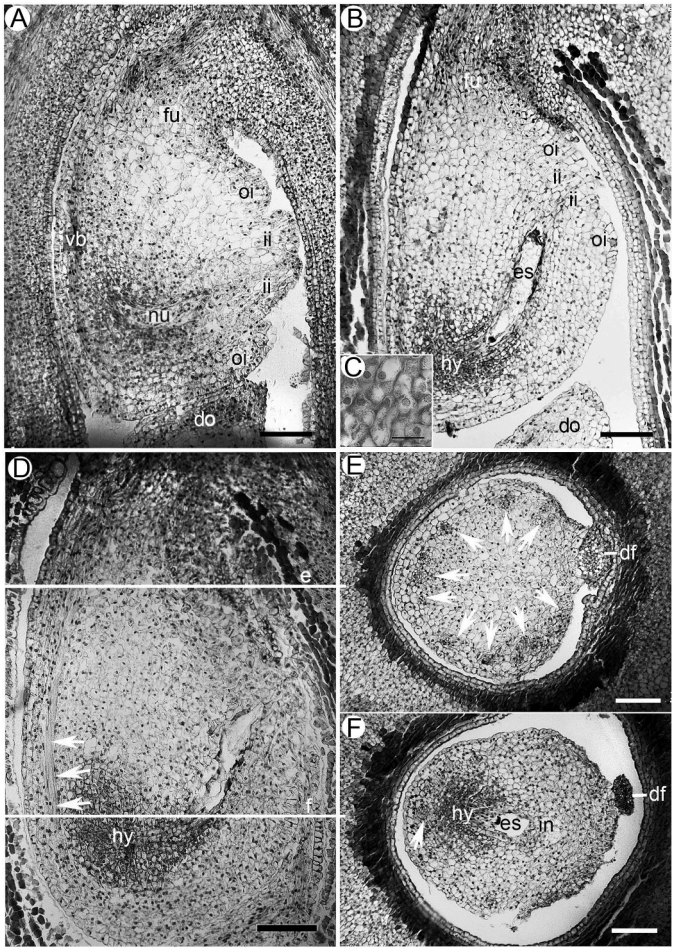

Fig. 5. Older and mature ovules in *Pittosporopsis kerrii*. A, LS through an ovule at the 4-nuclear embryo sac stage; note outer integument shorter than inner integument forming the micropyle, and one ovular vascular bundle entering the chalaza. **B,** LS through the mature ovule; note outer and inner integument tightly appressed, and heavily stained hypostase. **C,** Detail of the hypostase. **D,** LS through the mature ovule showing vascular bundle in the raphe (arrows) ending in the chalaza, next to the hypostase. **E,** Transverse section (TS) through the plane marked by line e (on longitudinal section shown in d); note at least nine vascular bundles (arrows) in the funiculus of the proximal ovule and only one vascular bundle (circled by the white dotted line) in the funiculus of the distal ovule. **F,** TS through the plane marked by line f in d; note vascular bundle in the raphe adjacent to the hypostase, and the two integuments with nearly 15 cell layers in total. Abbreviations: *df* distal funiculus; *do* distal ovule; *es* embryo sac; *fu* funiculus; *hy* hypostase; *ii* inner integument; *in* integument; *nu* nucellus; *oi*, outer integument; *vb*, vascular bundle. Scale bars = 100 μm.

Only the chalazal tetrad cell is functional while three micropylar ones degenerate (Fig. 6E). The functional megaspore undergoes three successive mitoses, producing the two-nucleate (Fig. 6F), four-nucleate (Fig. 6G), and eight-nucleate embryo sac successively. The nucellar cells other than those in the embryo sac degenerate by the 2-nucleate embryo sac stage (Fig. 6F), and the mature embryo sac is enveloped by a crushed nucellus and the inner integument (Figs. 7A-C). No endothelium is present (Fig. 6G). The mature embryo sac has one egg cell, two synergids in the micropylar region (Figs. 7A, D), a central cell with a large secondary nucleus formed by the fusion of two polar nuclei (Figs. 7B, E), and three antipodals in the chalazal region that degenerate early (Figs. 7C, F). The development of the embryo sac of *Pittosporopsis kerrii* therefore corresponds to the Polygonum type. The embryo sac is slightly curved (Figs. 7A-C). It contains multiple small objects (Fig. 7F) that might be oil droplets, which cannot be confirmed through staining of embedded sections; tests for starch bodies were negative.

After fertilization, the proximal ovule develops into the seed and the distal ovule becomes arrested (Figs. 8A, B).

4. Discussion

Members of Metteniusaceae are poorly known embryologically, but data that permit some comparisons are available from investigation by Mauritzon (1936), González and Rudall (2010), and Endress and Rapini (2014). It is not yet possible to infer patterns of floral and embryological development within the family, but future investigators will be aided by our summary of data from genera that are now known to be closely related. We chose several anther and ovule characters to compare *Pittosporopsis* primarily with *Metteniusa* and *Emmotum*, which have been recently investigated. Characters of genera of Bruniaceae and Oncothecaceae (also early divergent within core asterids) and Stemonuraceae and Cardiopteridaceae (former Icacinaceae s.l., now the first diverging clade within lamiids; Zhang et al., 2020) are compared where possible to explore patterns within early campanulid and lamiid lineages.

4.1. Floral morphology

Most members of Metteniusaceae, including *Pittosporopsis*, have bisexual, pentamerous flowers, with the exception of *Calatola* and *Platea*, which have unisexual, tetramerous or pentamerous flowers (Dickison and Bittrich, 2016; Potgieter and Duno, 2016). Flowers of *Ottoschulzia* are sometimes functionally unisexual. Sepals are free in *Calatola* and united to various degrees in the other genera of Metteniusaceae. Petals are free in *Emmotum, Pittosporopsis, Poraqueiba*, and *Rhaphiostylis*; free or united at the base in *Platea* and *Rhaphiostylis*; and fused basally in *Calatola, Cassinopsis, Dendrobangia, Metteniusa* and *Ottoschulzia* (González and Rudall, 2010; Endress and Rapini, 2014; Dickison and Bittrich, 2016; Potgieter_ and Duno, 2016; the present study). Stamens are free in the flowers with free petals and are fused to the base of the corolla tube when the petals are fused (González and Rudall, 2010; Endress and Rapini, 2014; Dickison and Bittrich, 2016; Potgieter and Duno, 2016; the present study). As in *Pittosporopsis*, the connective of *Ottoschulzia* and *Poraqueiba* protrudes beyond the pollen sacs (Santiago-Valentín and Viruet-Oquendo, 2013; Potgieter et al., 2016). Anther dehiscence is reported as introrse in most genera of Metteniusaceae (Potgieter and Duno, 2016), including *Metteniusa* and *Emmotum* (González and Rudall, 2010; Endress and Rapini, 2014), extrorse in *Ottoschulzia* and *Pittosporopsis* (Dickison, 1986; present study), and lateral in *Oecopetalum* and *Ottoschulzia* (Santiago-Valentín and Viruet-Oquendo, 2013; Potgieter and Duno, 2016). Though the carpel number of *Pittosporopsis* remains unknown, the gynoecial morphology in the genus is very similar to that of *Metteniusa* in the slender style, inconspicuous stigma and unilocular ovary with two pendent ovules. The last character is present in all members of Metteniusaceae and Icacinaceae s.l. except *Emmotum*, which has a trilocular ovary with two ovules in each locule (Endress and Rapini, 2014).

4.2. Anther characters

The number of sporangia varies within Metteniusaceae. As in *Pittosporopsis*, tetrasporangiate anthers occur in *Poraqueiba* (Howard, 1942). *Metteniusa* also has fundamentally tetrasporangiate anthers, but each microsporangium is divided into numerous secondary sporangia by transverse parenchymatous septa, resulting in polysporangiate anthers (González and Rudall, 2010). In contrast, *Emmotum* has bisporangiate anthers and lacks dorsal pollen sacs (Endress and Rapini, 2014). Other genera in the family have not been closely studied, although Endress and Rapini (2014) speculated that *Ottoschulzia* and *Oecopetalum* might also have bisporangiate anthers. Bisporangiate anthers are also found in *Oncotheca* (Oncothecaceae) (Dickison, 1986), which was recovered as sister to all other core asterids. Anthers of Bruniaceae (Claßen-Bockhoff,

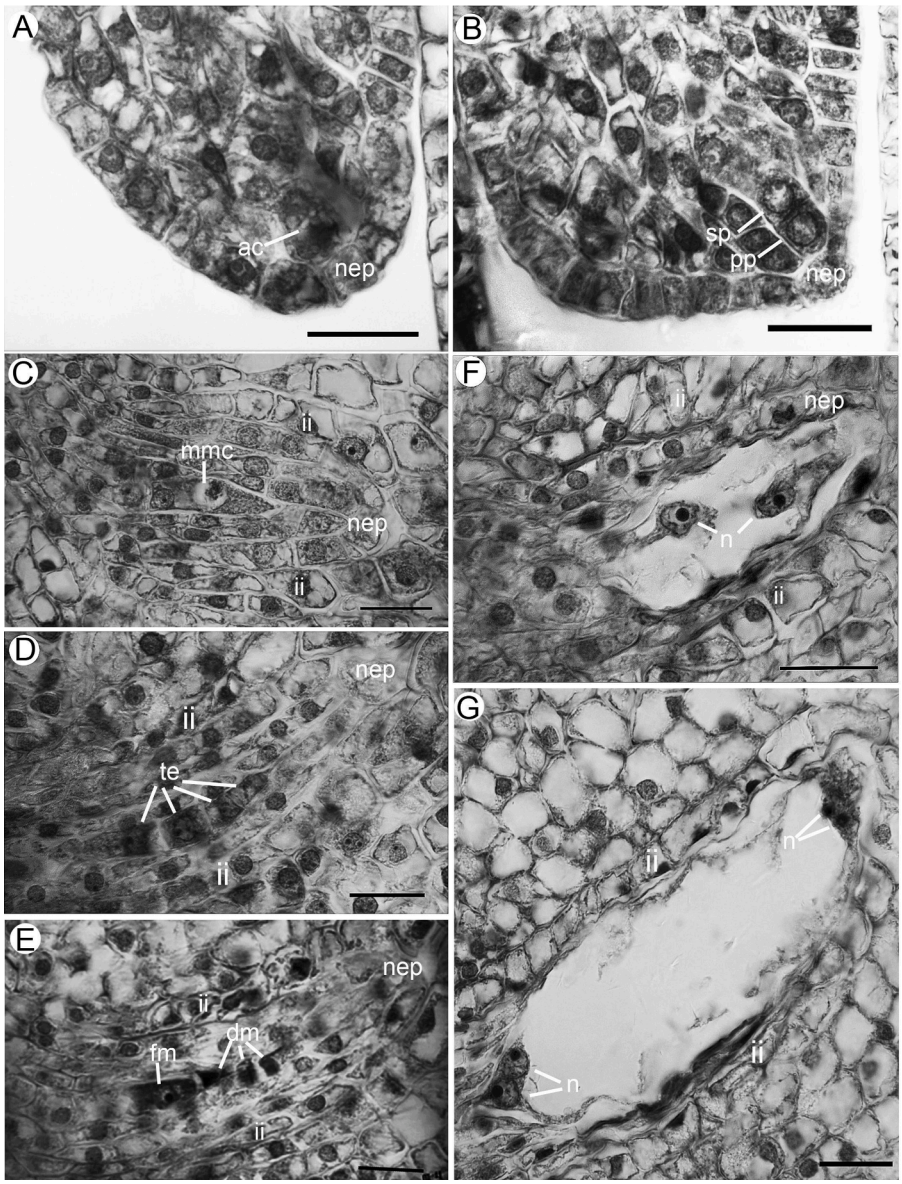

Fig. 6. Megasporogenesis and megagametogenesis in *Pittosporopsis kerrii*. **A,** LS through the ovule showing the archesporial cell. **B,** LS through the ovule showing the primary peripheral cell and the sporogenous cell. **C,** LS through the ovule showing the megaspore mother cell (MMC), with three cell layers between MMC and the nucellar epidermis on the micropylar side. **D,** LS through the ovule showing the linear tetrad. **E,** LS through the ovule showing the functional chalazal megaspore and three degenerated micropylar megaspores. **F,** LS through the ovule showing an embryo sac at a two-nucleate stage. **G,** LS through the ovule showing an embryo sac at a four-nucleate stage. Abbreviations: *ac* archesporial cell; *dm* degenerated megaspore; *es* embryo sac; *fm* functional megaspore; *ii* inner integument; *mmc* megaspore mother cell; *n* nucleus; *nep* nucellar epidermis; *oi*, outer integument; *pp*, primary peripheral cell; *sp*, sporogenous cell; *te* linear tetrad. Scale bars = 20 μm.

2016), *Gomphandra* Lindley (Stemonuraceae; Padmanabhan 1961), and *Cardiopteris* Wall. ex Royle and *Gonocaryum* Miq. (Cardiopteridaceae; Tobe, 2016; Kong and Li, 2017) are all tetrasporangiate, which is the ground pattern and most common type in angiosperms (Endress, 2011b). The tetrasporangiate anther is probably plesiomorphic in Metteniusaceae while polysporangiate and bisporangiate anthers are presumably derived from it through septation or reduction, respectively.

Longitudinal dehiscence is reported for all five families (Bruniaceae, Cardiopteridaceae, Metteniusaceae, Oncothecaceae and Stemonuraceae) under discussion (González and Rudall, 2010; Claßen-Bockhoff, 2016; Dickison and Bittrich, 2016; Potgieter and Duno, 2016; Schori, 2016). As in *Pittosporopsis*, the dorsal and ventral pollen sacs of each theca in *Metteniusa* are relatively far apart and each pollen sac has a separate dehiscence slit (González and Rudall, 2010). In their discussion of Icacinaceae s.l., Endress and Rapini (2014) noted distant pollen sacs with separate dehiscence as a specialized feature of *Metteniusa* but indicated that dehiscence in *Oecopetalum, Ottoschulzia,* and *Poraqueiba* (all now Metteniusoideae) might be similar based on earlier descriptions (e.g. Howard, 1942). Other former Icacinaceae s.l. do not have pollen sacs with separate dehiscence. In *Cardiopteris* and *Gonocaryum* (Cardiopteridaceae), two microsporangia of a theca share a common slit (Tobe, 2016; Kong and Li, 2017), a feature also observed in *Citronella* D. Don (Cardiopteridaceae), at least five genera of Stemonuraceae (M. Schori, unpublished data), and in Bruniaceae (Endress and Stumpf, 1991; Quint and Claßen-Bockhoff, 2006). Separate dehiscence slits may be a synapomorphy of Metteniusoideae if not of the family, but the character needs to be reexamined in the other eight genera of Metteniusaceae.

The connectives of *Pittosporopsis, Metteniusa*, and *Emmotum* all have cells with tannins, a condition not observed in *Gomphandra* (Stemonuraceae) or *Cardiopteris* and *Gonocaryum* (Cardiopteridaceae) (Padmanabhan, 1961; Tobe, 2016; Kong and Li, 2017). Tannins are abundant in the connective in *Oncotheca* (Dickison, 1986), and tanniferous tissue is present in the vascular bundle and epidermis in *Brunia* Lam. (Endress and Stumpf, 1991). However, the presence of tanniferous tissue does not necessarily indicate close relationships because tannins are often abundant in stamens and have been documented from many families (Schmid, 1976). Therefore, the presence of tanniferous tissue in the connective may be homoplasious.

Two unusual features are present in the anthers of *Pittosporopsis*. First, part of the endothecium has two layers, with large parenchymatous cells that form a protuberance. Although the role of this

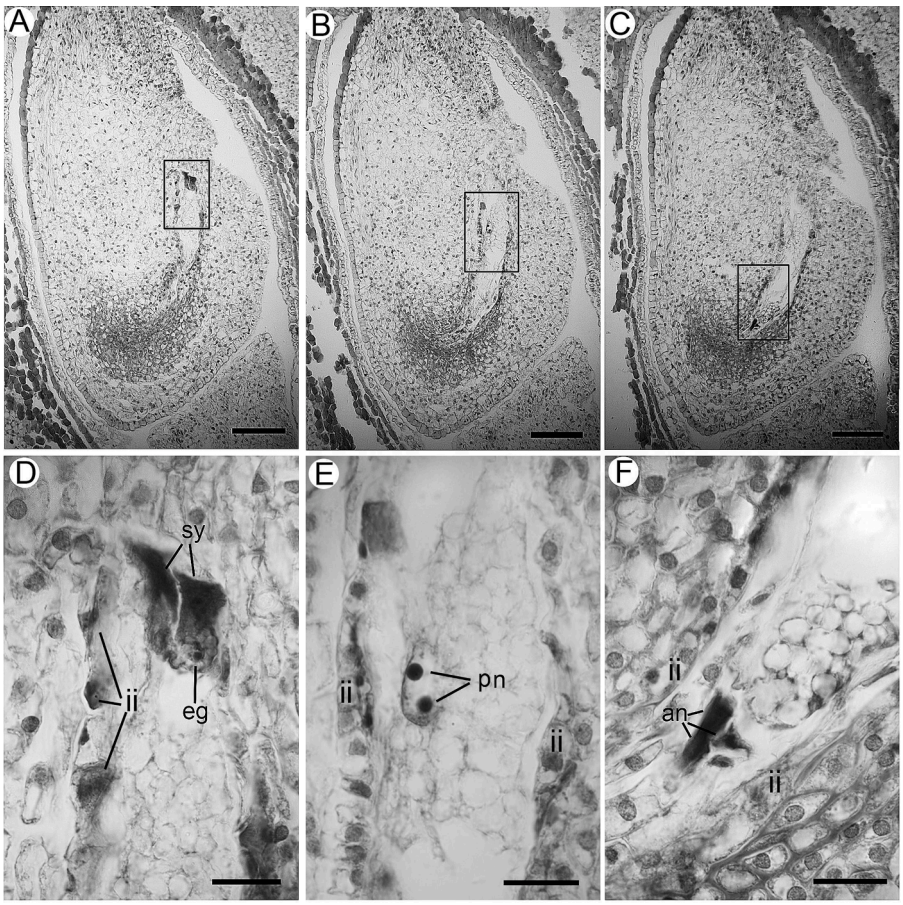

Fig. 7. Mature embryo sac in *Pittosporopsis kerrii*. A, B, C, LS successive sections through the ovule showing the mature embryo sac. **D,** Detail of a, showing the egg apparatus. **E,** Detail of b, showing the two polar nuclei before fusion. **F,** Detail of c, showing the degenerative antipodal cells and possible oil droplets. Abbreviations: *an* antipodals; *eg* egg; *ii* inner integument; *pn* polar nucleus; *sy* synergid. Scale bars = 200 μm in **A, C, E;** = 20 μm in **B, D, F**.

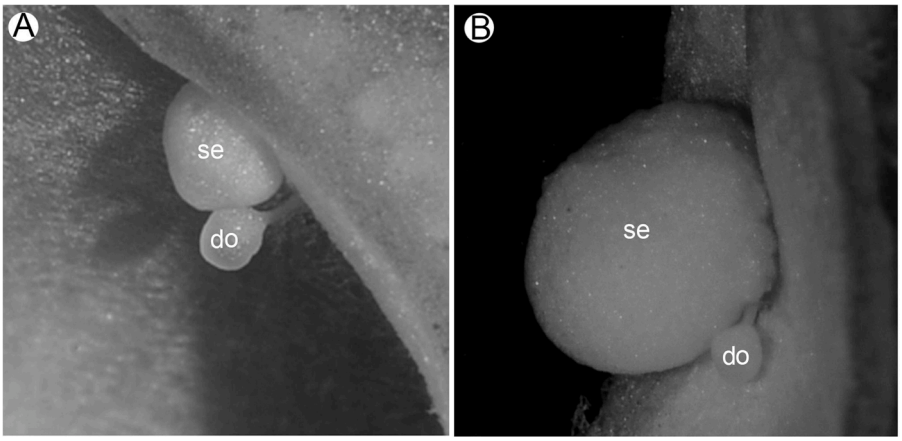

Fig. 8. Young seed and sterile ovule in *Pittosporopsis kerrii*. A, Inner view of a young fruit showing the proximal ovule developing into the seed and the abortive, distal ovule with long thin funiculus. **B,** Inner view of an older fruit, showing the developing seed and abortive distal ovule. Abbreviations: *do* distal ovule; *se* seed.

protuberance is unknown, it might be related to anther dehiscence. Second, the connective epidermis has multiple bulliform cells. Reports of these cells in anthers are limited to taxa that are not closely related, such as *Brassica napus* L. (Polowick and Sawhney, 1986), *Bunchosia* Rich. ex Kunth (Malpighiaceae, Dobson unpublished dissertation), *Justicia procumbens* L. and *Rungia repens* (L.) Nees (Acanthaceae, Labhane and Dongarwar, 2011), and Styracaceae (Dickison, 1993). Their function in anthers has not been determined, but researchers have hypothesized that they might be involved in dehiscence, functioning in a similar manner to bulliform cells in the leaves of graminoids, which influence leaf rolling during water stress (Alvarez et al., 2008; Grigore and Toma, 2017; Mader et al., 2020). The position of the cells in *Pittosporopsis* would allow the connective to roll inward, facilitating dehiscence. Large epidermal cells on the connective are present in *Metteniusa* (González and Rudall, 2010, see Fig. 5F, G), but those authors did not identify them as bulliform cells and reported that some of the cells appeared to be

secretory. Bulliform cells on the filament and anther appear to be uncommon, as they were not mentioned in a survey of taxa from 145 families (Schmid, 1976).

4.3. Ovule characters

One character used to define Icacinaceae s.l. was two ovules pendent from the locule apex (Kårehed, 2001). However, the relative position of the ovules within the locule varies and a closer examination reveals that it is not a uniform character among the families that are now recognized. In *Pittosporopsis, Metteniusa*, and *Emmotum* (Metteniusaceae), the ovules within one locule are positioned one above the other (superposed) (González and Rudall, 2010; Endress and Rapini, 2014; the present study). The proximal ovule is fertile in *Pittosporopsis* whereas the distal one is fertile in *Metteniusa* (González and Rudall, 2010). In Cardiopteridaceae and Stemonuraceae, the two ovules are collateral (usually back to back) (Mauritzon, 1936; Fagerlind, 1945; Padmanabhan, 1961; Tobe, 2012, 2016; Kong et al., 2014, 2018). Observations from two species of *Nothapodytes* Blume (Icacinaceae s.s.) indicate that the relative position of the two ovules is transitional between collateral and superposed, with oblique adjoining surfaces (D.-R. Kong, unpublished data). The superposed arrangement of the two ovules within one locule may be a synapomorphy of Metteniusaceae. The ovular arrangement in members of Icacinaceae s.s and *Oncotheca* needs investigation before conclusions can be drawn about whether the ovule position among former members of Icacinaceae s.l. is consistently different within clades under the most recent phylogenetic analyses.

Ovule vasculature varies considerably in Metteniusaceae and other genera previously considered part of Icacinaceae s.l. (Mauritzon, 1936; Fagerlind, 1945; Padmanabhan, 1961; Dickison, 1986; González and Rudall, 2010; Endress and Rapini, 2014; Tobe, 2016, the present study), although most genera have not been studied yet. In *Pittosporopsis*, the proximal ovule, which develops into the seed, has a thicker funiculus with many more vascular bundles (approximately nine) than in the slender funiculus of the distal ovule (one). The vascular bundles of *Pittosporopsis* do not enter the integuments, in contrast to *Metteniusa*, which has more than 10 vascular traces in the single integument of the distal ovule, which develops into the seed (González and Rudall, 2010). *Emmotum* has only one vascular bundle that does not reach the integuments (Endress and Rapini, 2014); similar vasculature is reported for *Oncotheca* (Dickison, 1986). In *Stemonurus* Blume and *Gomphandra* (Stemonuraceae), one vascular bundle extends from the raphe to the tip of the integument (Mauritzon, 1936; Fagerlind, 1945; Padmanabhan, 1961). In Cardiopteridaceae, a single vascular bundle divides in the chalaza and the branches enter the integument in *Leptaulus* Benth. and *Gonocaryum* (Mauritzon, 1936; Fagerlind, 1945), and in *Cardiopteris*, one vascular bundle is present in the raphe, which develops into a pseudo-integument after fertilization (Tobe, 2016). Vasculature can change as the ovule continues to develop after fertilization, as demonstrated in an extraordinary fashion in *Cardiopteris* (Tobe, 2016), but the other studies cited here reflect the ovule prior to fertilization. While additional data are needed before any patterns of ovule vascularization can be established, it may not be an informative character for inferring relationships.

Endress and Rapini (2014) presented an overview of integument number as known from Icacinaceae s.l. at the time and considered the bitegmic ovules of *Emmotum* to be very rare in euasterids and only known from *Vahlia* Thunb. (Vahliaceae) in lamiids before their discovery in *Emmotum*. Following the recent phylogenetic analyses of Zhang et al. (2020), concepts of lamiids have changed, and Metteniusaceae are now considered one of the first diverging clades of campanulids. However, bitegmic ovules are still quite rare in core asterids, and *Pittosporopsis* joins *Emmotum* and *Quintinia* (Paracryphiaceae, Friis et al., 2013) as the only genera documented with this condition in the campanulids. Within Metteniusaceae, unitegmic ovules have been recorded from five genera (*Apodytes, Cassinopsis, Poraqueiba*, and *Rhaphiostylis*, Mauritzon, 1936; *Metteniusa*, González and Rudall, 2010), but six genera have not been studied yet and one or more might also have bitegmic ovules. Even if no additional genera are found to have bitegmic ovules, their presence is quite unusual and provides evidence for a close relationship between *Pittosporopsis* and *Emmotum*.

The ovule of *Pittosporopsis* is crassinucellate, with three layers of cells between the megasporocyte and the nucellar epidermis. Comparisons with other genera are hampered by both imprecise terminology and the fact that the number of cell layers is often not reported or shown; weakly crassinucellate, crassinucellate, and almost tenuinucellate can indicate that one layer of cells is present, depending on the glossary. Endress (2011a) presented a more precise classification, but it has not necessarily been adopted by more recent authors. According to previous studies, *Emmotum* has (weakly) crassinucellate ovules (Endress and Rapini, 2014), and *Metteniusa* might have tenuinucellate ovules (González and Rudall, 2010). *Apodytes* and *Rhaphiostyles* have weakly crassinucellate to almost tenuinucellate ovules, whereas *Cassinopsis* is reportedly semicrassinucellate (Potgieter and Duno, 2016). Ovules are crassinucellate in *Oncotheca* (Dickison, 1986), *Gomphandra* and *Stemonurus* (Stemonuraceae; Mauritzon, 1936; Padmanabhan, 1961), and *Gonocaryum* (Cardiopteridaceae; Fagerlind, 1945), and tenuinucellate in *Phytocrene* Wall. (Icacinaceae s.s; Fagerlind, 1945) and *Citronella* and *Cardiopteris* (Cardiopteridaceae; Mauritzon, 1936; Tobe, 2016). In Bruniaceae, *Audouinia* Brongn., *Berzelia* Brongn., *Brunia*, and *Staavia* Dahl have crassinucellate ovules with approximately two cell layers between the megasporocyte and nucellar epidermis (Saxton, 1910). These reports indicate that (weakly) crassinucellate ovules are prevalent in the basal clades of the core asterids and may be a plesiomorphic character state in Metteniusaceae.

The other notable feature present in the ovule of *Pittosporopsis* is a hypostase, which has not been reported in other members of Metteniusaceae or Icacinaceae s.l. The hypostase does not appear to be composed of lignified or suberized cells, but rather has small cells with dense cytoplasm and a conspicuous nucleus. Given its position between the vascular supply and the embryo sac, it could facilitate the transport of nutrients, a suggested function of the hypostase (Bouman, 1984). Interestingly, a hypostase has been reported for several genera in Bruniaceae (Saxton, 1910; de Lange et al., 1993), which may be the sister lineage to Metteniusaceae as the earliest diverging clades of campanulids.

5. Conclusions

Pittosporopsis shares several floral and embryological features with *Metteniusa* and *Emmotum*, including pollen sacs with an independent dehiscence slit, connective tissue with tanniferous cells, and two superposed ovules within a locule, which support its inclusion in Metteniusaceae. In addition, *Pittosporopsis* shares separated pollen sacs within a theca with *Metteniusa* and a bitegmic, crassinucellate ovule with *Emmotum*. Another unusual feature not yet documented in other genera of Metteniusaceae but present in the campanulid family Bruniaceae is the formation of a hypostase. Additional developmental and embryological studies are needed on other genera of Metteniusaceae and former members of Icacinaceae s.l. to evaluate potential synapomorphies and reevaluate families in light of recent rearrangements of the campanulids and lamiid clades.

CRediT authorship contribution statement

Dong-Rui Kong: Conceptualization, Visualization, Data curation, Writing – original draft. **Melanie Schori:** Writing – review & editing. **Lu Li:** Resources, Conceptualization. **Yan Luo:** Resources, Visualization. **De-Chang Hu:** Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Dr. Julien B. Bachelier (Institut für Biologie /DCPS, Berlin, Germany) for his valuable suggestions and encouragement. We thank two anonymous reviewers for their constructive suggestions. This work was supported by the Natural Science Foundation of Shandong Province (No. ZR2019MC055).

References

Alvarez, J.M., Rocha, J.F., Machado, S.R., 2008. Bulliform cells in Loudetiopsis chrysothrix (Nees) Conert and Tristachya leiostachya Nees (Poaceae): structure in relation to function. Braz. Arch. Biol. Technol. 51, 113–119. https://doi.org/10.1590/S1516-89132008000100014.

APG [Angiosperm Phylogeny Group], 2016. An update of the Angiosperm phylogeny group classification for the orders and families of flowering plants: APG IV. Bot. J. Linn. Soc. 181, 1–20. https://doi.org/10.1111/boj.12385.

Bouman, F., 1984. The ovule. In: Johri, B. (Ed.), Embryology of Angiosperms. Springer-Verlag Press, Berlin, pp. 123–157. https://doi.org/10.1007/978-3-642-69302-1.

Claßen-Bockhoff, R., 2016. Bruniaceae. Flowering Plants. Eudicots. In: Kubitzki, K. (Ed.), The Families and Genera of Vascular Plants, 14. Springer, Cham, pp. 103–115.

Cronquist, A., 1981. An Integrated System of Classification of Flowering Plants. Columbia University Press, New York.

Davis, G., 1966. Systematic Embryology of the Angiosperms. John Wiley & Sons Press, New York.

de Lange, J.H., van der Walt, J.J.A., Boucher, C., 1993. Autecological studies on Audouinia capitata (Bruniaceae). 6. Nutritional aspects of the developing ovule. S. Afr. J. Bot. 59, 168–177. https://doi.org/10.1016/s0254-6299(16)30750-5.

Dickison, W.C., 1986. Further observations on the floral anatomy and pollen morphology of Oncotheca (Oncothecaceae). Brittonia 38, 249–259. https://doi.org/10.2307/2807350.

Dickison, W.C., 1993. Floral anatomy of the Styracaceae, including observations on intra-ovarian trichomes. Bot. J. Linn. Soc. 112, 223–255.

Dickison, W.C., Bittrich, V., 2016. Metteniusaceae (incl. Dendrobangia incert. sed.). In: Kubitzki, K. (Ed.), Flowering Plants. Eudicots, 14. Springer, Cham, pp. 263–267. https://doi.org/10.1007/978-3-319-28534-4_23.

Endress, P.K., 2011a. Angiosperm ovules: diversity, development, evolution. Ann. Bot. 107, 1465–1489. https://doi.org/10.1093/aob/mcr120.

Endress, P.K., 2011b. Evolutionary diversification of the flowers in angiosperms. Am. J. Bot. 98, 370–396. https://doi.org/10.3732/ajb.1000299.

Endress, P.K., Rapini, A., 2014. Floral structure of Emmotum (Icacinaceae sensu stricto or Emmotaceae), a phylogenetically isolated genus of lamiids with a unique pseudotrimerous gynoecium, bitegmic ovules and monosporangiate thecae. Ann. Bot. 114, 945–959. https://doi.org/10.1093/aob/mcu166.

Endress, P.K., Stumpf, S., 1991. The diversity of stamen structures in 'Lower' Rosidae (Rosales, Fabales, Proteales, Sapindales). Bot. J. Linn. Soc. 107, 217–293. https://doi.org/10.1111/j.1095-8339.1991.tb00225a.x.

Fagerlind, F., 1945. Bau des Gynöceums, der Samenanlage und des Embryosackes bei einigen Repräsentanten der Familie Icacinaceae. Sven. Bot. Tidskr. 39, 346–364.

Friis, E.M., Pedersen, K.R., Endress, P.K., 2013. Floral structure of extant Quintinia (Paracryphiales, campanulids) compared with the late cretaceous Silvianthemum and Bertilanthus. Int. J. Plant Sci. 174, 647–664. https://doi.org/10.1086/669908.

González, F., Maurin, O., Freudenstein, J.V., Chase, M., 2007. Metteniusaceae, an early-diverging family in the Lamiid clade. Taxon 56, 795–800. https://doi.org/10.2307/25065861.

González, F.A., Rudall, P.J., 2010. Flower and fruit characters in the early-divergent lamiid family Metteniusaceae, with particular reference to the evolution of pseudomonomery. Am. J. Bot. 97, 191–206. https://doi.org/10.3732/ajb.0900194.

Grigore, M.N., Toma, C., 2017. Bulliform cells, in: Anatomical Adaptations of Halophytes. Springer, Cham, pp. 325–338. https://doi.org/10.1007/978-3-319-66480-4_8.

Howard, R.A., 1942. Studies of the Icacinaceae. IV. Considerations of the New World genera. Contrib. Gray Herb. 142, 3–60.

Kårehed, J., 2001. Multiple origin of the tropical forest tree family Icacinaceae. Am. J. Bot. 88, 2259–2274. https://doi.org/10.2307/3558388.

Kong, D.-R., Schori, M., Lu, S.G., Li, L., Peng, H., 2014. Floral development of Cardiopteris, with emphasis on gynoecial structure and ovular morphology. J. Syst. Evol. 52, 629–642. https://doi.org/10.1111/jse.12081.

Kong, D.-R., Li, L., 2017. Microsporogenesis, male gametogenesis and pollen morphology of Gonocaryum lobbianum. Bull. Bot. Res. 37, 181–184 [In Chinese].

Kong, D.-R., Schori, M., Li, L., Peng, H., 2018. Floral development of Gonocaryum with emphasis on the gynoecium. Plant Syst. Evol. 304, 327–341. https://doi.org/10.1007/s00606-017-1479-7.

Labhane, N.M., Dongarwar, N.M., 2011. Anther and ovule development in some Acanthaceae. Int. J. Plant Reprod. Biol. 3, 165–166.

Li, H.-T., Luo, Y., Gan, L., Ma, P.-F., Gao, L.-M., Yang, J.-B., Cai, J., Gitzendanner, M.A., Fritsch, P.W., Zhang, T., Jin, J.-J., Zheng, C.-X., Wang, H., Yu, W.-B., Zhang, R., van der Bank, M., Olmstead, R.G., Hollingsworth, P.M., Chase, M.W., Soltis, D.E., Soltis, P.S., Yi, T.-S., Li, D.-Z., 2021. Plastid phylogenomic insights into relationships of all flowering plant families. BMC Biol 19, 232. https://doi.org/10.1186/s12915-021-01166-2.

Mader, A., Langer, M., Knippers, J., Speck, O., 2020. Learning from plant movements triggered by bulliform cells: the biomimetic cellular actuator. J. R. Soc. Interface 17, 20200358. https://doi.org/10.1098/rsif.2020.0358.

Mauritzon, J., 1936. Embryologische Angaben über Stackhousiaceae, Hippocrateaceae und Icacinaceae. Sven. Bot. Tidskr. 30, 541–550.

Padmanabhan, D., 1961. A contribution to the embryology of Gomphandra polymorpha. Proc. Natl. Acad. Sci. India B 27, 389–398.

Peng, H., Howard, R.A., 2008. Icacinaceae. In: Wu, C.-Y., Raven, P., Hong, D.-Y. (Eds.), Flora of China. Science Press; Missouri Botanical Garden Press, Beijing, St. Louis, pp. 505–514, 11. Science Press.

Polowick, P.L., Sawhney, V.K., 1986. A scanning electron microscopic study on the initiation and development of floral organs of Brassica napus (cv. Westar). Am. J. Bot. 73, 254–263.

Potgieter, M.J., Duno, R., 2016. Icacinaceae. Flowering Plants. Eudicots. In: Kubitzki, K. (Ed.), The Families and Genera of Vascular Plants, 14. Springer, Cham, pp. 239–256.

Potgieter, M.J., Schori, M., Utteridge, T.M.A., 2016. Stemonuraceae. Flowering Plants. Eudicots. In: Kubitzki, K. (Ed.), The Families and Genera of Vascular Plants, 14. Springer, Cham, pp. 367–376.

Quint, M., Claßen-Bockhoff, R., 2006. Floral ontogeny, petal diversity and nectary uniformity in Bruniaceae. Bot. J. Linn. Soc. 150, 459–477. https://doi.org/10.1111/j.1095-8339.2006.00482.x.

Santiago-Valentín, E., Viruet-Oquendo, E., 2013. Notes on the flower, fruit, and the reproductive phenology of the elusive Ottoschulzia rhodoxylon. Harvard Pap. Bot. 18, 61–65. https://doi.org/10.3100/025.018.0109.

Saxton, W.T., Péringuey, L., 1910. The ovule of the Bruniaceae. Trans. R. Soc. S. Afr. 2, 27–31. https://doi.org/10.1080/00359191009519358.

Schmid, R., 1976. Filament histology and anther dehiscence. Bot. J. Linn. Soc. 73, 303–315. https://doi.org/10.1111/j.1095-8339.1976.tb01811.x.

Schori, M., 2016. Cardiopteridaceae. Flowering Plants. Eudicots. In: Kubitzki, K. (Ed.), The Families and Genera of Vascular Plants, 14. Springer, Cham, pp. 131–136.

Sleumer, H.O., van Steenis, C.G.G.J., 1971. Icacinaceae. In: Flora Malesiana, 7. Noordhoff International Publishing, Leiden, pp. 1–87.

Stevens, P.F., 2001. onwards.. Angiosperm Phylogeny Website, 12. Missouri Botanical Garden. version 12, July 2012. http://www.mobot.org/MOBOT/research/APweb/ (accessed 20 July 2021).

Stull, G.W., de Stefano, R.D., Soltis, D.E., Soltis, P.S., 2015. Resolving basal lamiid phylogeny and the circumscription of Icacinaceae with a plastome-scale data set. Am. J. Bot. 102, 1794–1813. https://doi.org/10.3732/ajb.1500298.

Stull, G.W., Soltis, P.S., Soltis, D.E., Gitzendanner, M.A., Smith, S.A., 2020. Nuclear phylogenomic analyses of asterids conflict with plastome trees and support novel relationships among major lineages. Am. J. Bot. 107, 790–805. https://doi.org/10.1002/ajb2.1468.

Tobe, H., 2012. Floral structure of Cardiopteris (Cardiopteridaceae) with special emphasis on the gynoecium: systematic and evolutionary implications. J. Plant Res. 125, 361–369. https://doi.org/10.1007/s10265-011-0450-x.

Tobe, H., 2016. Embryology of cardiopteris (Cardiopteridaceae, Aquifoliales), with emphasis on unusual ovule and seed development. J. Plant Res. 129, 883–897. https://doi.org/10.1007/s10265-016-0845-9.

Zhang, C., Zhang, T., Luebert, F., Xiang, Y., Huang, C.H., Hu, Y., Rees, M., Frohlich, M. W., Qi, J., Weigend, M., Ma, H., 2020. Asterid phylogenomics/phylotranscriptomics uncover morphological evolutionary histories and support phylogenetic placement for numerous whole-genome duplications. Mol. Biol. Evol. 37, 3188–3210. https://doi.org/10.1093/molbev/msaa1600.