Serial Section-Based 3D Reconstruction of *Anaxagorea* Carpel Vasculature and Implications for Integrated Axial-Foliar Origin of Angiosperm Carpels

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Running Title: Integrated Axial-Foliar Origin of Carpel

Abstract

The carpel is the basic unit of the gynoecium in angiosperms and one of the most important morphological features differentiating angiosperms from gymnosperms; therefore, carpel origin is of great significance in the phylogeny of angiosperms. However, there are conflicting theories regarding its origin. According to the traditional view, angiosperm carpels are derived from structures similar to the macrosporophylls of pteridosperms or Bennettitales, which bear ovules on the surfaces of their foliar organs. Conversely, the other hypothesis suggests that the carpel originated from foliar appendages enclosing the ovule-bearing axis. Since it has been confirmed by morphological and molecular evidence that foliar homologous are involved in carpel evolution, if axial homologs can be traced in the carpel, it would more likely be derived from an integrated axial-foliar structure.

This study aimed to reveal the axial structures in carpels by analyzing the continuous changes in vasculature from the receptacle to the carpels and ovules. *Anaxagorea* is the most basal genus of the primitive angiosperm Annonaceae. The conspicuous carpel stipe at the base of each carpel makes it an ideal material for exploring the possible axial homologous structure in the carpel. In this study, floral organogenesis and the topological vasculature structure were delineated in *A. luzonensis* and *A. javanica*, and a 3D-model of the carpel vasculature was reconstructed based on the serial sections.

The results show that (1) At the flowering stage, the number of vascular bundles entering each *Anaxagorea* carpel from the receptacle was significantly more than three, arranged in a radiosymmetric pattern, forming a basal ring at the base of each carpel. (2) All the carpel bundles originated from the central stele. (3) At the slightly upper part of the carpel, all the lateral bundles from the basal ring were reorganized into two groups, each forming a lateral bundle complex below each placenta. Bundles in each lateral bundle complex are also ring-arranged. (4) The ovule bundles were composed of non-adjacent bundles in the lateral bundle complex directly.

It was suggested that the anatomical receptacle structures, the carpel stipe, and the placenta of *Anaxagorea* are the consistent preferences of axial homologs in support of the theory that the carpel originates from an integrated axial-foliar structure. Despite this, more evidence is still needed to confirm the homology of the ring-arranged bundles in the floral organ and stems.

Key words: 3D reconstruction; *Anaxagorea*; angiosperms; organogenesis; origin of the carpel; vascular anatomy

INTRODUCTION

Since Darwin's time, the elucidation of angiosperm origin and its evolutionary success has been a primary goal of plant science (Kennedy and Norman, 2005). The term "angiosperm" is derived from the Greek words angeion, meaning "container," and sperma, meaning "seed." Therefore, the carpel, an angiosperm-specific "seed container", is the definitive characteristic of angiosperms. The carpel is the basic unit of the gynoecium; it protectively surrounds the ovules by enclosing and sealing off their rims or flanks (Dunal, 1817; Robinson-Beers, 1992; Endress, 2015). The evolution of the carpel sets angiosperms apart from other seed plants, which develop exposed ovules. Previous studies have attempted to identify the potential angiosperm ancestors through phylogenetic analyses based on fossil, morphological, and molecular data. In these studies, particular emphasis was placed on assessing which ovule-bearing structures of various seed plants could be transformed into carpels. However, due to early differentiation, the extant angiosperms and gymnosperms underwent a long independent evolutionary process that resulted in the reproductive structure of the basal angiosperms and extant gymnosperms being significantly different, although some species of the two groups may have undergone convergent evolution (Winter et al., 1999; Soltis et al., 2002; Magallon et al., 2015). As a result, the origin of the carpel has not been solved.

The two contradictory hypotheses on carpel origination can be summarized as whether the ovule is axial- or foliar- borne before carpel formation. The macrosporophyll origin theory suggests that ovules were borne on the margin of the foliar organs or ectopic expression on the phyllome, and the carpel was formed by the folding of these reproductive leaves (Arber and Parkin, 1907; Eyde, 1975; Cronquist, 1988; Takhtajan, 1991; Frohlich and Parker, 2000). Conversely, the stachyosporous origin theory (with its further elaborations) proposes that the carpel originated from a compound shoot from the integration of the ovular axis and the foliar appendage (Stebbins, 1974; Retallack and Dilcher, 1981a; Crane, 1985; Doyle and Donoghue, 1986; Nixon et al., 1994; Hickey and Taylor, 1996; Doyle, 1978, 2006, 2008; Wang, 2010, 2018).

Owing to the different hypotheses, it is important to find clear evidence of an axial homolog of the carpel. Since it has been confirmed by morphological and molecular evidence that foliar homologs are involved in the evolution of the carpel, if axial homologs can be found in the carpel, it would more likely have been derived from an integrated axial-foliar structure. Progress has been made in morphological and functional genetics studies in angiosperms. The ovules/placenta and ovary wall are served by distinct vascular bundles (Guo et al., 2013; Liu et al., 2014; Guo et al., 2017; Zhang et al., 2017) and the ovules and ovary wall are regulated by different genes associated with branch and leaf organs, respectively (Roe et al., 1997; Skinner et al., 2004; Mathews and Kramer, 2012). In distributed lines of angiosperms, the ovule primordia originate in the axillary position between the flower apex and carpel wall (e.g., *Gyrostemon* [Hufford, 1996]; *Illicium* [Zhang et al., 2019]; *Phytolacca* [Zhang

et al., 2018]). These are key clues to whether the ovules/placenta and the carpel wall have different evolutionary origins.

In this study, two *Anaxagorea* (Annonaceae) species were selected for floral organogenesis and vascular anatomic examination. Annonaceae represents one of the largest families in the Magnoliales that is one of the most important lineages in the early radiation of angiosperms (Sauquet et al., 2003), while *Anaxagorea* is the most basal genus of Annonaceae (Doyle and le Thomas, 1996; Doyle et al., 2004; Chatrou et al., 2012; Chatrou et al., 2018). *Anaxagorea* carpels are apocarpous (free) throughout their life history (Deroin, 1988), and each has a notably long carpel stipe (Endress and Armstrong, 2011). The microscopic observation of *Anaxagorea* tissue sections could determine whether there are "axial homologs" in the carpel and provide additional carpel origin evidence.

MATERIALS AND METHODS

Scanning Electron Microscopy and Paraffin Sectioning

Anaxagorea luzonensis flower samples at different floral stages (from early bud to young fruit) were collected from the Diaoluo Mountain, Hainan, China, in July 2017 and *Anaxagorea javanica* from the Xishuangbanna Tropical Botanical Garden, Yunnan, China in May 2017. The gynoecia were isolated and preserved in 70% formalin-acetic acid-alcohol (5:5:90, v/v), and the fixed specimens were dehydrated in a 50% to 100% alcohol series. To delineate the structure and development of the carpel, carpels were removed from the gynoecia, passed through an iso-pentanol acetate series (SCR, Shanghai, China), critically point-dried, sputter-coated with gold, observed, and photographed under a scanning electron microscope (Tescan VEGA-3-LMU, Brno, Czech Republic). Flowers and carpels were embedded in paraffin, serially sectioned into 10–12-µm thick sections, and stained with Safranin O and Fast Green to illustrate the vasculature. The transverse sections were examined and photographed using a bright-field microscope (Olympus BX-43-U, Tokyo, Japan). In addition, longitudinal hand-cut sections were made and observed for a rough check and better understanding of the vasculature.

Topological Analysis of Carpel Vasculature

Consecutive paraffin sections, 12-µm each, of *A. javanica* were stained with aniline blue, examined and photographed after excitation at 365 nm using an epifluorescence microscope (Olympus BX-43-U, Tokyo, Japan) and a semiconductor refrigeration charged coupled device (RisingCam, MTR3CMOS). Forty-five images were selected equidistant from the 423 sections taken for the 3D reconstruction. The figures were organized according to the vascular bundle outlines of the sections by using Adobe Photoshop CC 2017 and Illustrator CC 2017. The xylem and phloem contours were manually drawn, extracted as paths with the pen tool, and exported in DWG format. The DWG files were imported into 3Ds Max 2016 and sorted according to the distance and order of the sections. The paths were converted to Editable Spline curves to generate the basic modeling contour. The Loft command of Compound Objects was used to get the shape of the Editable Spline, and a complete 3D carpel vasculature model was generated.

RESULTS

Gynoecium Structure and Carpel Organogenesis

The flowers of two study species were trimerous with a whorl of sepals, two morphologically distinct whorls of petals, and numerous stamens (and inner staminodes of *A. Javanica*) (**Figures 1A–D**).

A. luzonensis usually exhibits two to four completely separate carpels (**Figures 1A, G**). The carpel primordia are almost hemispherically initiated and larger than the stamen primordia (**Figure 1F**). Each carpel consists of a plicate zone, a very short ascidiate zone (**Figures 3G, 5I, J**), and a long, conspicuous stipe (**Figure 2F**). The carpel stipe ontogenesis at the early stages of the carpel (**Figure 2B**). The continuous growth of the flanks on the ventral side of the young carpel triggers its early closure; however, the closure does not extend to the base of the carpel, where the carpel stipe occupied (**Figure 2C**). Subsequently, the dorsal region of each carpel thickens markedly, and the stigma forms (**Figures 2D, E**). At anthesis, the carpels are widest at the basal region with an arch on the abaxial side. The carpel stipe remains elongate, accounts for approximately a quarter of the carpel length at anthesis, and continues to elongate during the fruiting stage (**Figure 1F**). Each carpel has two lateral ovules with the placentae at the ovary base (**Figures 3H, 5L**).

A. Javanica exhibits a multicarpellate gynoecium (**Figures 1B, J**). The carpels are completely separate and appear whorled at initiation (**Figure 1I**); as the carpel volume increases, the whorled structure becomes less obvious because the space in floral apex becomes limited. Each carpel consists of a plicate zone and a conspicuous carpel stipe (**Figure 2J**) but lacks the short ascidiate zone. The carpel stipe ontogenesis in the early stages (**Figure 2H**) and remains elongate during the flowering and fruiting stages (**Figures 1D, 2I–J**). Each carpel has two lateral ovules.

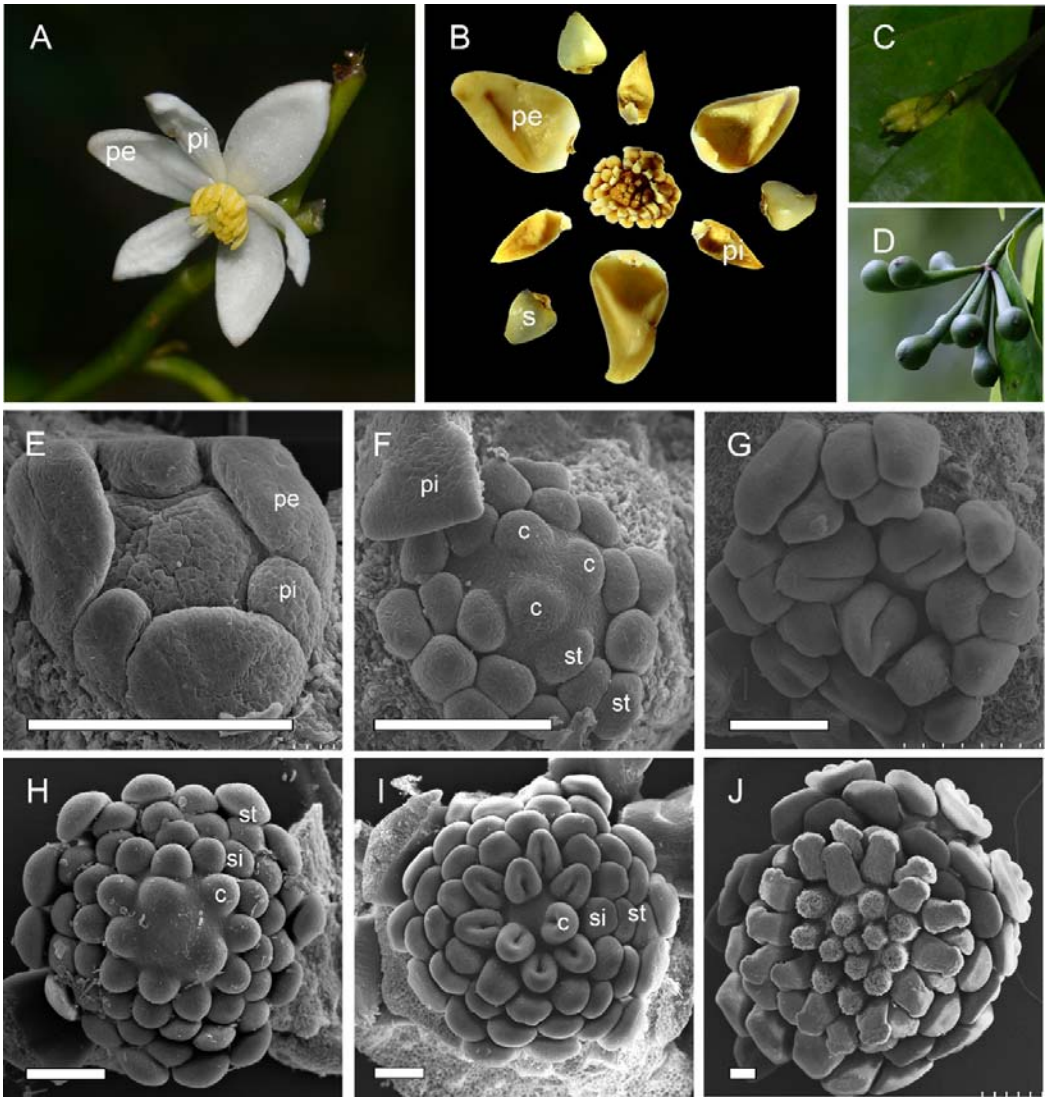

FIGURE 1. Floral morphology and gynoecium development in two *Anaxagorea* species. **(A)** *Anaxagorea luzonensis* flower. **(B)** *Anaxagorea javanica* flower. **(C)** Young *A. luzonensis* fruit. **(D)** Mature *A. javanica* fruit. **(E–G)** *A. luzonensis* floral development. **(H–J)** *A. javanica* gynoecium development. s, sepal; pe, outer petal; pi, inner petal; st, stamen; si, staminode; c, carpel. Scale bars =200 μm.

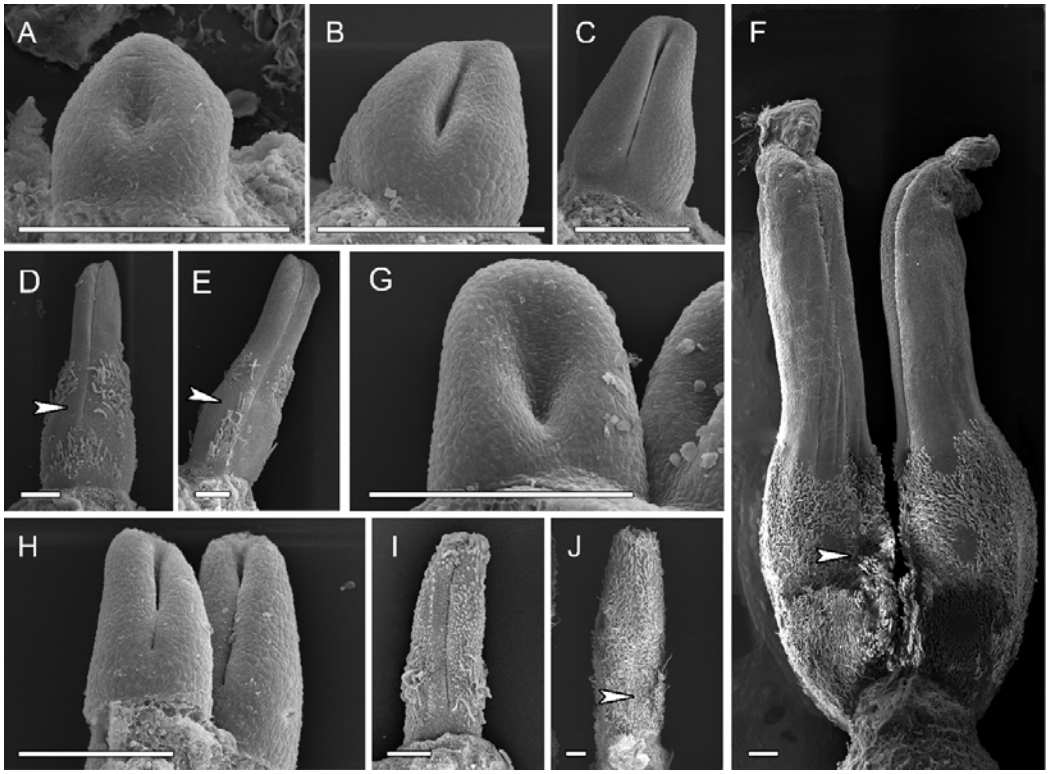

FIGURE 2. Carpel organogenesis in two *Anaxagorea* species.
(A–F) *A. luzonensis.* **(A)** Carpel primordia. **(B–C)** Carpel stipe emergence. **(D–E)** Carpel thickening and stigma formation, showing carpel stipe elongation. **(F)** Mature carpels. **(G–J)** *A. javanica* shows similar carpel developmental features to changes depicted in **A–E, F**. Ventral slit end indicated by arrows. Scale bars = 200 μm.

Vasculature from Receptacle to Carpel

In the *A. luzonensis* cross-sections, the receptacle base presented a hexagon of 18 bundles from the pedicel stele (**Figure 3A**). The hexagon had six breaks, which built up a crown of the cortical vascular system to supply the sepals and the two whorls of petals and the stamens (**Figures 3B**). The central stele, composed of 18 bundles, finally broke into two 9-bundle groups at the floral apex and ran into the 2-carpel gynoecium (**Figures 3C, D**). Each group of nine bundles assembled as a basal ring around the parenchyma at each carpel base (**Figures 3E**). At the slightly upper part of each carpel, several bundles emerged on the lateral side, and the basal ring broke, from which the dorsal bundle separated and the lateral bundles reorganized into two groups of lateral bundle complexes (**Figures 3F**). In each of the lateral bundle complexes, the adjacent bundles tended to join, assembling into an amphicribral pattern (the xylem surrounded by the phloem) **Figure 3G**. Below each placenta, each of the amphicribral lateral bundle complexes transformed into a set of "C"-shaped lateral bundle complexes, from which the ovule bundles separated, while the other bundles ran into the ovary wall. There were no horizontal connections between the dorsal and other bundles (**Figure 3H**).

The pseudosteles at the base of the *A. Javanica* receptacle were triangular, with ~ 45 bundles. The outer six cortical traces were cylindrical and served the sepals and petals (**Figures 4A, B**). At a slightly higher level, the androecial bundles emerged and served the stamens by repeated branching, and the staminode bundles emerged as a crown around the central stele (**Figure 4C)**. Before entering the gynoecium, the central stele enlarged and broke up into ~ 70 bundles to supply the nine carpels, and each carpel was served by 7–10 bundles (**Figures 4D–E**). The vascular bundle arrangement was similar to ascending sections in *A. luzonensis*, with the basal ring and amphicribral lateral bundle complexes presented in each carpel (**Figures 4F–H**).

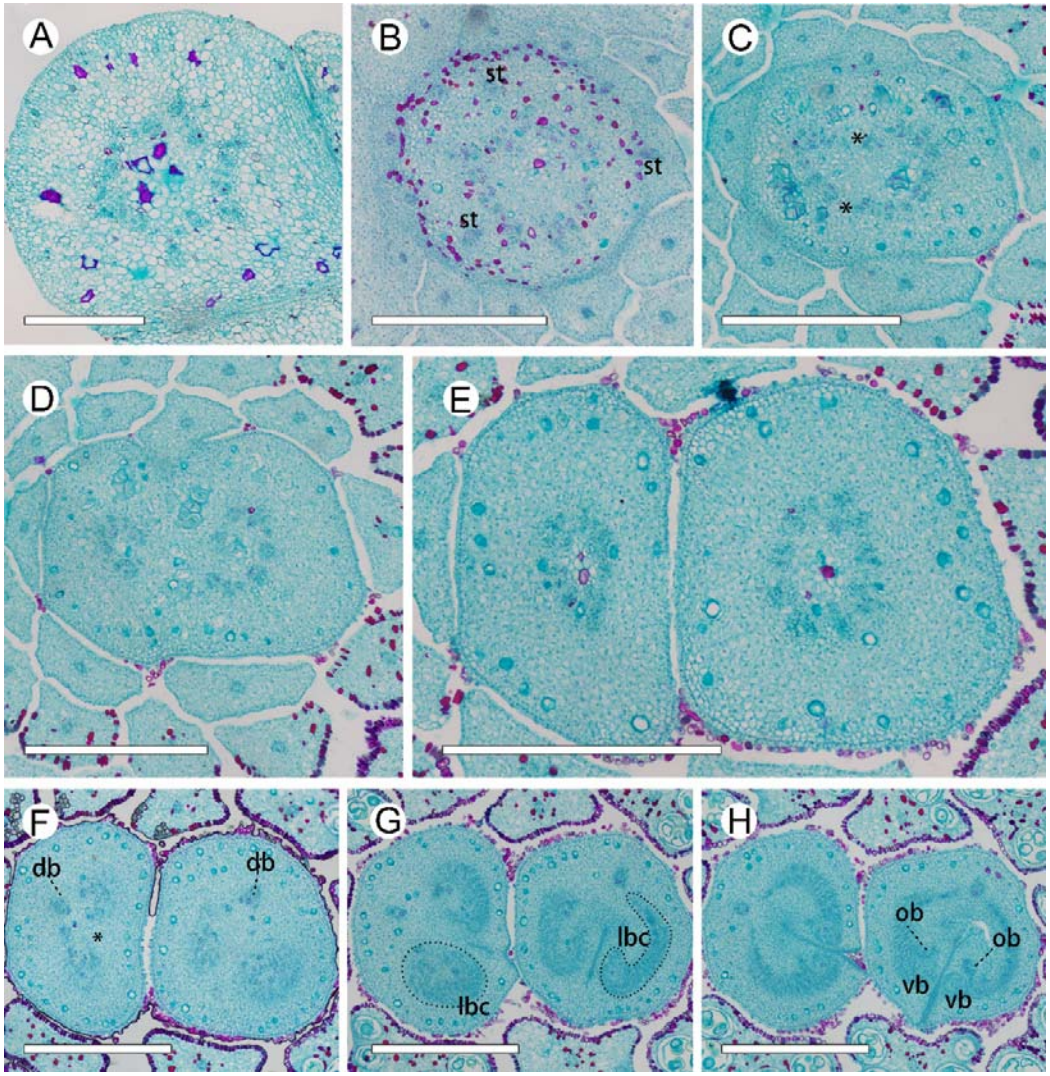

FIGURE 3. Ascending paraffin transections of *A. luzonensis* flower. **(A)** Base of receptacle. **(B)** Mid-section of androecia, showing stamen bundles and central stele. **(C)** Top of receptacle, showing central stele divided into two groups (* marked the breaks). **(D)** Bundles from the central stele enter carpels. **(E)** Base of carpels, showing basal ring. **(F)** Upper part of carpel stipes, showing the basal ring breaks (marked as *). **(G)** Bottom of ovary locule, showing amphicribral lateral bundle complexes (left) and "C"-shaped lateral bundle complexes (right). **(H)** Base of ovary locule. st, stamen; db, dorsal bundle; lbc, lateral bundle complex; vb, ventral bundle; ob, ovule bundle. Scale bars = 500 μm.

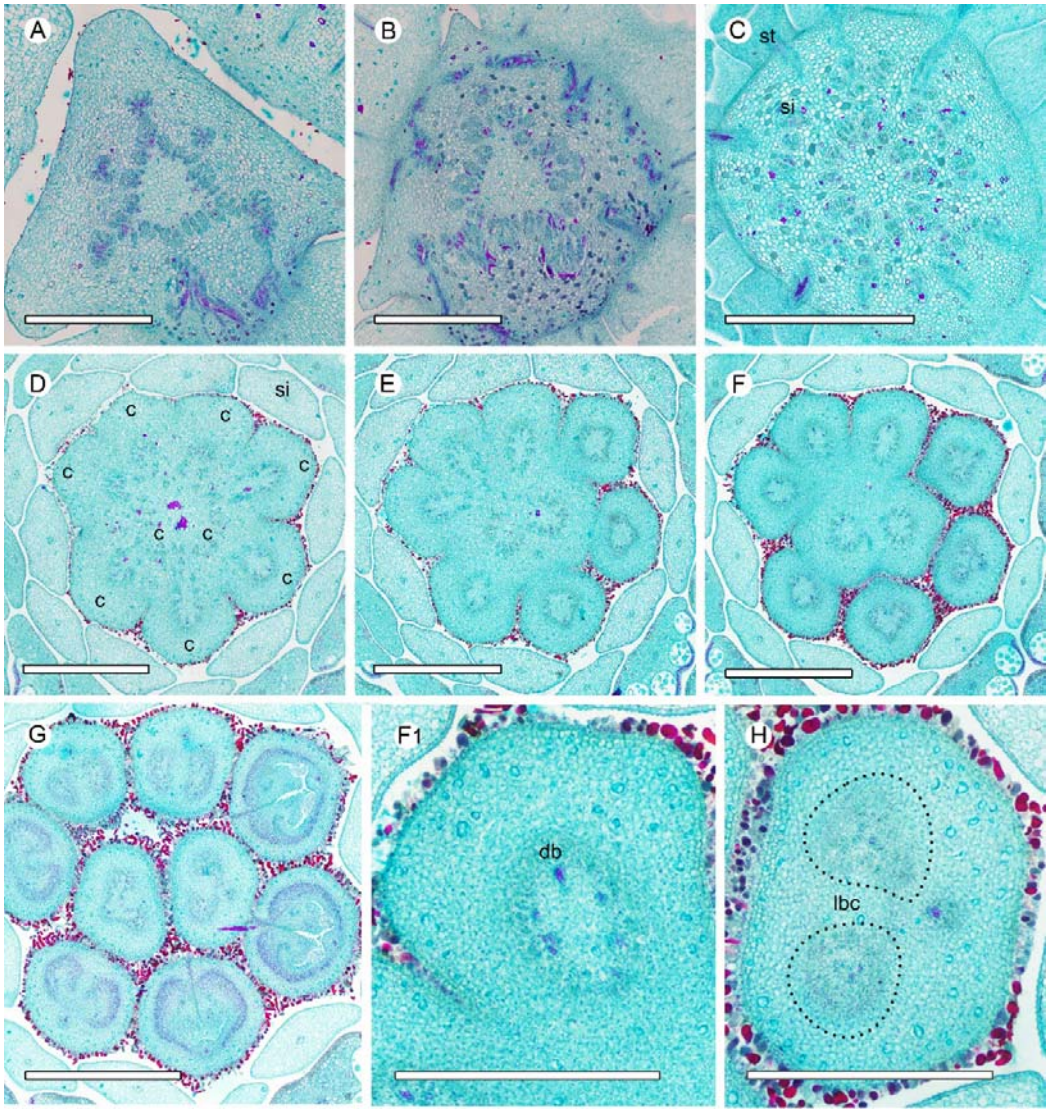

FIGURE 4. Ascending paraffin transections of *A. javanica* flower. **(A)** Base of receptacle, showing six groups of vascular bundles and sepal connections. **(B)** Points of petal connection to receptacle, showing perianth bundles. **(C)** Androecial bundles serving stamens by repeated branching. **(D–E)** Base of gynoecium, showing enlarged central stele breaks and bundles distributed into carpels. **(F–G)** Carpel vasculature at different positions. **(F1)** Detailed view of **(F)**, showing basal ring of carpel. **(H)** Amphicribral lateral bundle complexes in carpel. st, stamen; si, staminode; c, carpel; db, dorsal bundle; lbc, lateral bundle complex. Scale bars = 500 μm.

3D-Reconstruction of Carpel Vascular Topology

At the base of a mature *A. luzonensis* carpel, 15 discrete bundles were arranged in a radiosymmetric pattern, forming a basal ring around the central parenchyma (**Figure 5A**). At the slightly upper part, the basal ring curved inward on the ventral side and broke away from the invagination (**Figures 5B, C**). The bundles (except the dorsal) divided into two groups on each side of the carpel, each forming a lateral bundle complex, which was also ring-arranged. At the flowering stage, the lateral bundle complexes corresponded to the above-mentioned sections of the amphicribral complexes (**Figures 5D–F**). Below each placenta, bundles of each lateral bundle complex broke up on the dorsal side and transformed into a "C"-shaped lateral bundle complex (**Figures 5G, H**). The bundles on the ventral side of each lateral bundle complex gathered together (excluding the ventral bundle) and entered each ovule, while other bundles entered into the ovary wall. The ovule bundles are amphicribral. (**Figures 5I–L**).

Consecutive cross-sections of *A. Javanica* were similar in vasculature to those of *A. luzonensis* (**Figures 6A–D**). The base of the mature *A. Javanica* carpel exhibited 16 distinct bundles forming the basal ring (**Figure 6A, F**). The 3D model showed that (1) the basal ring and lateral bundle complex were cylindrical (**Figures 6F, H)**. (2) The ovules were fed directly by bundles from the base of the carpel. (3) Each ovule bundle was formed from several non-adjacent lateral bundles distributed both relatively dorsally and laterally, and in which two bundles of them that fed each ovule were joined on the ventral side (**Figures 6G–I)**. (4) The dorsal bundle remained independent throughout ontogenesis, without any link to other bundles (for details, please refer to the supplemental data).

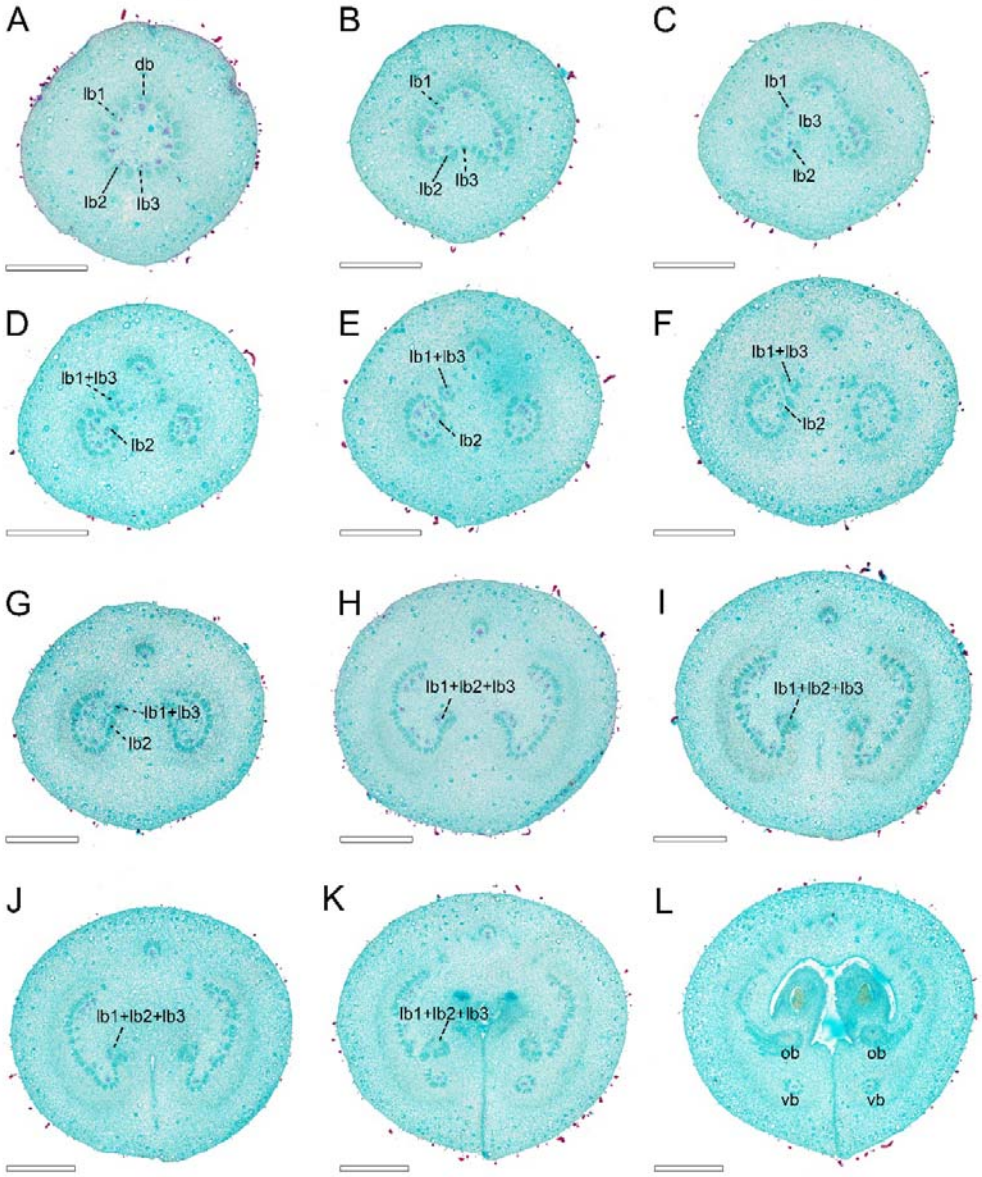

FIGURE 5. Ascending paraffin transections of mature *A. luzonensis* carpel. **(A)** Carpel base, showing basal ring. **(B–C)** Basal ring breaks on ventral side. **(D–F)** Ascending carpel stipe sections, showing lateral bundles reconstituted to two sets of ring-arranged lateral bundle complexes. **(G–H)** Top of carpel stipe, showing "C"-shaped lateral bundle complex. **(I–K)** Below ovary locule, showing formation of ovule bundles. **(L)** Base of ovary locule. db, dorsal bundle; lb, lateral bundle; vb, ventral bundle; ob, ovule bundle. Scale bars = 500 μm.

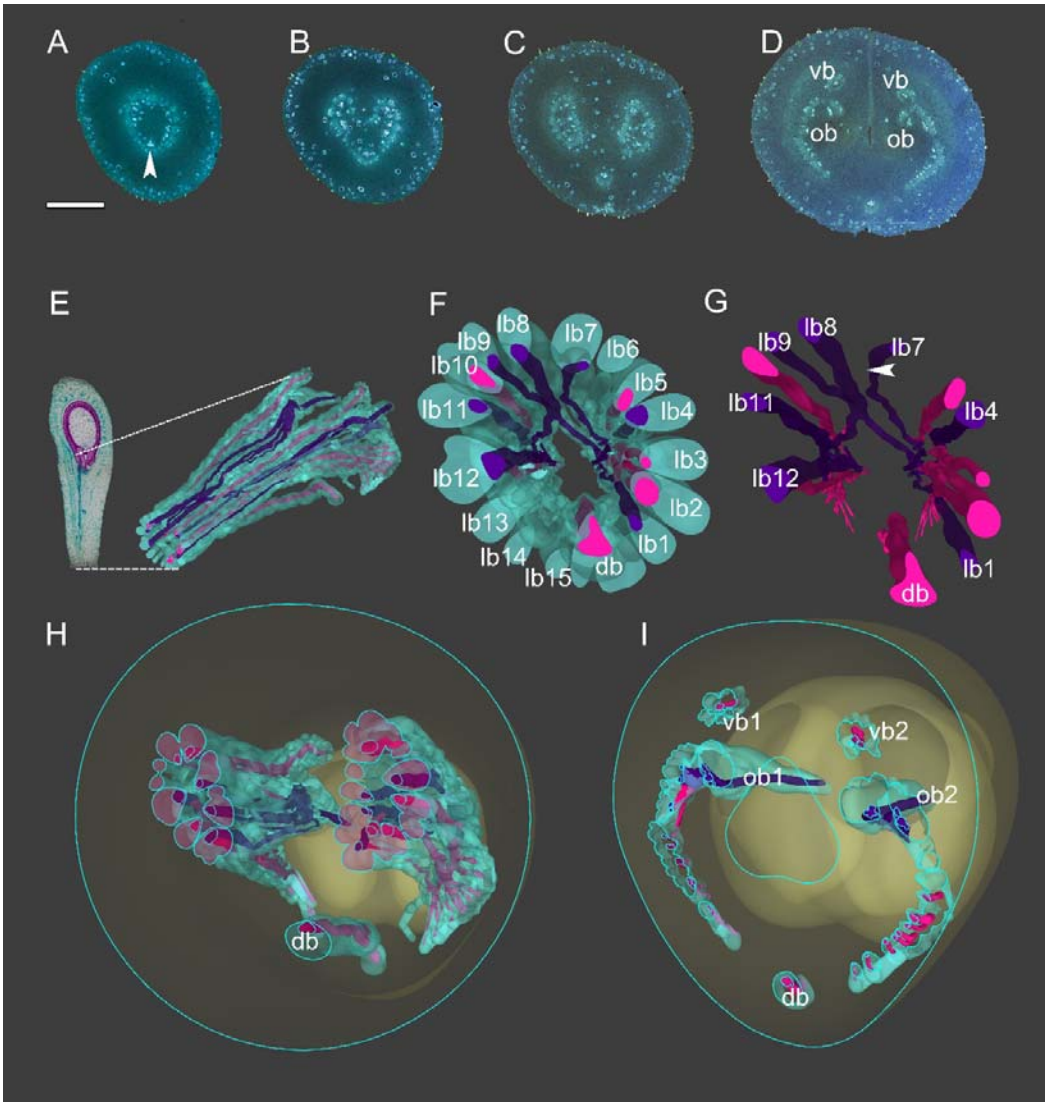

FIGURE 6. 3D construction of *A. javanica* vasculature.

Bundle outlines colored green, xylem red, and purple, among which bundles associated with ovule bundles are colored purple. **(A–D)** Aniline blue-stained *A. javanica* sections for modeling. **(E)** Longitudinal section of mature *A. javanica* carpel (left) and 3D vasculature model, dotted lines on longitudinal section indicate vasculature position in carpel. **(F)** Perspective from base of carpel vasculature. **(G)** Perspective from base of carpel (xylem only). The arrow indicates the intersection of two lateral bundles which fed two ovules. **(H)** Cross-section of 3D model corresponding to **(C)**, showing ring-arranged lateral bundle complexes. **(I)** 3D model section showing distribution of vascular bundles at base of ovary. db, dorsal bundle; vb, ventral bundle; ob, ovule bundle, lb, lateral bundle. Scale bars = 500 μm.

DISCUSSION

In this study, 3D reconstruction was used for the first time to reveal the *Anaxagorea* carpel vasculature, providing an analytical solution to the complex spatial relationship of floral organ vasculature. It is also the first time that the basal ring and the ring-arranged lateral bundle complex in the carpel have been reported. Observations on the continuous changes in vasculature from the receptacle to the carpel showed that the ring-arranged vasculature pattern was topologically continuous and repeatedly presented in the pedicel, the receptacle, the base of the carpel, and the placenta.

Anaxagorea Carpel Organogenesis

Peltate carpels have been suggested to be plesiomorphic in Annonaceae (Deroin, 1988; Igersheim and Endress, 1997; Surveswaran et al., 2010; Couvreur et al., 2011) and in some studies, *Anaxagorea* carpels have been reported to exhibit an ascidiate base (Deroin, 1988), while they have been described as completely plicate in others (Endress and Armstrong, 2011). In this study, floral organogenesis revealed that the carpel stipe emerges from the base of *A. luzonensis* and *A. javanica* carpels in the early stages of carpel development and elongate with the development of the carpel. In the flowering stage, the ventral slit of *A. luzonensis* terminates close to the base of the ovary locule, resulting in a very short ascidiate zone, while in *A. javanica*, it may continue below the ovary locule. These variations might suggest a transformation from peltate to plicate carpels in this genus. The specific carpel stipe of *Anaxagorea* provides a buffer for the drastic changes in carpel base vasculature and makes it an ideal material for exploring the possible axial homologous structure in the carpel; whereas in most apocarpous angiosperms, the carpel base is very close to the ovary locule and the vascular bundle in this area tends to be simplified [e.g., *Michelia* (Tucker, 1961); *Sagittaria* (Kaul, 1967); *Drimys* (Tucker, 1975); *Illicium* (Robertson and Tucker, 1979); *Brasenia* (Endress, 2005)].

Anaxagorea Floral Vasculature

Previous studies have reported that the Annonaceae gynoecium is fed by an enlarged central stele, and each carpel is usually fed by three bundles, one median and two lateral (Deroin, 1989; de Craene, 1993; Deroin and Norman, 2016; Deroin and Bidault, 2017). However, in *A. luzonensis* and *A. javanica*, the number of vascular bundles that fed the carpel during anthesis is significantly more than 3, regardless of the number of carpels. The bundles entering the carpel are arranged in a radiosymmetric pattern, and this pattern maintains a spatiotemporal continuity throughout the carpel stipe. Considering that radiosymmetric vasculature is a universal feature in vascular plant stems (Metcalfe and Chalk, 1979; Evert, 2006; Beck, 2010; McKown and Dengler, 2010; Evert and Eichhorn, 2011), and the vascular bundles enter the *A. luzonensis* gynoecium is consistent with the central stele in numbers, it is plausible that the basal ring is homologous to the axial structures, such as the pedicel bundles and the central stele.

It has been reported that in Annonaceae, the ovules are served by separate vascular bundles branching directly from the dorsal bundles [e.g., *Cananga* (Deroin and Le

Thomas, 1989); *Deeringothamnus* (Deroin and Norman, 2016); and *Pseudartabotrys* (Deroin and Bidault, 2017)] or from relatively dorsally positioned bundles of the lateral network of bundles [e.g., *Meiocarpidium* (Deroin, 1987); and *Ambavia* (Deroin and Le Thomas, 1989)]. Whereas in *Anaxagorea*, the ovules have been described as being served by the lateral bundle complex from the base of the carpel [e.g., *A. luzonensis* (Deroin, 1997); *A. crassipetala* (Endress, 2011)]. Our study showed that the topological structure of the ring-arranged lateral bundle complexes plays a key role in forming the ovule bundles and that it causes the non-adjacent bundles from the relatively dorsal and ventral sides to approach each other and merge. The dorsal bundle remained independent throughout, and there were no horizontal connections between it and the lateral bundle complexes. The ventral bundle took part in forming the spatial ring- arrangement of the lateral bundle complexes; however, it did not connect with the other bundles. These results indicate that the ovule and ovary wall bundles are relatively independent in origin. This conclusion is also supported by the findings of previous studies on floral meristems and chromosomal chimera cells, indicating that the placental tissue developed independently of the septum and carpel wall (Satina and Blakeslee 1941, 1943; Satina 1944, 1945). Another previous study reported that the ovule/placenta and ovary wall are controlled by two distinct, exclusive sets of genes (Roe et al. 1997; Wynn et al. 2014; Angenent et al. 1995).

Observation of the different development stages of the *Anaxagorea* carpel showed that the amphicribral bundle complexes in the placenta developed into the ring-arranged lateral bundle complexes with carpel maturation. In the vasculature development, the amphicribral bundles could be discrete inversely collateral bundles near the point of fusion, because their xylem portions need to approach each other before they become concentric (Endress, 2019). Based on derivation, the amphicribral bundles are frequently observed in small branches of early land plants, monocots, or immature young branches of dicots (Fahn, 1990). In *Magnolia* (Liu et al., 2014) and *Michelia* carpels (Zhang et al., 2017), the amphicribral bundles were observed supplying the ovules, and they emerged from the cortical amphicribral bundles. In *Actinidia* (Guo et al., 2013) and *Dianthus* pistils (Guo et al., 2017), the amphicribral bundles have been reported in the placenta and are distinct from the collateral bundles in the carpel wall. If the carpels are indeed derived from the integrated axial-foliar complex, that could explain why the amphicribral bundles are widely present in angiosperm placentae and funiculi [e.g., *Papaver* (Kapoor, 1973), *Psoraleae* (Lersten and Don, 1966), *Drimys* (Tucker, 1975), *Nicotiana* (Dave et al., 1981), *Whytockia* (Wang and Pan, 1998), *Pachysandra* (Von Balthazar and Endress, 2002)].

Based on previous studies on the comparison of flower organ morphology and vascular anatomy, our study further supports the view that the carpel originates from the integrated axial-foliar complex. However, the investigations in the present study were limited to a single genus. It is difficult to find similar morphological features in most angiosperms because the carpel vasculature is often simplified. Under natural conditions, terminal or axillary meristems can produce leaves and branches; however, the leaf meristem can only produce leaflets. If the carpel evolved from secondary reproductive shoots with foliar appendages, how does the female reproductive identity

program interact with the meristems, what genetic pathways might interact with the development of the placenta and ovule formation, and are they shared in seed plants? To determine the structure from which carpels originated, we need to further understand the molecular mechanisms of the various characteristics of carpel evolution.

AUTHOR CONTRIBUTIONS

YL planned and designed the research, performed the experiments, collected the images, drew the illustrations, and wrote the article; WD performed the experiments and complemented the writing; YC developed the 3D model; SW complemented the writing; X-FW supervised the experiments and complemented the writing.

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