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Research Article

Gynoecial diversity in the Annonaceae and the evolution of functional traits that overcome the limitations of apocarpy

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Abstract Most species in the early divergent angiosperm family Annonaceae are apocarpous, with very diverse gynoecial morphologies. Although several Annonaceae genera with apocarpous flowers are known to possess an extragynoecial compitum (EGC) that enables intercarpellary pollen-tube growth to enhance fertilization success, the occurrence of EGC across the whole family remains obscure. Twenty Annonaceae species from all four subfamilies (Anaxagoreoideae, Ambavioideae, Annonoideae, and Malmeoideae) are examined here, with anatomical evidence revealing the occurrence of stigmatic exudate-mediated suprastylar EGC in all four subfamilies. Histological and ontogenetic studies furthermore indicate that trichomes in *Cananga* and *Drepananthus* form a confluent zone that connects adjacent stigmas, providing a structural premise for suprastylar EGC. Infrastylar EGC are reported in the Annonaceae for the first time in several genera, including *Artabotrys*, *Annona*, and *Miliusa*, associated with the opening on the ovary ventral groove and/or basal placentation. In addition to the sister genera *Isolona* and *Monodora* that are clearly syncarpous, flowers of the distantly related genus *Cyathocalyx* with a unicarpellate gynoecium have also been hypothesized to be syncarpous. Evidence of carpel vasculature and primordium development does not support that the solitary *Cyathocalyx* carpel is originated from carpel fusion, however, although the increased number of ovules renders it functionally similar to syncarpy. Gynoecial features, including the extensive occurrence of EGC and the increased number of ovules per carpel (consonant with reduction to a solitary carpel) in *Cyathocalyx*, may have evolved to overcome limitations associated with apocarpy and possibly contribute to the reproductive success and diversification of the family.

Key words: Annonaceae, apocarpy, carpel development and vasculature, extragynoecial compitum, floral anatomy, pollen-tube growth, reproductive success, syncarpy.

1 Introduction

Gynoecial structure can significantly impact the reproductive success of flowering plants (Endress, 1982; Armbruster et al., 2002). The majority of angiosperm flowers possess multiple carpels, with usually two to five carpels in core eudicots, three carpels in monocots, and further variations in early divergent angiosperms (Endress, 2014), whereas only a small proportion (11%) possess unicarpellate gynoecia (Endress, 1982). Syncarpy (congenital fusion of carpels) and apocarpy (free carpels) are contrasting gynoecial morphologies in multicarpellate plants. Approximately 83% of the angiosperm species are syncarpous, in which all carpels are fused to form a collective internal compitum (Endress, 1982) that allows pollen tubes to reach any ovule irrespective of which stigma the pollen was deposited on (Carr & Carr, 1961; Armbruster et al., 2002). This contrasts markedly with apocarpy, which only accounts for 6% of extant flowering plants but is common in early divergent angiosperms and is widely considered as a plesiomorphic trait (Endress, 1982, 2010). Uneven pollen deposition on the stigmas of apocarpous flowers will theoretically often result in incomplete fertilization, as apocarpy constrains intercarpellary growth of pollen tubes. Despite the potential wastage of ovules and reduced pollination efficiency, some angiosperms have nevertheless preserved apocarpy, most notably in the ANA (Amborellales, Nymphaeales, and Austrobaileyales) grade, the magnoliids and many early divergent eudicots, with relatively few derived angiosperms having undergone evolutionary reversals to apocarpy (Armbruster et al., 2002; Endress, 2011).

Previous studies have suggested that the evolution of an extragynoecial compitum (EGC) can compensate for the disadvantage of apocarpy (Endress, 1982). This mechanism creates additional routes that enable pollen-tube growth between separate carpels, thereby allowing a greater proportion of ovules to be fertilized. An EGC is functionally similar to syncarpy due to the creation of a compitum that connects different carpels. Extragynoecial compita can be classified into three types according to gynoecial morphology and pollen-tube pathway: (1) suprastylar EGC, in which pollen tubes cross between adjacent stigmas or styles when the stigmas are contiguous and/or covered by stigmatic exudate; (2) infrastylar EGC, in which pollen tubes grow out of an ovary and penetrate other carpels after traversing the stigmas and styles; and (3) extrastylar EGC, in which pollen tubes grow towards the ovules through other routes (such as filaments and receptacle), instead of growing through the styles (Wang et al., 2012). Suprastylar and infrastylar EGC therefore differ in the locations of intercarpellary pollen-tube growth, with the former occurring before entering the style, and the latter after leaving the style. All three mechanisms can quantitatively enhance pollination success, with infrastylar EGC probably also boosting pollination success qualitatively as it enhances pollen competition in the styles (Wang et al., 2012).

The retention of apocarpy as a plesiomorphic trait in early divergent angiosperms (Endress, 1982) renders these lineages ideal models for studying the occurrence and evolutionary significance of EGC. The Annonaceae is the largest magnoliid family, comprising 110 genera and approximately 2430 species (Couvreur et al., 2019). Annonaceae flowers lack distinct styles (Saunders, 2020), and more than 95% of species have apocarpous gynoecia. This raises the question why apocarpy has been so extensively retained in this species-rich family. Several Annonaceae genera have previously been found to possess suprastylar EGC, including *Annona* (Lora et al., 2011), *Asimina* (Losada et al., 2017), *Boutiquea* (Deroin, 1991; Endress, 2010), *Goniothalamus* (Lau et al., 2017), *Hexalobus*, *Neostenanthera*, and *Uvaria* (Deroin, 1991; Endress, 2010). In each case, the pollen tubes cross to adjacent stigmas through aggregated viscous stigmatic exudate. The report of suprastylar EGC in *Goniothalamus parallelivenius* suggests that the pollen tubes only enter the carpel through the apex of the elongated stigma, leading to the hypothesis that the stigma might function as a "pseudostyle" that parallels the functions of a true style in more derived angiosperms to promote pollen competition (Lau et al., 2017; Saunders, 2020). These genera are phylogenetically closely related, all belonging to the tribes Annoneae, Monodoreae, and Uvariaeae in the subfamily Annonoideae (Guo et al., 2017); although it has been hypothesized that EGC might be widespread across the family (Lau et al., 2017), its occurrence beyond the seven genera listed remains unknown.

The repeated evolution of syncarpy is a conspicuous feature of flowering plant evolution and has widely been regarded as a key evolutionary innovation in angiosperms (Endress, 2001), increasing the proportion of ovules fertilized, reinforcing pollen competition, economizing investment for ovary construction and enhancing spatial precision for pollination (Endress, 1982; Armbruster et al., 2002; Wang et al., 2012). *Isolona* and *Monodora* are two African genera in the Annonaceae (subfamily Annonoideae, tribe Monodoreae) that are fully syncarpous, with multiseriate ovules and a thick dimerous or trimerous involute stigma (Couvreur et al., 2008; Couvreur, 2009). Two alternative hypotheses concerning gynoecial development have been proposed to explain the evolutionary origins of syncarpy (Endress, 1990), including the "multiplication hypothesis", involving the branching of a carpel primordium, and the "fusion hypothesis", following the fusion of multiple carpels. Ontogenetic studies of carpel development in *Monodora crispata* revealed patterns that correspond with those of a single carpel, supporting the multiplication hypothesis (Leins & Erbar, 1982). A subsequent study of the carpel vasculature of *Isolona* and *Monodora* (Deroin, 1997), however, suggested that the alternating arrangement of thick and thin vascular bundles in the carpel supported the fusion hypothesis. *Isolona* and *Monodora* are collectively sister to a multicarpellate apocarpous clade (Couvreur et al., 2008), providing circumstantial support for the latter.

Cyathocalyx (Annonaceae subfamily Ambavioideae) is also potentially syncarpous because each flower has a single pistil with a large peltate stigma (0.9–3.5 mm in diameter) that often appears to be trimerous (Saunders, 2020). The number of ovules in *Cyathocalyx* species ranges from 8 to 38, and the ovules are arranged in 1–4 rows (Wang & Saunders, 2006). *Cyathocalyx* is nested within the *Cyathocalyx-Drepananthus-Cananga* clade (Surveswaran et al., 2010), in which *Drepananthus* is apocarpous and *Cananga* is inferred to be partially syncarpous (Deroin, 1997). In *Cananga*, the stigmas of the two central carpels are reported to be fused to form a partial compitum (Deroin, 1997; Endress, 2010). Transverse sections of the carpel of *Cyathocalyx martabanicus* show multiple vascular traces (Endress, 2008) that resemble that of a compound carpel. Although this evidence is potentially indicative of an independent origin of carpel fusion in *Cyathocalyx*, this hypothesis remains untested. As an alternative to carpel fusion, multiseriate ovules in the solitary carpel of *Cyathocalyx* may also have resulted from an increase in the number of ovules—a possible evolutionary strategy to enhance seedset (Saunders, 2020). As the fruit-carrying capability of an individual plant is probably limited, an increase in the number of ovules per carpel might be associated with a decline in carpel or flower number (Saunders, 2020).

The first aim of the present study is to explore the occurrence of EGC across the Annonaceae by investigating pathways of pollen-tube growth in 20 selected species from all four subfamilies (Anaxagoreoideae, Ambavioideae, Annonoideae, and Malmeoideae). We specifically aim to infer the correlation between gynoecial features and EGC type, hypothesizing that Annonaceae flowers with wet-type or closely appressed stigmas are very likely to possess suprastylar EGC, whereas flowers with basal placentation and/or incompletely sealed carpels are more likely to possess infrastylar EGC. The second aim is to investigate the hypothesis of an independent origin of syncarpy in *Cyathocalyx* by comparing the vascular anatomy of carpels across the *Cyathocalyx-Drepananthus-Cananga* clade, as well as the distantly related species *Alphonsea monogyna*, which is unicarpellate, and *Monodora myristica*, which is syncarpous. We also investigate the development of carpel primordia in the putatively syncarpous genus *Cyathocalyx* and the multicarpellate genus *Cananga*. Our study of the occurrence of EGC and the evolutionary origin of the *Cyathocalyx* carpel structure reveals various gynoecial strategies in the species-rich family Annonaceae that overcome the limitations of apocarpy, promoting its reproductive success and potentially contributing to its high diversity.

2 Material and Methods

2.1 Occurrence of EGC

The occurrence of EGC was assessed using field-based artificial pollination experiments with the following 20 Annonaceae taxa: *Anaxagorea javanica*, *Annona squamosa*, *Artabotrys blumei*, *Artabotrys hainanensis*, *Artabotrys hexapetalus*, *Artabotrys pilosus*, *Cananga odorata* var. *odorata*, *Desmos chinensis*, *Fissistigma oldhamii*, *Fissistigma polyanthum*, *Fissistigma wallichii*, *Goniothalamus calvicarpus*, *Goniothalamus laoticus*, *Goniothalamus peduncularis*, *Goniothalamus saccopetaloides*, *Goniothalamus yunnanensis*, *Miliusa balansae*, *Uvaria kweichowensis*, *Uvaria littoralis*, and *Uvaria tonkinensis*. The floral morphology and tribal affiliations of the 20 taxa are shown in Fig. 1. The 20 taxa were selected based on phylogenetic diversity (encompassing representatives of all subfamilies and various tribes), stigma shape/orientation, and the presence/absence of copious stigmatic exudate. The location and the voucher information of each taxon are shown in Table S1. Field observations of the general floral phenology were undertaken before the artificial pollination experiments. Artificial pollination of *Ana. javanica* was carried out under permit SBG-MTA 2022/0003 from the Singapore Botanic Gardens, National Parks Board, and that of *Ar. blumei* was carried out under permit (6) in AF CON 11/13 pt. 6 from the Agriculture, Fisheries and Conservation Department of Hong Kong.

Pollen was collected from staminate-phase flowers with paint brushes and deposited immediately onto the stigmas of pistillate-phase flowers of other conspecific, sympatrically occurring individuals. Pollinated flowers were collected ca. 24 h later to ensure sufficient time for pollen germination and pollen-tube growth. Floral samples were fixed in freshly prepared FAA solution (a 50:35:10:5 mix of 100% ethanol, distilled water, formaldehyde, and glacial acetic acid) for 24 h, and then stored in 70% ethanol prior to examination. Sepals, petals, and stamens were removed and the remaining pistils and receptacles were transferred to 5% KOH solution until they became translucent. Samples were then immersed in glacial acetic acid for 1 min and washed with distilled water. Fresh aniline blue solution (0.1% aniline blue powder in 0.03 mol/L aqueous potassium phosphate) was used to observe pollen tubes (Lau et al., 2017). Samples were stained for ca. 24 h, rinsed with distilled water, then compressed in glycerin on glass slides using cover slips. Prepared slides were observed under an upright brightfield and fluorescent microscope (Eclipse 80i; Nikon, Tokyo, Japan) with a DAPI (4′,6-diamidino-2-phenylindole) filter, and pollen-tube pathways photographed using the DS-Ri2 image system (Nikon).

2.2 Carpel vasculature

Carpel vasculature was examined using serial sections of paraffin wax-embedded flower buds of the following 10 taxa: *Alphonsea monogyna*, *Cyathocalyx harmandii*, *Cyathocalyx martabanicus*, *Cyathocalyx sumatranus*, *Cyathocalyx zeylanicus*, *Cananga odorata* vars. *odorata* and *fruticosa*, *Drepananthus ramuliflorus*, *Drepananthus ridleyi*, and *Monodora myristica*. The floral morphology and tribal affiliations of the 10 taxa are shown in Fig. 1. The location and the voucher information of each taxon are shown in Table S1. Sample collection of *D. ramuliflorus* and *D. ridleyi* was carried out under permit NP/RP20-079a from the National Parks Board, Singapore.

Freshly collected buds were fixed in FAA solution for 24 h and preserved in 70% ethanol until examination. Floral specimens were dehydrated using a graded ethanol series and ethanol-HistoChoice tissue fixative solutions (Sigma-Aldrich, St. Louis, USA), after which they were vacuum infiltrated in Paraplast Plus (Leica Biosystems, Wetzlar, Germany) for 3–5 days (Ruzin, 1999), embedded using a Leica EG1150 embedding center and sectioned at 12–16 µm using a Leica RM2135 rotary microtome. Sections were mounted sequentially on glass slides coated with gelatin, dewaxed, and stained with Safranin O and Fast Green (both Sigma-Aldrich) solutions (Ruzin, 1999). Slides were mounted with Neo-Mount anhydrous mounting medium (Merck KGaA, Darmstadt, Germany) and photographed using the DS-Ri2 image system (Nikon) coupled to an upright brightfield microscope (Eclipse 80i; Nikon). The slides are stored in The University of Hong Kong herbarium (HKU).

2.3 Carpel primordium development

The development of carpel primordia was examined in three taxa: *Cy. martabanicus*, and *Ca. odorata* vars. *odorata* and *fruticosa* (Table S1). Flower buds of different sizes at early developmental stages were studied using standard scanning electron microscopy (SEM) techniques. Flower buds were fixed in FAA solution for 24 h and stored in 70% ethanol for long-term preservation. Flower buds were dissected under a Stemi 305 stereomicroscope (Zeiss, Oberkochen, Germany) to remove the sepals and petals, dehydrated using a graded ethanol series, and critical point dried with a Leica EM CPD300 Critical Point Dryer. Specimens were attached to SEM stubs using carbon tape and coated with a layer of iridium or Au/Pd using a Q150R ES Plus Rotary Pumped Coater (Quorum Technologies, Lewes, UK). Prepared samples were observed and photographed using an S-3400N variable pressure SEM (Hitachi, Tokyo, Japan) at 20 kV or an S-4800 FEG SEM (Hitachi) at 5–12 kV. The SEM stubs have been deposited in HKU herbarium.

3 Results

3.1 Intercarpellary growth of pollen tubes

All the species studied revealed evidence of intercarpellary growth of pollen tubes through suprastylar EGC, with the pollen tubes crossing from one stigma to another across

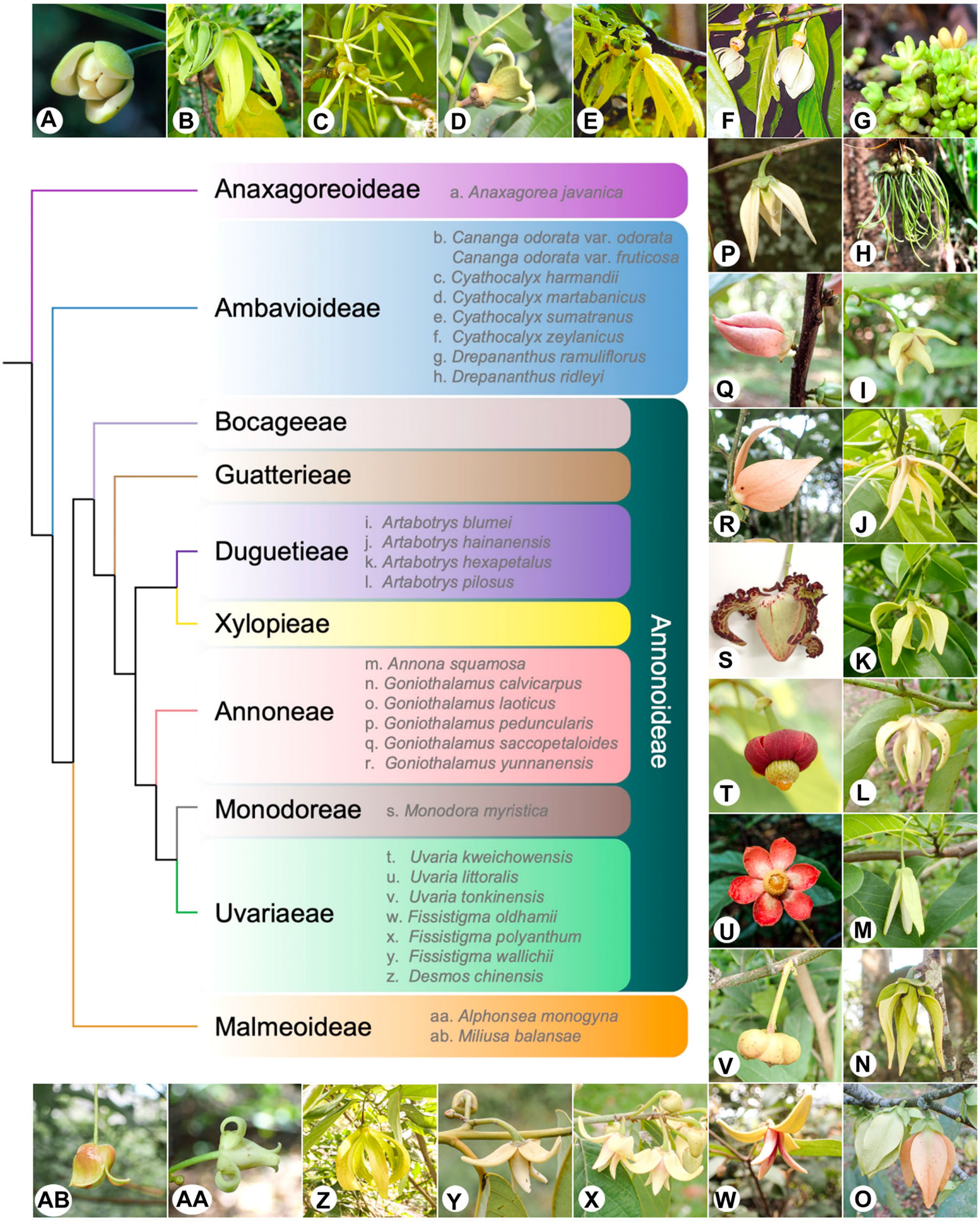

Fig. 1. Floral morphology and phylogenetic affiliations of the 28 Annonaceae species studied to investigate the occurrence of extragynoecial compita, carpel vasculature, and development. Reproduced from the maximum likelihood tree published by Guo et al. (2017). Photographs: **A**, S. Gardner, https://annonaceae.myspecies.info/file-colorboxed/709; **B**, **I**–**R**, **T**–**X**, **Z**–**AB**, Y. Chen; **C**, **F**, R. Wang, reproduced from Surveswaran et al., 2010; **D**, **E**, P. Chalermglin, reproduced from Surveswaran et al., 2010; **G**, **H**, **S**, J. Chen & D. Thomas; **Y**, S. Chen.

their surfaces (Figs. 2, 3). Suprastylar EGC in *Artabotrys blumei* (Fig. 2F), *Ar. hainanensis* (Fig. 2H), *Ar. hexapetalus* (Fig. 2J), *Ar. pilosus* (Fig. 2L), *Cananga odorata* var. *odorata* (Fig. 2N), *Goniothalamus laoticus* (Fig. 3F), *G. yunnanensis* (Fig. 3L), and *Miliusa balansae* (Fig. 3N) not only occurs on the upper parts of the stigmas, but also at the base of the stigmas. Thus the pollen-tube entry into the ovary is not constrained to the apex of the stigmas in these species.

It is noteworthy that the pollen tubes in *Ca. odorata* var. *odorata* initially grew downwards towards the ovules, before forming horizontal loops as they grew into the groove of an adjacent stigma (Figs. 2N, 4A, 4B); this distinct intercarpellary pollen-tube pathway differs from the other species studied. The stigmas of *Drepananthus ramuliflorus* (Figs. 4D, 4E), *D. ridleyi* (Figs. 4F, 4G), and *Ca. odorata* var. *odorata* (Figs. 4H–4K) contain an area where the ventral grooves of the carpels are oriented centripetally and mutually connected by trichomes, forming a common space among adjacent stigmas that enables intercarpellary growth of pollen tubes. The stigmas of *Ca. odorata* var. *fruticosa* do not show such a connecting area, but are closely packed instead (Figs. 4L–4O)—although this was only examined for five flowers in a single cultivated population. Densely packed trichomes form on the apical surface of the carpel of *Ca. odorata* var. *odorata* (Figs. 4P, 4Q) late in development, consistent with the anatomical evidence of trichomes interconnecting stigmas, unlike *Ca. odorata* var. *fruticosa* (Figs. 4R, 4S).

Infrastylar EGC was observed to occur in *Ar. pilosus* in two different ways: pollen tubes that crossed from one ovary to another (Figs. 5A, 5B), and those that penetrated the base of the ovaries and passed through the receptacle to reach

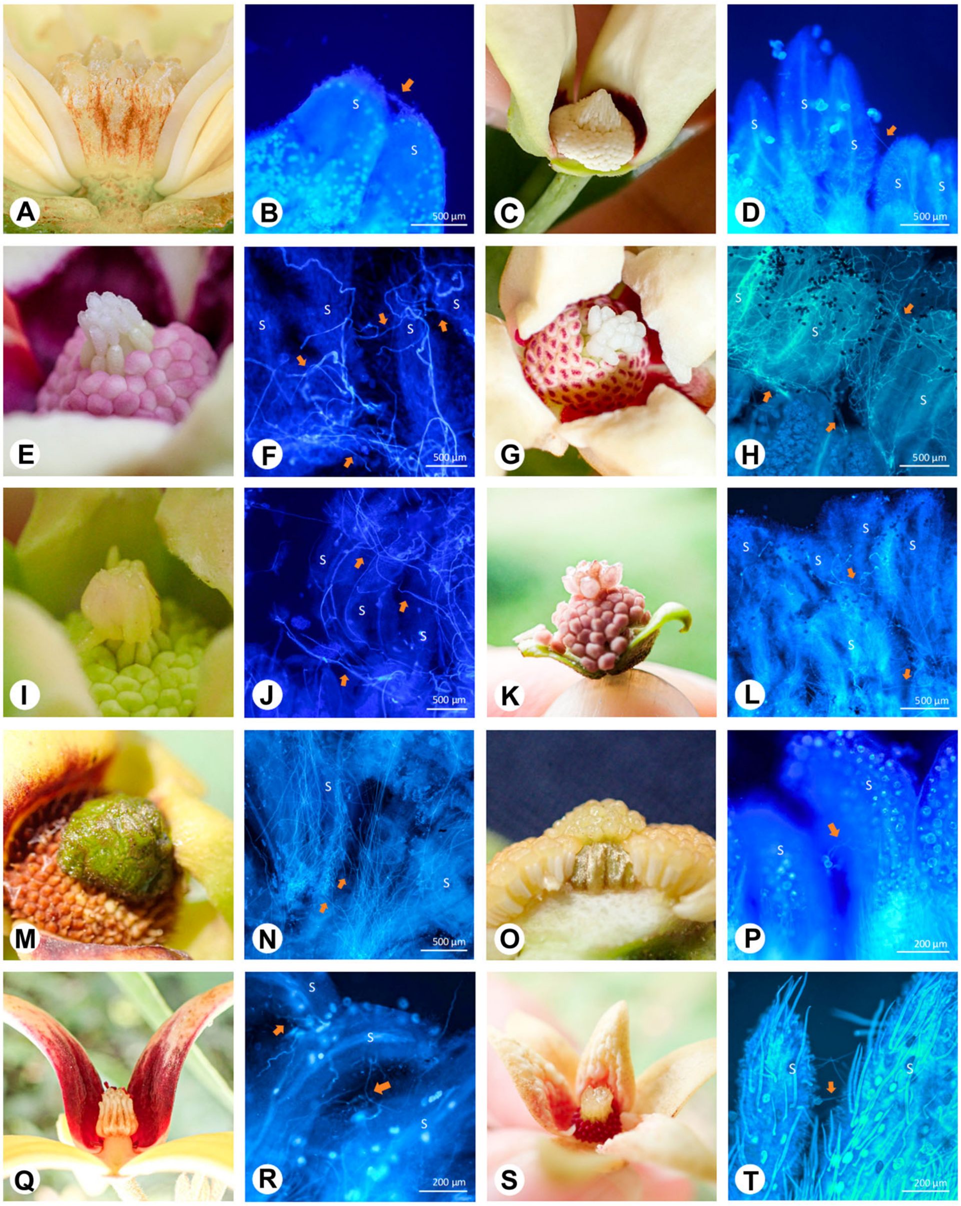

Fig. 2. Stigmatic morphology and evidence of suprastylar extragynoecial compita in *Anaxagorea javanica* (**A**, **B**), *Annona squamosa* (**C**, **D**), *Artabotrys blumei* (**E**, **F**), *Artabotrys hainanensis* (**G**, **H**), *Artabotrys hexapetalus* (**I**, **J**), *Artabotrys pilosus* (**K**, **L**), *Cananga odorata* var. *odorata* (**M**, **N**), *Desmos chinensis* (**O**, **P**), *Fissistigma oldhamii* (**Q**, **R**), and *Fissistigma polyanthum* (**S**, **T**), with pollen tubes crossing from one stigma (S) to another across their surfaces. Arrows indicate intercarpellary growth of pollen tubes. Photographs: **A**, J. Chen; **B**–**T**, Y. Chen.

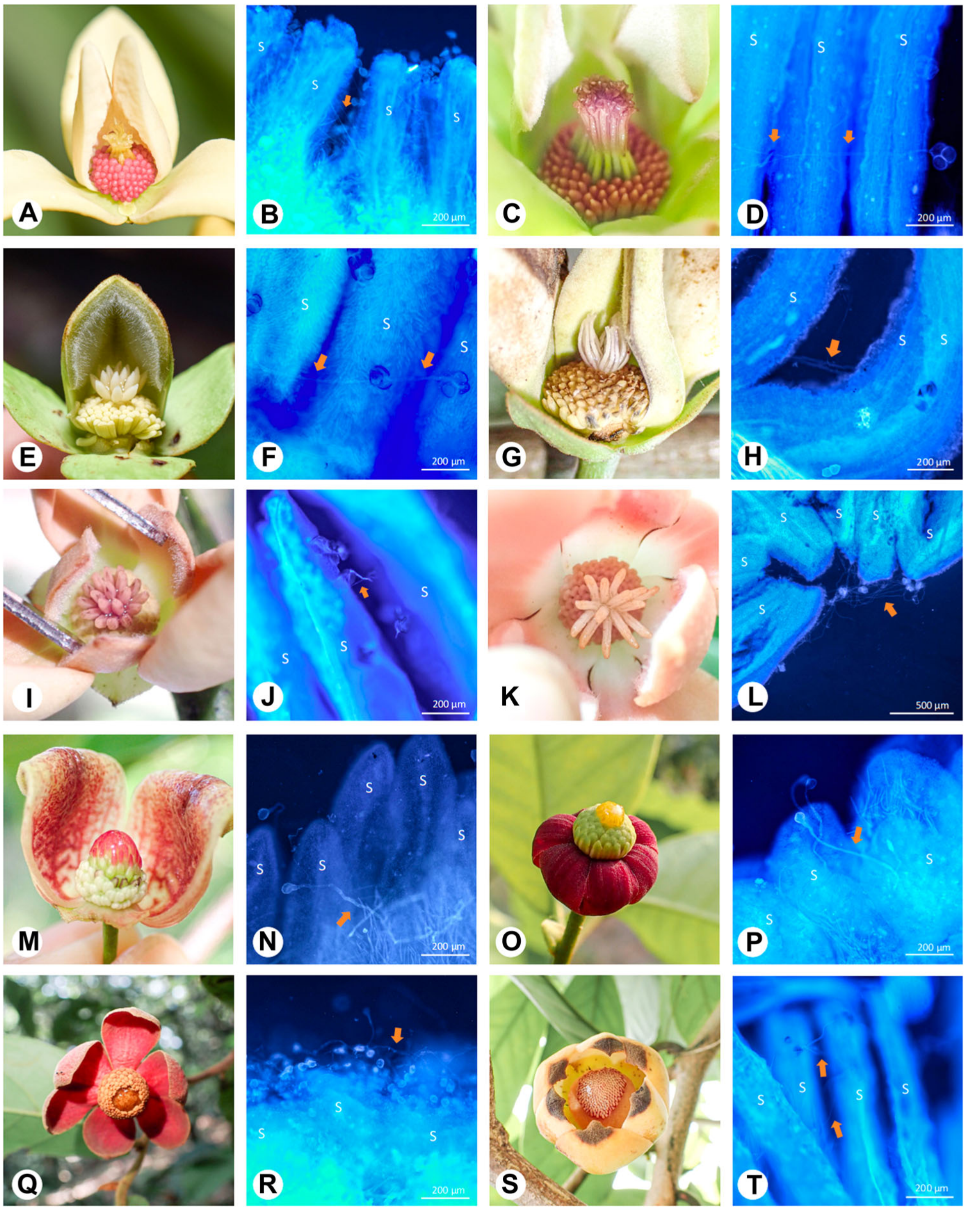

Fig. 3. Stigmatic morphology and evidence of suprastylar extragynoecial compita in *Fissistigma wallichii* (**A**, **B**), *Goniothalamus calvicarpus* (**C**, **D**), *Goniothalamus laoticus* (**E**, **F**), *Goniothalamus peduncularis* (**G**, **H**), *Goniothalamus saccopetaloides* (**I**, **J**), *Goniothalamus yunnanensis* (**K**, **L**), *Miliusa balansae* (**M**, **N**), *Uvaria kweichowensis* (**O**, **P**), *Uvaria littoralis* (**Q**, **R**), and *Uvaria tonkinensis* (**S**, **T**), with pollen tubes crossing from one stigma (S) to another across their surfaces. Arrows indicate intercarpellary growth of pollen tubes. Photographs: **A**, B. Yang; **B**–**D**, **F**–**T**, Y. Chen; **E**, G. Jiang.

adjacent ovaries (Figs. 5C, 5D). The former pathway is also recorded in *Annona squamosa* (Fig. 5E) and *Mi. balansae* (Figs. 5F, 5G).

3.2 Stigma morphology and anatomy, gynoecial vasculature, and ovule arrangement

The species examined for the occurrence of EGC are all apocarpous with stigmas that are highly variable in shape (Figs. 2, 3). The stigmas of all the species studied are covered in exudate, which is particularly copious in *Fissistigma polyanthum* (Fig. 2S), *Mi. balansae* (Fig. 3M), *Uvaria kweichowensis* (Fig. 3O), *U. littoralis* (Fig. 3Q), and *U. tonkinensis* (Fig. 3S). Moreover, enlarged, elongated, and appressed stigmas are observed in several species studied here, including *Ar. hexapetalus* (Fig. 2I), *Ca. odorata* var. *odorata* (Fig. 2M), *Fissistigma oldhamii* (Fig. 2Q), *F. polyanthum* (Fig. 2S), *Goniothalamus calvicarpus* (Fig. 3C), and *G. peduncularis* (Fig. 3G).

Only one suture is observed on the solitary carpel of *Cyathocalyx harmandii* (Figs. 6A–6D), *Cy. martabanicus* (Figs. 6E–6H), *Cy. sumatranus* (Figs. 6I–6L), and *Cy. zeylanicus* (Figs. 6M–6P). The single carpel in *Cyathocalyx* (Figs. 6A–6P), *Alphonsea* (Fig. 6T), *Drepananthus* (Figs. 7A–7H), and *Cananga* (Figs. 7I–7P) contains one median and two lateral vascular

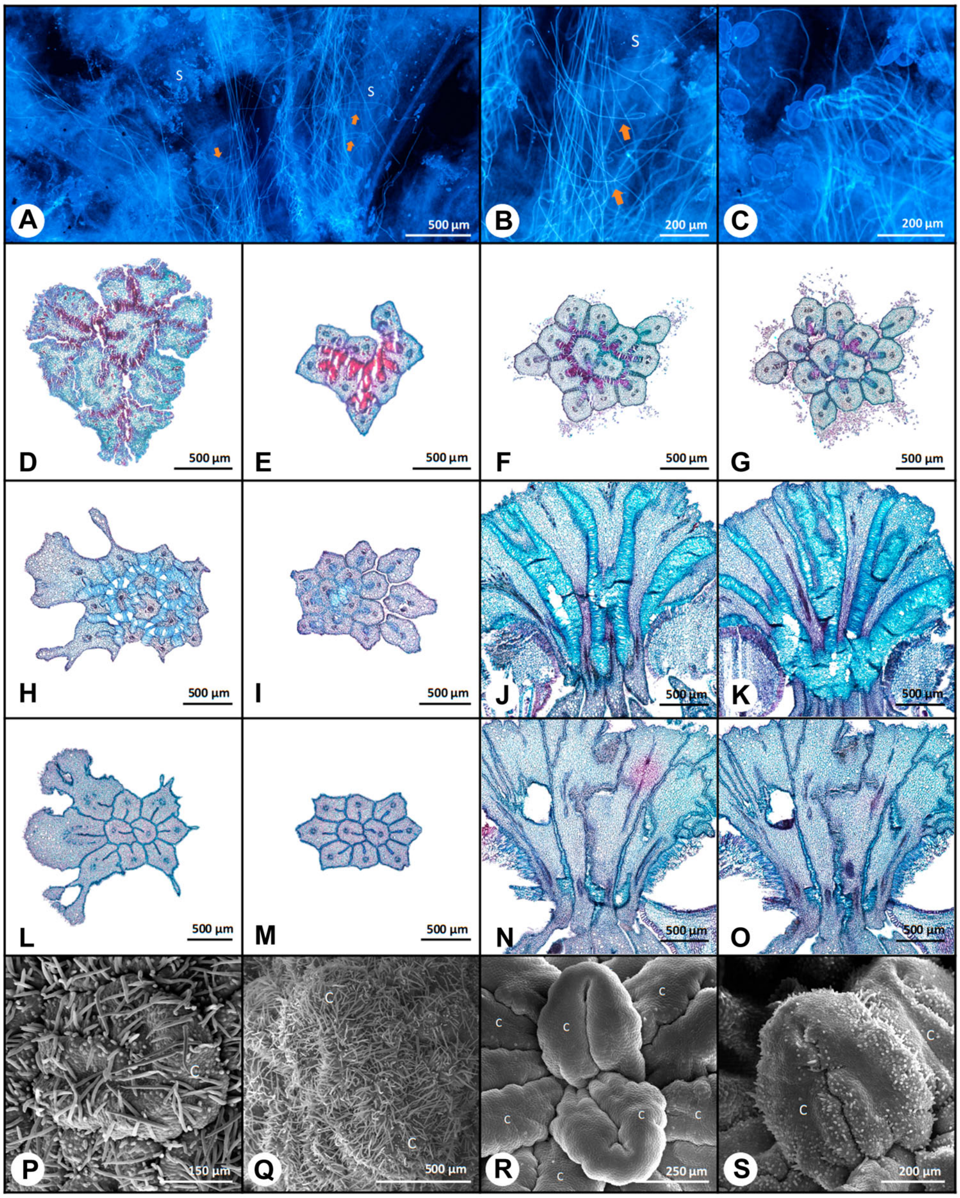

Fig. 4. Anatomical evidence (**A**–**C**), transverse (**D**–**I**, **L**, **M**) and vertical (**J**, **K**, **N**, **O**) sections through the stigmas, as well as scanning electron micrographs (**P**–**S**) showing trichome-mediated suprastylar extragynoecial compita in *Cananga odorata* var. *odorata* (**A**–**C**, **H**–**K**, **P**, **Q**), *Drepananthus ramuliflorus* (**D**, **E**), and *Drepananthus ridleyi* (**F**, **G**) in contrast to *Cananga odorata* var. *fruticosa* (**L**–**O**, **R**, **S**). (**C**) Germinated pollen and pollen tubes. Arrows indicate horizontal loops of pollen tubes. C, carpel; S, stigma. Photographs: Y. Chen.

bundles. In *Cy. harmandii* (Fig. 6C), *Cy. martabanicus* (Fig. 6G), *Cy. sumatranus* (Fig. 6L), *Cy. zeylanicus* (Figs. 6N, 6P), *D. ramuliflorus* (Figs. 7C, 7D), *D. ridleyi* (Figs. 7G, 7H), *Ca. odorata* var. *odorata* (Figs. 7K, 7L), and *Alphonsea monogyna* (Fig. 6T), the ovules are supplied by the median bundle in each carpel, while the ovules are irrigated by the lateral bundles in *Ca.* var. *fruticosa* (Figs. 7O, 7P).

In each *Cyathocalyx* and *Drepananthus* carpel, there are three or four obvious vascular bundles that lie between the lateral and median bundles, with widths that are comparable to those of the lateral bundles. The ovules of some *Cyathocalyx* and *Drepananthus* species are compacted: although the ovules are known to be biseriate in *Cyathocalyx* species, transverse sections of *Cy. harmandii* revealed up to five ovules at the same height (Fig. 6D), with up to three in *Cy. martabanicus* (Fig. 6H) and four in *Cy. sumatranus* (Fig. 6L). A similar ovule arrangement is found in *D. ramuliflorus*, in which up to three ovules can be found at the same level in an ovary (Figs. 7A, 7B).

The stigmas of *Cy. harmandii* (Fig. 6A), *Cy. martabanicus* (Fig. 6E), *Cy. sumatranus* (Fig. 6I), *Cy. zeylanicus* (Fig. 6M), and *Monodora myristica* (Figs. 6Q, 6R) are large and peltate, with those in *Mo. myristica* consisting of two involute units. The carpel of *Mo. myristica* possesses multiseriate ovules and

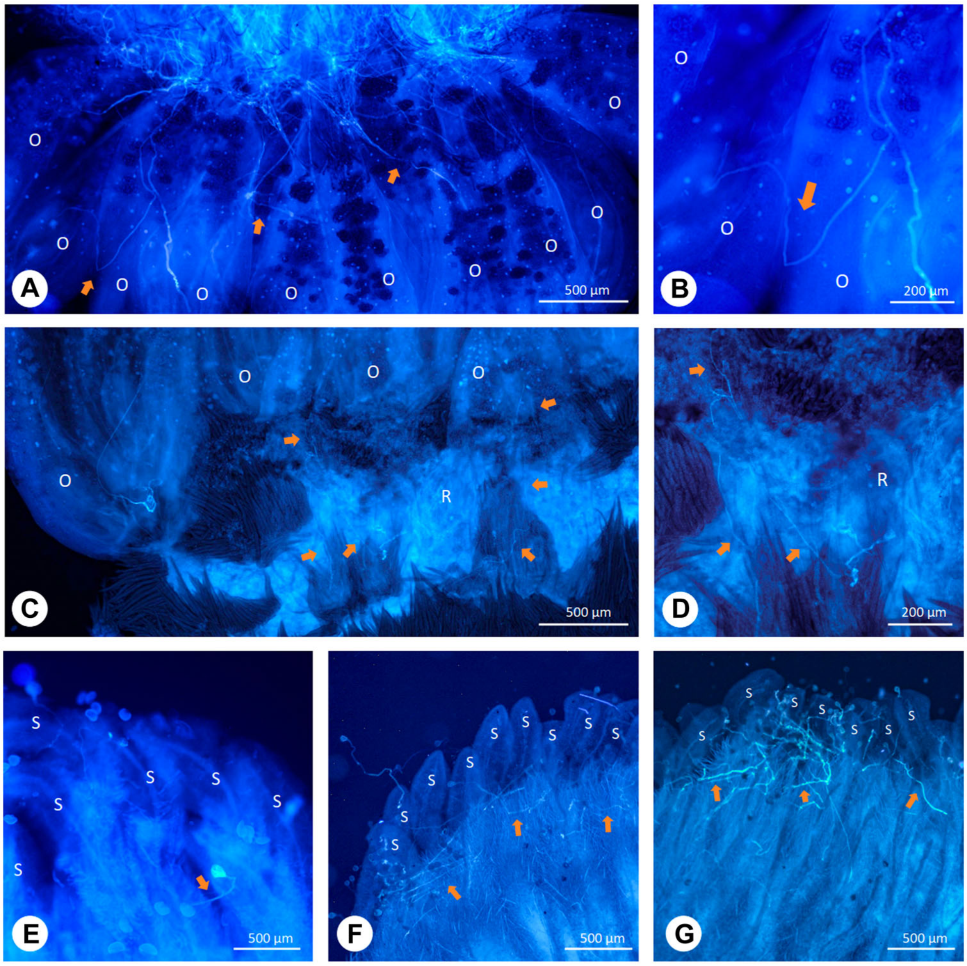

Fig. 5. Evidence of infrastylar extragynoecial compita in *Artabotrys pilosus* (**A**–**D**), *Annona squamosa* (**E**), and *Miliusa balansae* (**F**, **G**), with pollen tubes crossing between ovaries (**A**, **B**, **E**–**G**) and penetrating the receptacle (**C**, **D**). Arrows indicate intercarpellary growth of pollen tubes. O, ovary; R, receptacle; S, stigma. Photographs: Y. Chen.

numerous vascular bundles, in which thick and thin bundles are arranged alternately (Fig. 6S).

3.3 Gynoecial development

The gynoecial primordium of *Cy. martabanicus* develops congenitally as a single carpel (Figs. 8A–8H): a swelling is formed in the central part of the plate surrounded by stamen primordia, with the apex of the young carpel gradually developing into a heart shape and then becoming involute. Carpel development in *Ca. odorata* vars. *odorata* (Figs. 8I–8O) and *fruticosa* (Figs. 8P–8T) is similar: both form as swellings that become folded, with the outer carpel primordia formed earlier than the inner.

4 Discussion

4.1 Correlation between floral features and the occurrence of suprastylar EGC

Intercarpellary growth of pollen tubes is demonstrated in 20 Annonaceae species, with functional suprastylar EGC occurring in all cases (Figs. 2, 3). Annonaceae flowers possess wet-type stigmas (Lora et al., 2011; Lau et al., 2017), with stigmatic exudate observed in all species studied. In previous studies of EGC in Annonaceae, the occurrence of suprastylar EGC in *Annona cherimola* (Lora et al., 2011), *Asimina triloba* (Losada et al., 2017), *Goniothalamus parallelivenius*, and *G. tapisoides* (Lau et al., 2017) has been demonstrated to be mediated by stigmatic exudate. This type of suprastylar EGC is also discovered in other early divergent angiosperms such as *Amborella trichopoda* (Amborellaceae), *Austrobaileya scandens* (Austrobaileyaceae), and *Nymphaea tetragona* (Nymphaeaceae), with the stigmas connected by exudate (Endress & Igersheim, 2000). The stigmas in some *Artabotrys*, *Cananga*, *Fissistigma*, and *Goniothalamus* species are furthermore elongated and closely appressed, further enhancing opportunities for suprastylar EGC by increasing contact among stigmas (Saunders, 2020). Suprastylar EGC occurs in some *Artabotrys*, *Cananga*, *Goniothalamus*, and *Miliusa* species across the entire stigma, possibly due to the widespread occurrence of secretory papillae and associated exudate on the stigmas (Vithanage, 1984). As the extent of stigmatic exudate delimits the range of pollen-tube growth, the extensive exudate coverage enables intercarpellary pollen-tube growth anywhere on the stigma surface. Stigmas

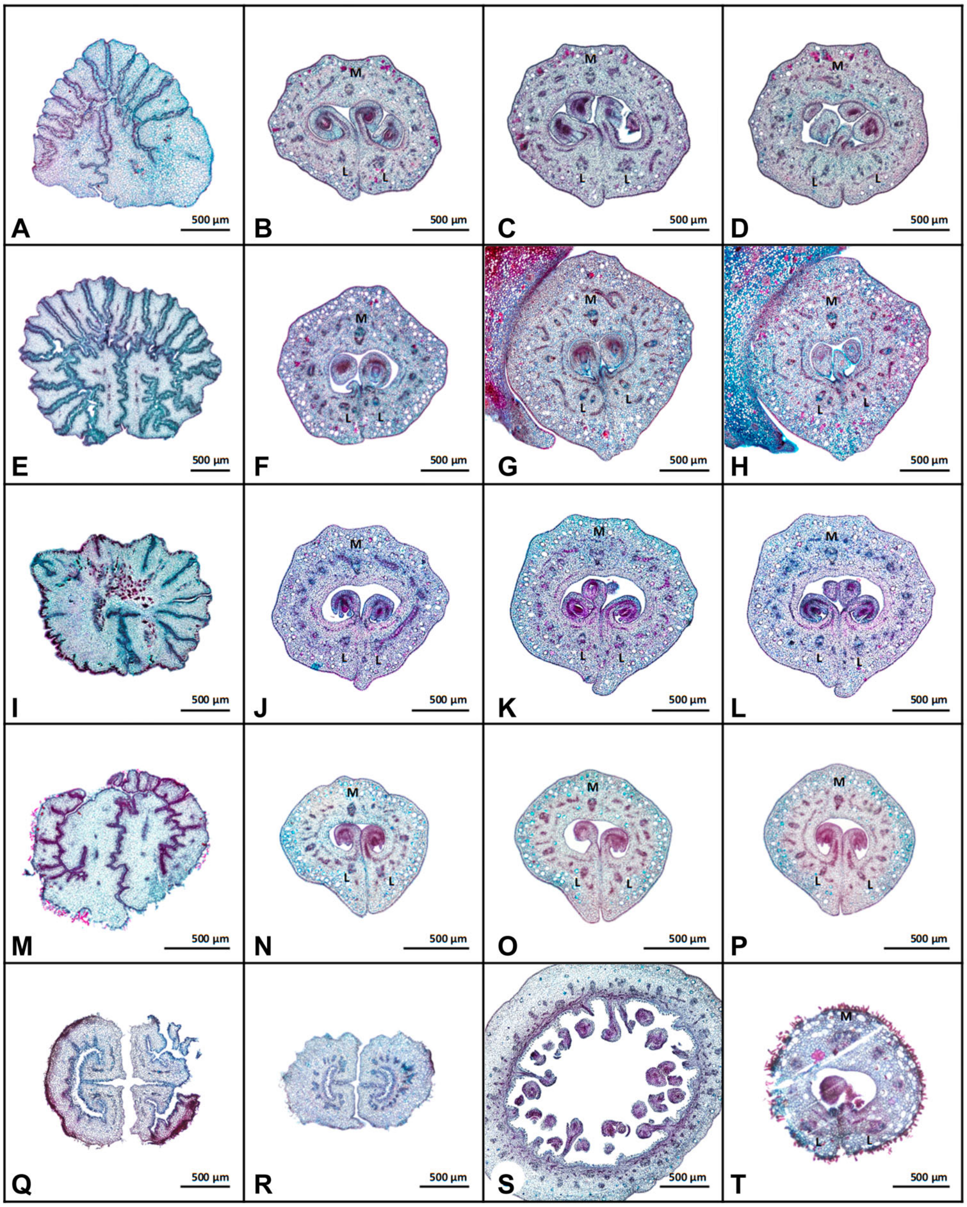

Fig. 6. Transverse sections through the stigmas (**A**, **E**, **I**, **M**, **Q**, **R**) and ovaries (**B–D**, **F–H**, **J–L**, **N–P**, **S**, **T**) of *Cyathocalyx harmandii* (**A–D**), *C. martabanicus* (**E–H**), *C. sumatranus* (**I–L**), *C. zeylanicus* (**M–P**), *Monodora myristica* (**Q–S**), and *Alphonsea monogyna* (**T**). L, lateral bundle; M, median bundle. Photographs: Y. Chen.

in these species can therefore be regarded as an interconnecting "stigmatic head."

In addition to the genera studied here, suprastylar EGC has also been recorded in the Annonaceae in *Asimina*, *Boutiquea*, and *Neostenanthera* (subfam. Annonoideae tribe Annoneae), as well as *Hexalobus* (subfam. Annonoideae tribe Monodoreae), which all possess stigmas with copious exudate (Deroin, 1991; Endress, 2010; Losada et al., 2017). As wet-type stigmas are prevalent in Annonaceae flowers (Lora et al., 2011; Lau et al., 2017), it can be surmised that other species with copious stigmatic exudate are highly likely to similarly possess suprastylar EGC. It is therefore inferred to be a key functional floral trait in the family (Saunders, 2020). In addition to Annonaceae, suprastylar EGC has been discovered in several other families in the order Magnoliales, including the sister family Eupomatiaceae and the closely related family Himantandraceae, as well as in the sister order Laurales, although EGC is absent in other Magnoliales families (Endress & Igersheim, 1997; Endress & Doyle, 2009; Wang et al., 2012). This suggests that the evolution of suprastylar EGC likely preceded the origin of the Annonaceae.

4.2 Structural evidence for suprastylar EGC in *Cananga* and *Drepananthus*

Cananga and *Drepananthus* species possess conduplicate carpels, in which the ventral groove in each ovary extends to the stigmas, forming a folded stigma head. Sections through

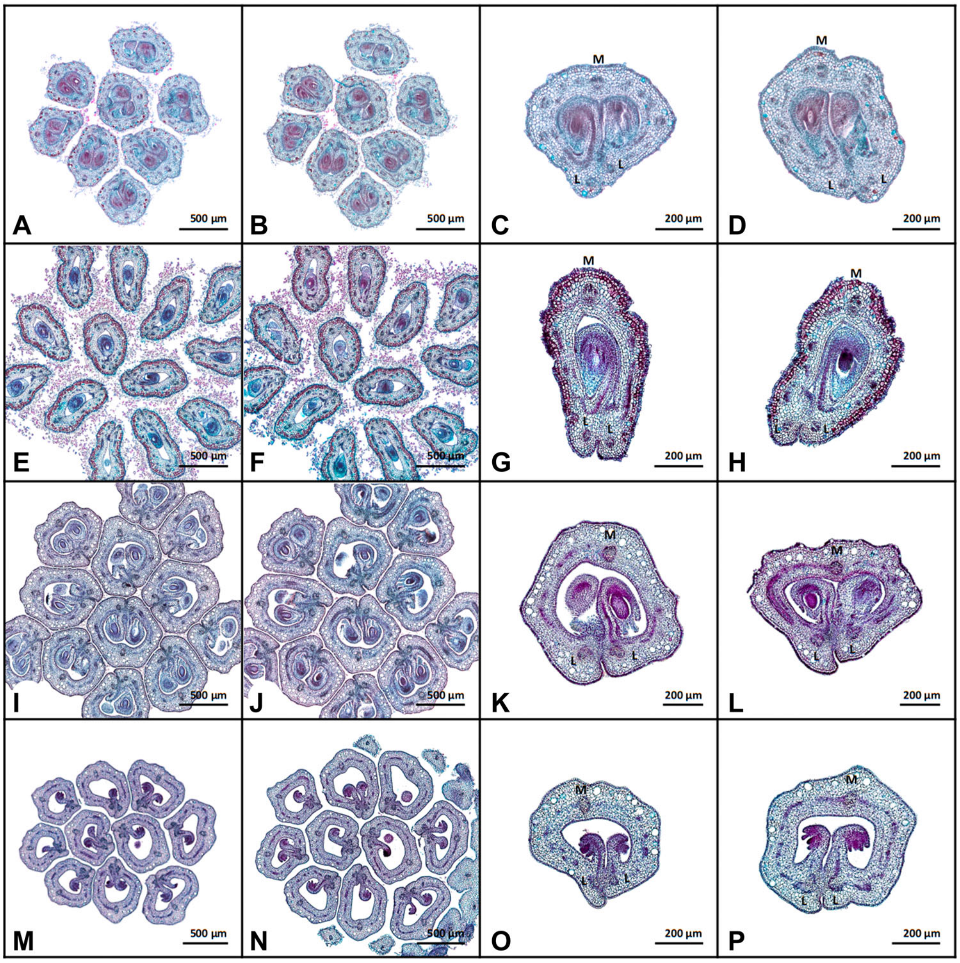

Fig. 7. Transverse sections through the ovaries of *Drepananthus ramuliflorus* (**A**–**D**), *Drepananthus ridleyi* (**E**–**H**), *Cananga odorata* var. *odorata* (**I**–**L**), and *Cananga odorata* var. *fruticosa* (**M**–**P**). L, lateral bundle; M, median bundle. Photographs: Y. Chen.

the stigmas of *Cananga odorata* var. *odorata*, *Drepananthus ramuliflorus*, and *D. ridleyi* reveal that the ventral grooves and stigmas are connected by trichomes. Previous studies of the gynoecium of *Ca. odorata* var. *odorata* defined such a structure as a "low cross zone" (Leins & Erbar, 1982, 1996) or a partial compitum (Deroin, 1991; Endress, 2010), in which fusion only occurred between the innermost two or three stigmas (Deroin, 1988, 1997). This phenomenon might be similar to the suprastylar EGC of *Eupomatia laurina* (Eupomatiaceae), in which the innermost carpels are confluent at the stigma level, although the stigmatic exudate is not copious (Endress, 1984; Igersheim & Endress, 1997). However, our anatomical study implies that previous interpretations of the confluent zone in *Ca. odorata* var. *odorata* might be erroneous for two reasons: first, the central two stigmas are not fused or sealed, but merely oppose each other with contiguous carpel margins; and second, the confluent zone involves all stigmas instead of only the central stigmas (*D. ramuliflorus*: Figs. 4D, 4E; *D. ridleyi*: Figs. 4F, 4G; and *Ca. odorata* var. *odorata*: Figs. 4H–4K). It is observed here that in *Ca. odorata* var. *odorata* the carpel sutures of the peripheral carpels are formed earlier and hence are taller than the carpel sutures of the younger central carpels. Previous studies on the gynoecium of *Ca. odorata* var. *odorata* were confined to the lower part of the stigmas, overlooking that the confluent zone ends higher among peripheral stigmas than the central stigmas due to the gradual closing of sutures, and that this might have led to the previous impression that the intercarpellary pollen-tube growth only occurs between the two central stigmas, although it is not the case in *D. ramuliflorus* or *D. ridleyi*.

The trichomes that are concentrated in the confluent zones of *Cananga* and *Drepananthus* stigmas are papillate glandular cells (Periasamy & Swamy, 1956), observed also in the development of the young carpels in *Ca. odorata* var. *odorata* (Figs. 4P, 4Q). *Cananga* and *Drepananthus* have wet-type stigmas that are covered with mucilaginous exudate, in which the trichomes not only function in exudate secretion but also interconnect the stigmas and act as a framework that enables stigmatic exudate to fill the space between and within the stigma slits (Leins & Erbar, 1996), thereby forming a suprastylar EGC. The existence of the trichomes enables the stigmatic exudate to extend across the entirety of the stigmas rather than aggregating at the base.

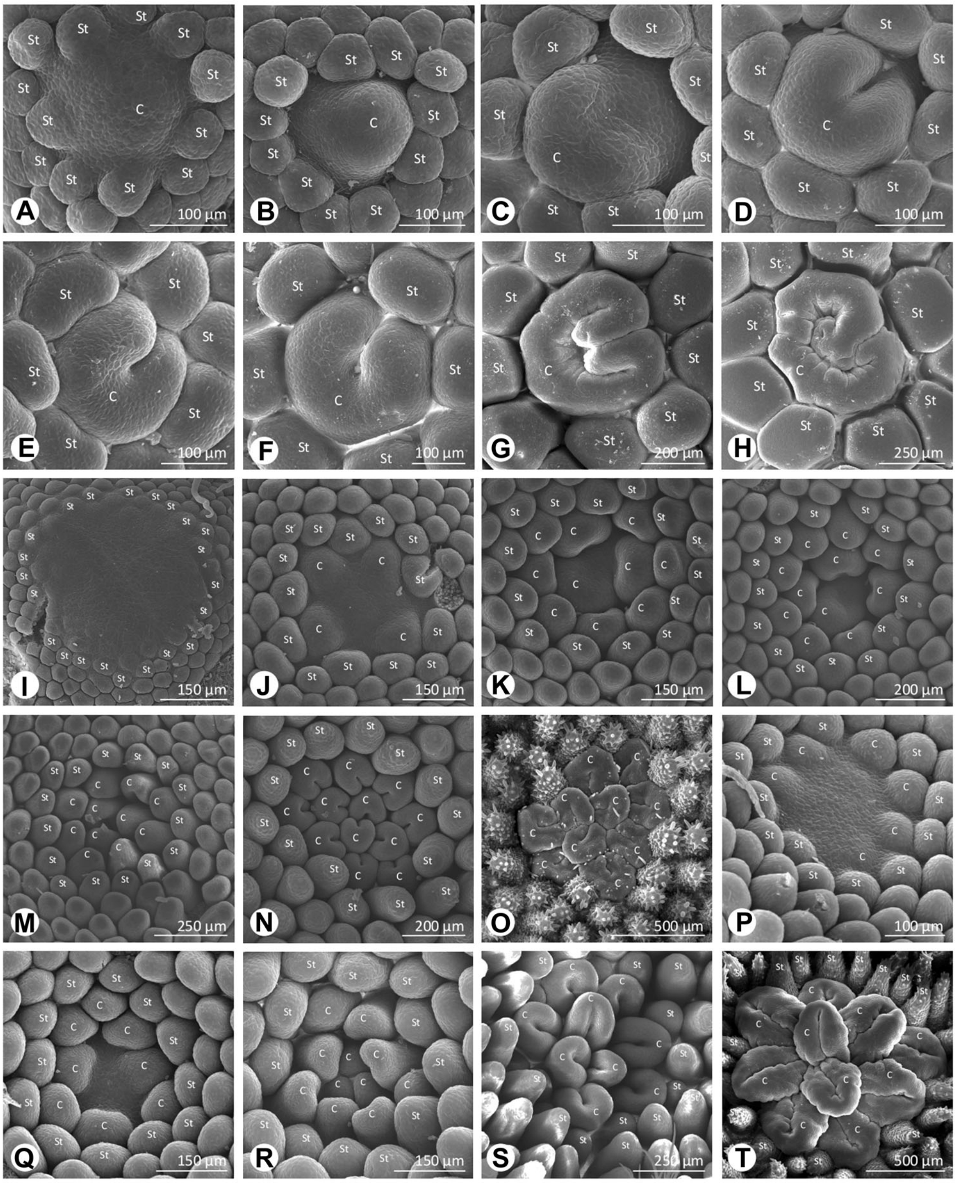

Fig. 8. Scanning electron micrographs of immature carpels of *Cyathocalyx martabanicus* (**A**–**H**), *Cananga odorata* var. *odorata* (**I**–**O**), and *Cananga odorata* var. *fruticosa* (**P**–**T**) (shown from young to old). C, carpel; St, stamen. Photographs: Y. Chen.

It is likely that the long trichomes also provide some shelter to the stigmatic exudate, thereby decreasing its evaporation rate and extending its longevity. In the intercarpellary pollen-tube growth of *Ca. odorata* var. *odorata* (Figs. 4A, 4B), horizontal loops were observed to form as pollen tubes grew into the groove of the adjacent stigmas, implying that pollen-tube entry into the ovaries is possibly restricted to the inner surface of the ventral groove. Such a limitation to pollen-tube entry might be associated with the folded stigma shape in *Cananga* and *Drepananthus* species.

4.3 Correlation between floral features and the occurrence of infrastylar EGC

Our study reports the occurrence of infrastylar EGC in the Annonaceae for the first time. In *Annona squamosa*, *Artabotrys pilosus*, and *Miliusa balansae*, intercarpellary growth of pollen tubes occurs after growing down the stigmas, as in the early divergent angiosperm *Kadsura longipedunculata* (Schisandraceae), which is associated with incompletely closed carpels (Lyew et al., 2007). Another early divergent angiosperm *Illicium floridanum* (Illiciaceae) has similarly been shown to possess infrastylar EGC between the marginal openings of neighboring carpels near the apical residuum (Williams et al., 1993). Infrastylar EGC has also been reported from eudicot taxa, such as *Sedum lineare* (Crassulaceae), which has marginal openings on the carpels (Wang et al., 2012). This phenomenon can be termed "ovarian EGC," as intercarpellary pollen-tube growth occurs between different ovaries. It was shown in a previous study that the stigmatic canal containing papillate secretory cells extended to the ovary in *Annona squamosa*, with the carpel wall suturing slightly below the junction between the stigma and the ovary (Vithanage, 1984) to enable ovarian EGC. Carpel margins are mostly sealed by secretion rather than postgenital fusion in the Amborellales-Nymphaeales-Austrobaileyales (ANA) grade (Doyle, 1998; Endress, 2004). Previous studies indicated that a combination of both arrangements occurs in the Annonaceae (Igersheim & Endress, 1997), with partial postgenital fusion and a continuous unfused secretory canal (Endress & Igersheim, 2000; Sauquet et al., 2003), implying the presence of incomplete carpel closure in the Annonaceae. Although histological evidence of this in *Ar. pilosus* and *Mi. balansae* is still lacking, the possession of a partial or complete opening to the ovary is very likely to be a structural feature as indicated by the occurrence of ovarian EGC.

Infrastylar EGC of a different type is demonstrated in *Artabotrys pilosus*, with pollen tubes penetrating the base of the ovary and traversing the receptacle to reach another ovary (Figs. 5C, 5D)—this form is termed "receptacular EGC" here. Receptacular EGC has previously been discovered among monocots such as *Sagittaria potamogetifolia* (Alismataceae), which has ovaries with a basal opening that enables pollen tubes to grow to neighboring ovaries by traversing the receptacle (Wang et al., 2012). It has been hypothesized that basal placentation of ovules might be correlated with receptacular EGC (Saunders, 2020), and hence is predicted to occur in several Annonaceae genera with basal placentation. Although *Ar. pilosus* has basal placentation, receptacular EGC has not been observed in other *Artabotrys* species studied or any of the other genera studied that have basal placentation (*Annona*, *Goniothalamus* p.p., and *Miliusa* p.p.); if it occurs, it is therefore likely to be rare.

The rarity of infrastylar EGC in the Annonaceae may be attributed to the uncommon occurrence of basal placentation and/or openings on the ovaries while the prevalence of suprastylar EGC may be linked to the ubiquity of wet-type stigmas in the family. Among the 110 genera in Annonaceae (Couvreur et al., 2019), basal placentation has primarily been recorded in *Annona*, *Artabotrys*, *Duguetia*, *Goniothalamus* p.p., *Guatteria*, and *Miliusa* p.p. (van Heusden, 1992). Among early divergent angiosperms, infrastylar EGC has previously only been reported from Illiciaceae (*Illicium floridanum*: Williams et al., 1993) and Schisandraceae (*Kadsura longipedunculata*: Lyew et al., 2007; *Schisandra sphenanthera*: Igersheim & Endress, 1997). As evidence of infrastylar EGC is lacking for Eupomatiaceae (sister to Annonaceae) and other closely related families, it might be a derived floral trait that has evolved independently in phylogenetically divergent clades in Annonaceae. Moreover, infrastylar EGC is shown here to co-occur with the ubiquitous suprastylar EGC, with the frequency of the former possibly constrained by the latter, as both reproductive strategies yield similar consequences.

4.4 Putative syncarpy in *Cyathocalyx*

Although *Cyathocalyx* flowers have been hypothesized to be syncarpous based on their external morphology (Saunders, 2020)—with a large peltate stigma and up to four rows of ovules, in contrast to the small ellipsoid or obconical stigmas and the uniseriate or biseriate ovules in the closely related genera *Drepananthus* and *Cananga* (Wang & Saunders, 2006)—the anatomical evidence gathered in the present study does not support this interpretation. The carpel anatomy of the four *Cyathocalyx* species studied all showed biseriate lateral placentation, contradicting the previous observations of up to three rows of ovules in *Cyathocalyx martabanicus*, and up to four in *Cy. harmandii* and *Cy. sumatranus* (Wang, 2004). Previous identification of ovular arrangements in *Cyathocalyx* carpels was based on vertical dissections that are potentially misleading because the funiculi are not clearly revealed, and ovule vascularization is neglected: transverse sections can compensate for this by revealing vasculature and the orientation of the funiculi. The transverse sections of the carpels of *Cy. harmandii*, *Cy. martabanicus*, and *Cy. sumatranus* appear to show three to five ovules at the same height (Figs. 6D, 6H, 6L), which might be interpreted as implying an equivalent number of rows of ovules and thus carpel fusion, but this appearance is actually due to the staggered orientation of the vertically contiguous funiculi on both margins of the carpel ventral suture. Ovules are generally formed along the carpel suture (Endress, 2019): as there is only one suture in *Cyathocalyx* carpels, what superficially appear to be three to five rows of ovules in each carpel are actually only two rows.

The vasculature of *Cyathocalyx* carpels demonstrates that ovules are supplied by the median bundle, with only one median bundle clearly seen from a single carpel, adding further support to the unicarpellate nature of the *Cyathocalyx* gynoecium. Moreover, the presence of only one obvious longitudinal suture in each *Cyathocalyx* carpel also suggests that it is unicarpellate, especially when compared to the unilocular gynoecium of *Takhtajania perrieri* (Winteraceae), which is formed by the fusion of two carpels as indicated by the existence of two sutures, two median bundles, and "double-crested" stigma (Leroy, 1977; Endress et al., 2000). Although the peltate stigma in a *Cyathocalyx* flower is large, the proportion of the *Cyathocalyx* stigma to its entire carpel is in keeping with the proportion of the ellipsoid or obconical stigmas in *Drepananthus* and *Cananga* to their respective smaller carpels. The apparently compound stigma, hypothesized to comprise three parts (Saunders, 2020), is merely due to the corrugation formed by compression during carpel development and involution.

Previous anatomical studies of Annonaceae carpels have demonstrated that each carpel is supplied by three vascular bundles, including one median and two lateral bundles (Periasamy & Swamy, 1956; Deroin, 1989). Possession of more than three vascular bundles in a single carpel in *Cyathocalyx* flowers (Endress, 2008; present study) has been considered a possible indicator of syncarpy (Saunders, 2020), as in *Isolona* and *Monodora* flowers (Deroin, 1997). In contrast to the vascularization of *Isolona* and *Monodora*, in which the large median bundles and the smaller lateral bundles are alternately arranged (Deroin, 1997; present study), vasculature in *Cyathocalyx* carpels clearly contains a thick median bundle and two lateral bundles, with three or four additional bundles appearing between the median and lateral bundles. The *Cyathocalyx* pattern parallels that of a single carpel in its closely related genus *Drepananthus*. The origin of multiple vascular traces in *Cyathocalyx* and *Drepananthus* carpels might have arisen from the need for an increased vascular supply during fruit development

(Endress, 2019): the complexity of the vascular system in a carpel can increase in relation with the number of seeds and the size of the fruit (Carlquist, 1969). The maximum fruit size in *Cyathocalyx* has been recorded as 83 × 68.5 mm (*Cy. sumatranus*; Wang, 2004). In another unicarpellate Annonaceae species, *Alphonsea monogyna*, the vasculature is simpler than that of *Cyathocalyx*, with fewer vascular traces between the median and lateral bundles (Fig. 6T); significantly, however, the fruits of *Al. monogyna* are smaller (ca. 35 × 25 mm) than those of *Cyathocalyx* (Keßler, 1995).

The carpel ontogeny of *Cy. martabanicus* reveals that it develops as a single carpel. The stigmatic head typically becomes strongly involute, forming grooves on both the inner and outer surfaces of the carpel wall on the stigmas, resembling stigma formation in the syncarpous species *Monodora crispata* (Leins & Erbar, 1996). Although stigma development of these two species is similar, this does not provide any indication of carpel fusion, but only explains their external morphology.

Even though the new evidence provided here does not support the interpretation of the *Cyathocalyx* gynoecium as syncarpous, it is noted that the solitary *Cyathocalyx* carpel is likely to have undergone an evolutionary increase in the number of ovules: *Cyathocalyx* species have 8–38 ovules per carpel, compared to only 1–10 in *Drepananthus* and fewer than seven in *Cananga* (Wang & Saunders, 2006; Surveswaran et al., 2010). As the *Cyathocalyx-Drepananthus-Cananga* clade is collectively sister to the genus *Lettowianthus*, which only has approximately two ovules per carpel (van Heusden, 1992; Surveswaran et al., 2010), an increased number of ovules per carpel is likely an apomorphic trait for *Cyathocalyx*. The closely related genera *Cyathocalyx* and *Drepananthus* might have undergone evolutionary adaptations in opposing directions, with *Cyathocalyx* experiencing a reduction in carpel number but an increase in ovule number per carpel, while some *Drepananthus* lineages increased the number of carpels per flower (Wang, 2004).

4.5 Gynoecial diversity and adaptations that overcome the limitations of apocarpy in Annonaceae: potential reproductive significance

Annonaceae flowers are largely apocarpous with 6–20 carpels (van Heusden, 1992). Apocarpy is generally considered deleterious, restricting pollen-tube growth between different carpels and hence causing imbalanced fertilization when carpels are subject to uneven pollen deposition (Carr & Carr, 1961; Armbruster et al., 2002). While Annonaceae flowers are predominantly apocarpous, our study of 29 taxa from different tribes and subfamilies (Table S1) indicates that Annonaceae flowers show diverse gynoecial structures that compensate for potential limitations in pollination efficiency and quality associated with apocarpy. The first strategy is congenital carpel fusion in the African genera *Isolona* and *Monodora* (subfam. Annonoideae tribe Monodoreae) (Couvreur et al., 2008), thereby overcoming all the limitations of apocarpy. The second strategy is an evolutionary reduction in carpel number with a concomitant increased number of ovules per carpel, as in *Cyathocalyx* (subfam. Ambavioideae). This strategy structurally resembles syncarpy, and possibly overcomes most of the constraints of apocarpy, depending on the number of carpels and ovules. It is likely that some *Hexalobus* species (subfam. Annonoideae tribe Monodoreae) might also adopt such a strategy: *Hexalobus bussei*, for example, contains 1–5 carpels with up to 36 ovules per carpel, and *Hexalobus mossambicensis* possesses only one carpel with up to 14 ovules (Botermans et al., 2011). The third strategy is the occurrence of EGC—the most phylogenetically widespread adaptation and a key functional floral trait of Annonaceae. Evolution of EGC that avoids the limitations of apocarpy seems to be the most parsimonious route as it only requires the secretion of stigmatic exudate to interconnect the stigmas instead of changing carpel morphology and structure.

The selective advantages of an increased number of ovules per carpel in *Cyathocalyx* might mirror that of syncarpy, as both strategies presumably enable an increase in the proportion of ovules that can be fertilized (Endress, 1982; Armbruster et al., 2002), despite their contrasting origins. Both strategies (syncarpy and solitary carpel with increased ovule number) are furthermore likely to enhance the spatial precision of pollen deposition during flower–pollinator interaction compared to apocarpous gynoecia with scattered stigmas (Armbruster et al., 2002). The advantages mentioned above are associated with improvements in pollination efficiency. In addition, due to the existence of a compitum enclosing all ovules, a solitary carpel with an increased number of ovules might possibly reinforce pollen competition compared to apocarpy and hence improve pollination quality (Endress, 1982). However, seeds in a large multiseeded monocarp derived from carpel fusion or a solitary carpel would probably be dispersed together and potentially lead to overcrowding between seedlings, whereas smaller monocarps with fewer seeds would lead to further spatial distance and less intense competition between seedlings (Saunders, 2020).

Extragynoecial compita have been suggested as an evolutionary innovation in apocarpous angiosperms, enabling pollen tubes to cross between carpels, thereby compensating for the limitations of apocarpy (Endress, 1982). The reproductive significance of EGC in enhancing fruit set has been reported in a previous study on *Schisandra sphenanthera* (Schisandraceae) (Du & Wang, 2012). Armbruster et al. (2002) pointed out that the fusion of the stigmas and the upper part of the styles merely facilitates pollen dispersion, while the fusion of lower regions of the carpels boosts both pollen dispersion and competition. Likewise, it was suggested that suprastylar and infrastylar EGC differ slightly in their reproductive significance, probably due to their distinctions in structural and spatial features: pollen tubes in different styles can compete with one another in infrastylar EGC, but this is unlikely with suprastylar EGC (Wang et al., 2012). Infrastylar EGC has therefore been considered more advantageous than suprastylar EGC as the former allows greater pollen competition, increasing opportunities for genetically superior pollen to fertilize eggs.

Nonetheless, styles are either very short or lacking in early divergent angiosperms, including Annonaceae (Saunders, 2020), Schisandraceae (Lyew et al., 2007), Cabombaceae (Galati et al., 2016), and Nymphaeaceae (Orban & Bouharmont, 1995), and so the differences between suprastylar and infrastylar EGC need to be re-evaluated in these families. When pollen-tube penetration is not restricted to the top of the stigmas or styles, their growth to reach the ovules is not constrained within a long and enclosed channel, thus the effect of pollen competition is possibly weakened. In Annonaceae flowers, for example, stigmatic exudate covers the entire stigmas, which sometimes tend to be lanceolate, cylindrical, and linear, and are thus vertically elongated. The random deposition of pollen grains by the pollinators might result in pollen grains that are closer to the ovules being at an advantage; hence fast- and slow-growing pollen grains are less likely to be differentiated, reducing the efficacy of pollen competition (Mulcahy & Mulcahy, 1975). In such cases, the elongated stigmas might be an adaptation to enhance the opportunities for pollen capture, rather than to facilitate pollen competition, in contrast to the pseudostyles of *G. parallelivenius*, in which the pollen tubes enter the pistils only through the apex of the stigmas (Lau et al., 2017; Saunders, 2020).

A combination of the lack of styles, full coverage of stigmas by their secretion, and openings on the ovaries does not encourage pollen competition and thus obscures the functional distinctions between suprastylar and ovarian EGC in Annonaceae, as pollen-tube growth is not restricted in a long and closed channel in either type of EGC. Nevertheless, pollen competition in the Annonaceae can be enhanced by receptacular EGC, in which a greater distance is required for all pollen tubes growing towards an unfertilized ovule. Given the coexistence of suprastylar and infrastylar EGC and their similar functions in Annonaceae, with the former invariably occurring prior to the latter, the latter is likely to play a complementary role to the former by further redistributing pollen tubes to unfertilized carpels and ovules. In general, the broadly occurring EGC is presumably a crucial evolutionary strategy that functions in increasing the quantity and quality of offspring in Annonaceae, as well as other early divergent angiosperms with wet-type stigmas, openings on the ovaries, and basal placentation.

5 Conclusions

Our study provides evidence of diverse gynoecial adaptations that can offset the limitations of apocarpy in the Annonaceae. First, both suprastylar and infrastylar EGC occur in the family, although with different frequencies and reproductive significance, with suprastylar EGC very likely to be a key functional floral trait. Second, an increased number of ovules per carpel is observed in *Cyathocalyx*, which is another gynoecial adaptation in the family that can enhance pollination efficiency and quality. Both adaptations may contribute to the reproductive success and high diversity of the Annonaceae.

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Supplementary Material

The following supplementary material is available online for this article at http://onlinelibrary.wiley.com/doi/10.1111/jse.13143/suppinfo:

Table S1. Taxa sampled and the corresponding locations, voucher information, and analysis.