STUDIES

Homology and functions of inner staminodes in Anaxagorea javanica (Annonaceae)

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Abstract

Inner staminodes are widespread in Magnoliales and present in Anaxagorea and Xylopia, but were lost in the other genera of Annonaceae and have no counterparts in derived angiosperms. The coexistence of normal stamens, modified stamens and inner staminodes in Anaxagorea javanica is essential to understand the homology and pollination function of the inner staminodes. Anaxagorea javanica was subjected to an anatomical study by light and scanning electron microscopy, and the chemistry of secretions was evaluated by an amino acid analyser. Inner staminodes have a secretory apex, but do not have thecae. They bend towards either tepals or carpels at different floral stages, and function as a physical barrier preventing autogamy and promoting outcrossing. At the pistillate phase, the exudates from the inner staminodes have high concentration of amino acid, and provide attraction to pollinating insects; while abundant proline was only detected in stigmas exudates, and supply for pollen germination. Modified stamens have a secretory apex and one or two thecae, which are as long as or shorter than that of the normal stamens. As transitional structures, modified stamens imply a possible degeneration progress from normal stamens to inner staminodes: generating a secretory apex first, shortening of the thecae length next and then followed by the loss of the thecae. The presence of modified stamens together with the floral vasculature and ontogeny imply that the inner staminodes are homologous with stamens.

Keywords: Anaxagorea javanica; Annonaceae; homology; inner staminode; modified stamen; pollination function.

Introduction

The Annonaceae is a pantropical family comprising 107 genera and about 2400 species, which is the most species-rich group of the basal angiosperms (Guo et al. 2017). In Annonaceae, two distinct types of staminodes (sterilized stamens) are consequently identifiable based on their position within the flower (Ronse De Craene and Smets 2001; Ronse De Craene 2007): the inner staminodes situated between the functional stamens and carpels, and the outer staminodes, situated between the petals and stamens, are much more common in Annonaceae (Saunders 2010), appearing in Monanthotaxis (Ronse De Craene and Smets 1990), Fusaea, Uvaria (Van Heusden 1992), Xylopia, Orophea (Keßler 1988) and Pseuduvaria (Su et al. 2008). Inner staminodes are rare in derived groups, but they are widespread in basal angiosperms (Schodde 1969; Endress 1980, 1984; Endress and Lorence 2004; Staedler et al. 2007, 2009; Rohwer 2009; Staedler and Endress 2009), and the presence of inner staminodes may be plesiomorphic within Magnoliales,
although they do not occur in Magnoliaceae, Myristicaceae or Annonaceae other than Anaxagorea and Xylopia (Maas and Westra 1984; Van Heusden 1992; Doyle and Le Thomas 1996; Sauquet et al. 2003; Doyle and Endress 2010). Xylopia is unique in having both outer and inner staminodes and probably represents an independent evolution of staminodes within the Annonaceae, as the genus is not closely allied to any of the other genera with staminodes (van Heusden 1992; Doyle and Le Thomas 1996; Johnson and Murray 1988).

Anaxagorea, the only genus in Anaxagoreoeideae, comprises around 30 species with a disjunct distribution in tropical America and Southeast Asia (Maas and Westra 1984, 1985; Maas et al. 1986; Van Heusden 1992; Chatrou et al. 2012). Studies combining morphological and molecular analysis indicated that Anaxagorea is the sister group of the remaining taxa in Annonaceae (Doyle and Le Thomas 1994, 1996; Doyle et al. 2000; Sauquet et al. 2003). Most species in Anaxagorea (except for A. breuipedicellata and A. luzonensis) possess inner staminodes, which may or may not have a secretory apex (Maas and Westra 1984, 1985; Scharaschkin and Doyle 2005, 2006; Endress and Armstrong 2011).

Since the inner staminodes have no counterparts in more advanced clades of angiosperms (Endress 1984), there have been few reports concentrating on the origin, function and evolution of the inner staminodes. In Anaxagorea, the closed pollination chamber during anthesis makes it difficult to directly observe the inner staminodes and movements of pollinators inside (Webber 2002; Braun and Gottsberger 2011; Gottsberger 2016). Saunders (2010) interpreted that inner staminodes with secretory apex were homologues of stigmas, by comparing the scanning electron micrographs of stamens, inner staminodes and carpels, based on research of Scharaschkin and Doyle (2006). However, it seemed insufficient to define the homology of the extremely specialized inner staminodes without structural data. Although some reports postulated that inner staminodes act as a physical barrier and prevent the possibility of autogamy, experiments to clarify their function were very few and indirect (Maas-van de Kamer 1993; Webber 2002; Saunders 2010; Gottsberger 2016). Based on only the absence of gnawing marks on the staminodes and stigmas and without any analysis of the chemical composition of the exudate, Gottsberger (2016) speculated that mucus secreted by the inner staminodes or stigmas might be involved in offering rewards for pollinators (Webber 1996; Braun and Gottsberger 2011; Teichert et al. 2011).

In the present studied species Anaxagorea javanica, the outer stamens are normal with two thecae and lack a secretory apex. The inner stamens are sterile with a secretory apex and lack thecae, which is consistent with the previous reports (Maas and Westra 1985; Scharaschkin and Doyle 2006). Furthermore, A. javanica has one or two modified stamens with a secretory apex and one or two thecae, located between the normal stamens and the inner staminodes. Anaxagorea javanica proves to be an excellent species to investigate the homology and functions of the inner staminodes. In the present study, we carefully documented floral phenology and pollinators of A. javanica, and analysed the content of exudates from inner staminodes and stigmas, respectively, at the pistillate and staminate phase, with the aim to elucidate the pollination function of the inner staminodes in A. javanica. We also tried to clarify the homology of inner staminodes based on the floral anatomy and development, and histological tests comparing the inner staminodes with the modified stamens, normal stamens and carpels. The study provides new insights to understand the evolutionary trends of stamens in Annonaceae, even in Magnoliaceae.

Materials and Methods

Study site
Observations were made in Xishuangbanna Tropical Botanical Garden of the Chinese Academy of Sciences (21°52′N, 101°39′E) in China, Yunnan province. Mature flowers of A. javanica were fixed immediately in FAA (70 % alcohol, formaldehyde and glacial acetic acid in a ratio of 18:1:1).

Phenological observations
Field-based experiments of pollination ecology of A. javanica were conducted from September 2018 to October 2019, covering two flowering periods. Thirty flower buds from two individuals of A. javanica were tagged and monitored every day until petal opening. Photographic observations were then taken at 2-h intervals afterwards until the end of the staminate phase. The pollinators were observed and photographed using a Zeiss-Smartzoom5 ultra depth of field stereoscopic microscope (Zeiss, Germany).

Light microscopy
Fixed flower buds, stamens, modified stamens, inner staminodes and pistils were dehydrated in an ethanol series, embedded in paraffin wax and then sectioned at 8 μm. After staining with haematoxylin, Safranin O and Fast Green, iodine-potassium iodide, mercury bromophenol blue and Sudan black, these sections were observed and photographed using a Leica-DM5500B light microscope (Leica, Germany).

Scanning electron microscopy
Fixed series of flower buds, stamens, modified stamens, inner staminodes, carpels and pollen grains from the normal stamens and the modified stamens were dehydrated using an ethanol series to absolute ethanol. The materials were then critical point-dried, mounted onto scanning electron microscope (SEM) stubs using double-sided adhesive tape and coated with platinum using a JFC-1600 coater. The dried materials were examined using a SEM JSM-6360 cold field emission at 20 kV (JEOL, Tokyo, Japan).

Pollen germination
Pollen grains were collected from the normal and modified stamens in 10 staminate-phase flowers from two different individuals, and incubated in 100 μL of 10 % sucrose solution on cavity slides within closed Petri dishes for 24 h at ambient temperature (Dafni 1992).

Stigmatic exudate chemistry
Amino acid extraction methods were adopted from the procedure described by Lau et al. (2017). The amino acid composition of the exudates from stigmas and staminodes from the pistillate phase and staminate phase in A. javanica were similarly determined using exudate pooled from 10 pistillate-phase and staminate-phase flowers. After being vacuum dried and weighed, 300 μL 0.1M HCl was added to Eppendorf tube containing exudates and ethanol was added to achieve a final concentration of 80 % alcohol. The samples were then vortexed and incubated for 30 min at ambient temperature, and then centrifuged at 3000 rpm for 20 min. The supernatant was vacuum-dried and 200 μL 0.1 M HCl added, then.
filtered through a 0.22-μm membrane and injected into a Sykam S433 amino acid analyser (fitted with a 150 × 4.6 mm column).

**Results**

**Floral phenology**

*Anaxagorea javanica* exhibits an annual mode of flowering and has its flowering period from the beginning of September to the end of October. Here, observations are grouped into three phases, viz. pistillate phase, interim phase and staminate phase.

Pistillate phase (ca. 12 h) (**Fig. 1A–C**): The petals open just slightly by narrow slits at ca. 1400 h (**Fig. 1A**). Flowers enter the pistillate phase where stigmatic receptivity increases from late afternoon (ca. 1700 h) to the next day (ca. 500 h; **Fig. 2**). During this stage flowers emit a fruity, banana-like odour, which is particularly strong in the late afternoon to early evening, around 1700 to 2000 h (**Fig. 1B**). Stigmas turn greenish-yellow and the receptivity is indicated by the secretion of a sticky exudate upon the stigmas. At the same time, the staminodes are covered by a gelatinous exudate and bend towards the stamens and away from the pistils (**Fig. 1C**).

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**Figure 1.** Floral phenology of *A. javanica* during anthesis. (A) The petals of *A. javanica* are slightly opened. (B) Nitidulid beetles visiting gaps between petals at the pistillate phase. (C) The inner staminodes bend towards the stamens and away from the pistils at the pistillate phase. (D) Flower of *A. javanica* at the interim phase. (E) The inner staminodes start bending towards pistils at the interim phase. (F) Flower of *A. javanica* at the beginning of the staminate phase. (G) The inner staminodes are curved over the stigmas at the staminate phase. (H) The petals are widely opened at the end of staminate phase; pollinator (arrow) enters a gap between the petals. (I) There are several *Colopterus* spp. (Nitidulidae) dusted with pollen inside of the flower at the end of the staminate phase. C, carpel; S, stamen; St, staminode.
Interim phase (ca. 11 h) (Fig. 1D and E): The odour of the flowers decreases from ca. 500 h to ca. 1600 h and almost no odour is emitted towards the end of the interim phase (Fig. 2). The stigmatic receptivity of the flowers is reduced during this stage, and the stigmas turn to yellowish brown and become dry (Fig. 1F). The exudate of the inner staminodes slowly dries up as they start to bend towards and enclose the pistils (Fig. 1B).

Staminate phase (ca. 3 h) (Fig. 1F–I): Flowers begin to emit a sweet odour ca. 1600 h. The inner staminodes eventually enclose the pistils and then the thecae dehisce, providing large amounts of pollen for pollinators (Fig. 1G and H). Around 1900 h at the end of the staminate phase, the pollination chamber momentarily opens, and petals abscise from the receptacles in less than a minute, and then petals, stamens and inner staminodes are gradually lost (Figs 1H, I and 2).

One or two nitidulid beetles of the genera *Colopterus* or *Epuraea* were observed arriving on the petals at the staminate phase (around 1630 h), entering the pollination chamber, and then departing the flowers at the end of the staminate phase (around 1900 h; Figs 1F, I and 3A–D). They were later found on the petals of another flower at the pistillate phase (around 1800 h; Fig. 1B). Large numbers of pollen grains stick to the abdomens and legs of the *Colopterus* spp., which were covered with a great deal of bristly hairs (Fig. 3E–G). Abundant pollen was observed in *Epuraea* spp. on the dorsal shell and ventral tail covered with bristles (Fig. 3H–J).

Stamen type and morphology

Three types of stamens were observed in *A. javanica*, viz. normal stamens, modified stamens and inner staminodes. In an individual flower, around 33–36 normal stamens are located in the outer portion of androecium (Fig. 7A). They are laminar and have two thecae, extrorsely dehisce, and completely embedded within a tongue-shaped connective (Fig. 7P and Q). About 15–20 staminodes are located in the inner portion of the androecium (Figs 1C, E, 7B and C). The long and narrow inner staminodes...
are S-shaped and lack thecae (Fig. 7R and S). The apex of the inner staminodes is densely covered by secretory structures (Figs 7O and 8F). In each flower of A. javanica examined, only one or two modified stamens were found between the normal stamens and the inner staminodes, with the apex covered by secretory structures and the theca dehiscing extremely (Fig. 4A-J). In the individual flowers with only one modified stamen, the two thecae are as long as or one-fourth to three-fourths shorter than those in the normal stamens (Fig. 4A, D and E). In the flowers with two modified stamens, they are always adjacent to each other, one is similar in organization to a single modified stamen (Fig. 4B-E), the other has only one much smaller theca (usually one-fifth the length of the theca in the normal stamens) (Fig. 4B, C and F) and was not found on its own in any individual flower. Pollen grains from both the normal stamens and the modified stamens are ellipsoidal monads and the exine ornamentation is psilate (Fig. 4K-F). The pollen germination rate is 46 % in normal stamens, while the pollen germination rate can attain 51 % in modified stamens.

Floral vasculature

Serial sections through the receptacle of A. javanica reveal the pattern of vasculature supplying each perianth organ. The stele in the pedicel (Fig. 5A) diverges into six groups of vascular bundles at the base of the receptacle (labelled B1-6 in Fig. 5B). Three of these bundle clusters (B1, B3 and B5 in Fig. 5B) fuse with the median bundle of the sepal (MB in Fig. 5C-E) and the vasculature supplying the inner petals (Fig. 5H and I); the other three groups (B2, B4 and B6 in Fig. 5B) connect with the vasculature of the outer petals (Fig. 5F and G) and two lateral bundles feeding adjacent sepal (LB in Fig. 5D and E). Each stamen and staminode receives only one vascular trace (Fig. 5J-N). Each of the carpels has three distinct longitudinal vascular bundles, a dorsal bundle and two ventral ones, and there are horizontal connections between them (Fig. 5O and P).

The longitudinal sections of A. javanica show the positions of carpels, inner staminodes, stamens, petals and sepalas (Fig. 6A). The vasculature of the inner staminodes splits from the basal traces of the free stamen bundles (Fig. 6C-E), and then the inner staminode bundles fuse with middle bundles supplying carpels (Fig. 6F-H). With the ventral bundles ending in the apex of the ovary, the dorsal carpellary bundles follow the narrowing of the ovary and enter the style where they continue acropetally through the style as three distinct bundles before ending blindly in the base of the stigma (Fig. 6I-K). The median bundle supplies the ovary, which has two lateral ovules with the placenta at the base (Fig. 6F and I).

Staminal and pistillar ontogeny

Following the forming of normal stamen primordia, the six inner staminode primordia are initiated (Fig. 7A). Further initiation of the inner staminate (9-14) runs in an irregular sequence on the floral apex in a relatively rapid succession. After the initiation of inner staminodes, 9-15 carpels primordia develop rapidly in an irregular sequence at the centre of the floral apex (Fig. 7B-D), and the anthers of normal stamens begin to differentiate at the same time (Fig. 7B). When all flower organs have emerged, the carpel primordia become horseshoe-shaped with developing concavities on their ventral surface (Fig. 7C). The epidermal cells of the inner staminodes are unequal in size and somewhat protrude at the apex, making the surface uneven, while in the normal stamens, the surface of the connective appendage apex is smooth without trichomes (Fig. 7D and F). At this stage, both the capitate and peltate trichomes originate from a single protodermal cell, larger than the neighbouring ones of the stigmas (Fig. 7E). With the development of the flower, the differences between the secretory structures at the apex of the inner staminodes and carpels become more and more obvious (Fig. 7G-N). The cylindrical stigmas become densely covered with filamentous and capitate peltate trichomes (Fig. 7T).

Trichome development in inner staminodes and stigmas

At an early stage, the glandular cells of the inner staminodes consist of one basal cell, two stalk cells and one head cell, and present large nuclei and dense cytoplasm (Fig. 8A). After one periclinal division forms three stalk cells, the size of glandular cells in the inner staminodes continues to increase at a successive developmental stage (Fig. 8B-E). At maturity, the trichome cells of the inner staminodes are rectangular with a large nucleus and thin cytoplasm (Fig. 8F). The initial cells of capitate and peltate trichomes of stigmas are spherical and all derive from the epidermis (Fig. 8G). The glandular cells of stigmas continue protruding outward and gradually become oblong (Fig. 8H). Two sister cells are formed after the first periclinal division of the glandular cells (Fig. 8I). The top cell undergoes a periclinal division, producing two daughter cells: the stalk cell and the head cell (Fig. 8J). The secretory cells in the head continue to develop vacuoles, and the secretory cells begin to be filled with secretions (Fig. 8K-M). These trichomes of stigmas contain two types: capitate trichomes with one or two basal cells, one to three stalk cells and one head cell (Fig. 8M), together with peltate ones with one basal cell, one stalk cell and a multicellular head (Fig. 8N and O). The secreting multicellular head continues to increase in peltate trichomes (Fig. 8P and Q). The secretory structures at the top of inner staminodes are negative to iodine-potassium iodide but are positive to mercury bromophenol blue and Sudan black, showing them to be rich in proteins and lipids (Fig. 8R-T). Both capitate and peltate trichomes are negative to iodine-potassium iodide (Fig. 8U and V). Capitate trichomes are negative to mercury bromophenol blue (Fig. 8W), while peltate trichome heads are positive to mercury bromophenol blue for rich proteins (Fig. 8X). The subcuticular space of both capitate and peltate trichomes could be stained by Sudan black for total lipids (Fig. 8Y and Z). Because only one or two modified stamens were present per flower, the development of secretory structures could not be detected; only the mature structure was observed, which consists of one basal cell, three stalk cells and one head cell (Fig. 4J).

Exudate chemistry at the apex of staminodes and pistils

Total amino acid concentrations at the apex of the inner staminodes and stigmas are 7576.5 and 3594.5 µg g⁻¹ at the pistillate stage, and 3512.5 and 2161.1 µg g⁻¹ at staminate phase, respectively (Table 1). Nine essential and 22 other amino acids (along with urea) were recorded from the apex of inner staminodes at the pistillate phase: the most abundant of these was asparagine (1549 µg g⁻¹; 20.4 % of total amino acid content), followed by alanine (902.5 µg g⁻¹; 11.9 %). Nine essential and 23 other amino acids were recorded from the stigmas at the pistillate phase: the most abundant of these is alanine (542 µg g⁻¹; 15.1 % of total amino acid content), followed by hydroxyproline (518.5 µg g⁻¹; 14.4 %). Analysis of the apex of inner staminodes and the stigmas at the staminate phase revealed a similar range of amino acids, both with 9 essential and 22 other amino acids recorded. Alanine was found to be the most abundant (946.5 µg g⁻¹; 26.9 %) in the inner staminodes, while urea was found to be
Figure 4. The morphology of modified stamens and pollen grains from the normal stamens and the modified ones. (A–C) Modified stamens located between the normal stamens and the inner staminodes. (A) Flowers with one modified stamen possessing a secretory apex and two shorter thecae than in the normal stamens. (B, C) Flowers with two adjacent modified stamens covered by secretory structures at the apex: one with two thecae of the usual length or shorter thecae, the other with one shorter theca. (D, E) Modified stamens with two shorter thecae than in the normal stamens. (F) Modified stamen with one shorter theca. (G–I) The apex of modified stamens covered by secretory structures. (J) Longitudinal microtome sections of the apex of inner staminodes, showing one basal cell, three stalk cells and one head cell. (K, M) Pollen grains from a normal stamen. (L, N) Pollen grains from a modified stamen. (O) Germinating pollen from a normal stamen producing a pollen tube. (P) Germinating pollen from a modified stamen producing a pollen tube. BC, basal cell; C, carpel; HC, head cell; S, stamen; SC, stalk cell; MS, modified stamen; St, staminode. Scale bars: D–F = 500 μm; G–I = 100 μm; J–L = 50 μm; M–P = 10 μm.
the most abundant (316.8 μg g⁻¹; 14.7 %) in the stigmas. Serine is the second-most abundant amino acid in both inner staminodes (360 μg g⁻¹; 10.3 %) and stigmas (246.2 μg g⁻¹; 11.4 %). The modified stamens are covered by a small quantity of exudates at the pistillate phase (Fig. 4A–I). Additionally, because there are only one or two modified stamens per flower, the exudate is too scanty to be collected for chemistry tests.

Discussion

Staminodes as a physical barrier preventing autogamy

An 11-h non-reproductive interim phase separating the pistillate and staminate phases in *A. javanica* indicates that there is no temporal overlap between pollen presentation and stigmatic receptivity. At the pistillate stage, the inner staminodes bend towards the tepals and away from the pistils at a right angle, such that beetles penetrating the pollination chamber are canalized towards the region of the carpels, where they can deposit pollen stuck to their bodies. When the flowers come into the staminate stage, anthers shed their pollen, and the inner staminodes incline towards the carpels, sometimes grow a bit longer and even partly cover the stigmas. This makes the way free for the beetles to crawl down to the dehisced stamens and receive a new pollen load before leaving the flower. Owing to the movements of the inner staminodes, the beetles are always being concentrated in the right compartment of the pollination chamber. This has also been reported in other species of *Anaxagorea* (*A. brevipes, A. dolichocarpa, A. phaeocarpa*), as well as...
as in Eupomatiaceae, Himantandraceae and Degeneriaceae (Endress 1984; Maas-van de Kamer 1993; Webber 1996, 2002; Braun and Gottsberger 2011; Gottsberger 2016). In _A. javanica_, inner staminodes play a key role in flower–animal interactions and act as a physical barrier to prevent the stigmas from receiving their own pollen. Adding the non-sexual interim phase between stigmatic receptivity and pollen presentation as well, outcrossing in _A. javanica_ is undoubtedly promoted and the possibility of autogamy is limited.

**Exudate from staminodes and stigmas provides different needs**

In both the inner staminodes and stigmas, the content of the exudate is rich in proteins and lipids, but lacks starch. At the pistillate stage, more proteins are in the inner staminodes than in stigmas, and consequently, the total amino acid content in the inner staminodes (7576.5 μg g⁻¹) is more than twice that in the stigmas (3594.5 μg g⁻¹; Fig. 10). It has been reported that 10 amino acids are essential for insects and cannot be synthesized by the insects themselves, and hence, these amino acids must be obtained through their diet (Haydak 1970; Baker and Baker 1975; Baker 1977; Gottsberger et al. 1984). The 10 essential amino acids have previously been recorded in floral nectar. However, in this study, nine of these were detected both in the inner staminode and stigma exudates (only tryptophan is absent). At the pistillate phase, the concentration of the nine essential amino acids in the inner staminode exudate (2060.5 μg g⁻¹) is nearly three times higher than that in the stigma (765 μg g⁻¹; Fig. 10), with inner staminodes bending towards stamens and pollinators penetrating the pollination chamber. This implies that the exudate from inner staminodes could supply the energetic demands of nitrogen for flower pollinators (Nicolson and Thornburg 2007), because of its high amino acid content, without relying on the absence of gnawing marks on inner staminodes as Gottsberger speculated (2016), or the exudate being sticky (Endress 1984; Endress and Hufford 1989; Armstrong and Irvine 1990). Therefore, the exudate from stigmas may be avoided for consumption by flower visitors. At the staminate phase, the inner staminodes eventually enclose the pistils. The total amino acid content in the inner staminode exudate decreases halfway compared to the pistillate phase (7576.5 μg g⁻¹; Fig. 10), and may not be nutritious enough for the insects. The pollinators stayed around the dehisced stamens and nourished themselves on the large amounts of pollen, shed from the normal stamens. Obviously, during anthesis of _A. javanica_, a

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**Figure 6.** Longitudinal sections of a flower of _A. javanica_. (A) Longitudinal section of the _A. javanica_. (B) Petal and septal bundles located in the outer whorl. (C–E) Stamens and inner staminodes bundles have a common origin. (F–I) Inner staminode bundles fuse with lateral carpellary bundles. (J–K) Carpels are vascularized by synlateral and dorsal bundles. C, carpel; S, stamen; St, staminode; Se, sepal; OP, outer petal; IP, inner petal. Scale bars: A, C, F, I = 500 μm; D, E, G, H, J, K = 100 μm.
Figure 7. Staminal and pistillar ontogeny of *A. javanica* observed with a scanning electron microscopy. (A) Initiation of the first whorl of inner staminode primordia (asterisks). (B) Initiation of the second whorl of carpel primordia (asterisks). (C) Horseshoe-shaped carpel primordia with developing concavities on the ventral surface. (D–N) Comparative development of the gynoecium and the staminodes. (D) After the inception of the last carpels, the ventral depression deepens and extends to the tip of each carpel. (E) Carpels covered by trichomes (arrow). (F) The top cells of staminodes become bigger than others. (G) Mature flower bud. (H) Multicellular trichomes produced by division (arrow). (I) Cells at the top of inner staminodes start bulging out. (J) Nine carpels in the flower. (K) Glandular hairs on top of the carpels increase in size (arrow). (L) Cells at the top of the inner staminodes start bulging out. (M) Glandular hairs begin to seal the carpels (arrow). (N) Glandular hairs of the inner staminodes become distinct. (O) Inner staminodes have a secretory apex. (P) A stamen from the dorsal side. (Q) A stamen from the ventral side. (R) A staminode from the dorsal side. (S) A staminode from the ventral side. (T) The cylindrical stigma densely covered with trichomes. C, carpel; S, stamen; St, staminode. Scale bars: A–O, T = 50 μm; P–S = 500 μm.
Figure 8. The development of trichomes on the inner staminodes and carpels. (A–F) Longitudinal microtome sections of the apex of inner staminodes, showing the development of secretory structures with the one basal cell, three stalk cells and one head cell. (G–Q) Lateral view of capitate and peltate trichome. (R) The outgrowth of initial cells of stigmas. (S) A vacuolized initial cell. (T) Two sister cells formed after the first periclinal division of the initial cell. (U) Three-cell stage, a capitate trichome showing the vacuolized basal cell. (V) Four-cell stage, a capitate trichome showing the vacuolized stalk cells. (W–M) A capitate trichome with one basal cell, one or two stalk cells and one head cell. (N) A peltate trichome with one basal cell, one stalk cell and multicellular head. (O) A peltate trichome showing the secreted multicellular head. (P, Q) The multicellular head secretions increase. (R) The secretory apex in the inner staminodes is negative to iodine-potassium iodide. (S) The secretory apex in the inner staminodes stained by mercury bromophenol blue for abundance of proteins. (T) The secretory apex in the inner staminodes stained by Sudan black shows small amounts of total lipids. (U) Capitate trichomes are negative to iodine-potassium iodide. (V) Peltate trichomes are negative to iodine-potassium iodide. (W) Capitate trichomes are negative to mercury bromophenol blue. (X) Peltate trichomes head stained by mercury bromophenol blue for abundance of proteins. (Y, Z) Capitate trichomes and peltate trichomes subcuticular space stained by Sudan black for total lipids. BC, basal cell; HC, head cell; SC, stalk cell. Scale bars: A–Z = 10 μm.
Figure 9. The reductive processes in the androecium, showing the secretory apex generated and thecae reduced. (A) The normal stamen with two thecae, but without secretory apex. (B) The modified stamen with secretory apex and two thecae of usual length. (C) The modified stamen with secretory apex and two thecae one-fourth to three-fourths shorter than that in the normal stamens. (D) The modified stamen with secretory apex and one much smaller theca (usually one-fifth of the normal stamens). (E) The inner staminode with secretory apex, but without thecae.

Table 1. Amino acid (AA) composition of the exudates from the stigmas and staminodes.

| AA                  | Pistillate phase (μg g⁻¹) | Stigmas | Staminode phase (μg g⁻¹) | Stigmas |
|---------------------|---------------------------|---------|--------------------------|---------|
|                     | Inner staminodes | Stigmas | Inner staminodes | Stigmas |
| Threonine           | 440                      | 34.5    | 91                       | 186.3   |
| Leucine             | 182.5                    | 119.5   | 138.5                    | 102.8   |
| Histidine           | 111                      | 23      | 48                       | 10.3    |
| Isoleucine          | 811                      | 9       | 105                      | 77.8    |
| Valine              | 148.5                    | 35      | 106.5                    | 33.3    |
| Phenylalanine       | 99.5                     | 51      | 70                       | 26.2    |
| Methionine          | 342                      | 341     | 20                       | 2.2     |
| Arginine            | 46.5                     | 104     | 119                      | 10.7    |
| Lysine              | 79.5                     | 48      | 81.5                     | 37.7    |
| Alanine             | 902.5                    | 542     | 946.5                    | 48      |
| Serine              | 711                      | 271     | 360                      | 246.2   |
| Tyrosine            | 493                      | 109     | 306                      | 203.3   |
| Urea                | 173                      | 61.5    | 40                       | 316.8   |
| Glutamic acid       | 330                      | 131     | 55                       | 90.83   |
| γ-Aminobutyric acid | 403.5                    | 220     | 175                      | 148     |
| Asparagine          | 1549                     | 85.5    | 244.5                    | 87.3    |
| Phosphoserine       | 67.5                     | 76.5    | 165.5                    | 135     |
| Aspartic acid       | 205                      | 60.5    | 150                      | 77.3    |
| Glycine             | 41.5                     | 11.5    | 27                       | 8.7     |
| α-Aminobutyric acid | 45.5                     | 1       | 4.5                      | 1.7     |
| Cystine             | 93                       | 4       | 5                        | 10.8    |
| β-Aminoisobutyric acid | 11.5            | 13.5    | 6.5                      | 35.5    |
| β-Alanine           | 5.5                      | 23.5    | 4.5                      | 27.3    |
| Ornithine           | 10.5                     | 76      | 126.5                    | 80.5    |
| Taurine             | 58                       | 21      | 19                       | 7.5     |
| Phosphorus ethanolamine | 80                   | 121     | 69                       | 20.8    |
| α-Amino-adipic acid | 77                       | 161.5   | 7                        | 4.3     |
| Citrulline          | 10                       | 52.5    | 5                        | 0.2     |
| 3-Methylhistidine   | 164.5                    | 28.5    | 1                        | 26.7    |
| 1-Methylhistidine   | 25.5                     | 104     | 2.5                      | –       |
| Carnosine           | 59                       | –       | 13                       | 56.8    |
| Hydroxyproline      | –                        | 518.5   | –                        | –       |
| Proline             | –                        | 136     | –                        | 40      |
| Total AAs           | 7576.5                   | 3594.5  | 3512.5                   | 2161.1  |
insects, while that of stigma provides for the needs of pollen that the staminode exudate provides for the needs of pollinating at the pistillate nor the staminate phase. It can be hypothesized not suitable for pollen germination at this period. Interestingly, that the stigmatic receptivity decreased and the stigma became yellowish brown, indicating proline content decreased obviously with the reduction of at the pistillate phase. At the end of the staminate phase, the In Previous studies (Saunders 2010) suggested that the secretory proteins and lipids, providing needs for pollen germination. ones. The former contain lipids only, and the latter are rich in protodermal cell, larger than the neighbouring ones. In mature both the capitate and peltate trichomes originate from a single somewhat, forming a secreting surface at this region. In stigmas, are laminar, while the carpel primordia are horseshoe-shaped organ primordia are developed, the inner staminode primordia fused with carpellary bundles from the base. When all the flower thecae in the modified stamens but did not report the secretory result of stamen reduction, the secretory apex and absence of the modified ones). On the other hand, both the modified stamens and the inner staminodes have a similar secretory apex with one basal cell, three stalk cells and one head cell, and produce sticky exudate at the pistillate phase, although the exudate chemistry of the modified stamens was not available. Obviously, the modified stamens have some characters in common with normal stamens and inner staminodes, and hence could be recognized as the transitional station between the normal stamens and the inner staminodes. From normal stamens to inner staminodes, the degeneration is clear and progressive, generating a secretory apex first, shortening thecae length next and finally losing thecae, one after the other (Fig. 9).

Some morphological differences between the normal stamens and the inner staminodes are present during the developmental process. In normal stamens with two extrorse thecae, the surface at the level of the connective appendage apex is smooth without trichomes, while in the inner staminodes, the thecae abort, but trichomes appear and gradually protrude outward at the apex, forming the secretory structures. However, the inner staminodes are initiated as a regular whorl similar to normal stamen primordia, and the vasculature of the inner staminodes splits from the basal traces of the free stamen bundles. Additionally, because the modified stamens are transitional structures between the normal stamens and the inner staminodes, the inner staminodes should be recognized as homologous with stamens in A. javanica, even though they are morphologically and functionally different from normal stamens. As the end result of stamen reduction, the secretory apex and absence of thecae in the inner staminodes were recognized as extreme specializations, also observed in other families of Magnoliidae, namely Eucomatiaceae, Himantandraceae, Degeneriaceae (Endress 1984). Maas and Westra (1984) noted the reduction of thecae in the modified stamens but did not report the secretory apex. In the present study, A. javanica is the only example in Annonaceae with modified stamens possessing secretory apex, which not only strengthens the staminal origins of the inner staminodes in A. javanica, but also provides a clue to recognizing evolutionary trends of stamens in Magnoliidae.

Conclusions

This is the first observation of the occurrence of three types of stamens in A. javanica, viz. normal stamens, modified stamens and inner staminodes. Inner staminodes play a role as a physical barrier preventing autogamy and promoting outcrossing, and

The homology of inner staminodes

Anaxagorea javanica possesses a vascular anatomy that is typical of the Annonaceae, comprising an outer perianth cortical vascular system (CVS) with three whorls of vascular traces and the inner corolla whorls that contain the vascular bundle of the stamens, inner staminodes and carpels (Deroin 1989, 1999; Xue and Saunders 2013; Guo et al. 2017). The longitudinal sections show that the vascular bundles to the inner staminodes are derived from the basal traces of the free stamen bundles and fused with carpellary bundles from the base. When all the flower organ primordia are developed, the inner staminode primordia are laminar, while the carpel primordia are horseshoe-shaped with developing concavities on the ventral surface. In the inner staminodes, the epidermal cells at the apex protrude somewhat, forming a secreting surface at this region. In stigmas, both the capitate and peltate trichomes originate from a single protodermal cell, larger than the neighbouring ones. In mature flowers, the inner staminodes are S-shaped and the apex is densely covered by secretory structures, providing needs of pollinating insects, being rich in proteins and lipids. The stigmas are densely covered by capitate trichomes, together with peltate ones. The former contain lipids only, and the latter are rich in proteins and lipids, providing needs for pollen germination. Previous studies (Saunders 2010) suggested that the secretory apex of the inner staminodes resembles a modified stigma in Anaxagorea. There seems to be no support for this study, because there are obvious differences between the inner staminodes and stigmas in flower vasculature, staminal and pistillary ontogeny, and trichome development.

In the investigated individuals of A. javanica (about 50 in total), three types of stamens were observed in one flower. The normal stamens are laminar with smooth and tongue-shaped connective appendages and have two thecae, extrorsely dehiscing. Between the normal stamens and the inner staminodes, one or two modified stamens have a secretory apex and two thecae, as long as or shorter than that in the normal stamens, or sometimes only a single theca (Fig. 9B–D). Inner staminodes lack thecae, and the apex is densely covered by secretory structures. Both the normal stamens and the modified one(s) produce ellipsoidal pollen grains and the exine ornamentation is plicate. In addition, pollen germination rates are nearly equal (51 % for the normal stamens, and 46 % for the modified ones). On the other hand, both the modified stamens and the inner staminodes have a similar secretory apex with one basal cell, three stalk cells and one head cell, and produce sticky exudate at the pistillate phase, although the exudate chemistry of the modified stamens was not available. Obviously, the modified stamens have some characters in common with normal stamens and inner staminodes, and hence could be recognized as the transitional station between the normal stamens and the inner staminodes. From normal stamens to inner staminodes, the degeneration is clear and progressive, generating a secretory apex first, shortening thecae length next and finally losing thecae, one after the other (Fig. 9).
their exudate provides for the needs of pollinating insects. The presence of modified stamens implicates that the possible transition from normal stamens to inner staminodes may involve a sequence of origin of a secretory apex first, shortening of the thecae next and finally loss of the thecae, one after the other. It also provides a clue to recognize evolutionary trends of stamens in Magnoliidae. The transitional modified stamens together with the floral vasculature and ontogeny support the homology of the inner staminodes with normal stamens.

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**Contributions by the Authors**

B.L. performed the experiments and analyzed the data. B.L. and F.X. wrote and approved the manuscript.

**Conflict of Interest**

None declared.

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**Literature Cited**

Armstrong JE, Irvine AK. 1990. Functions of staminodia in the beetle-pollinated flowers of *Eupomatia laurina*. Biotropica 22:429–431.

Baker HG. 1977. Non-sugar chemical constituents of nectar. *Apidologie* 8:349–356.

Baker HG, Baker I. 1975. Studies of nectar-constitution and pollinator-plant coevolution. *Corollae of Animals and Plants* 100:591–600.

Braun M, Gotsberger G. 2011. Floral biology and breeding system of *Anaxagorea dolichocarpa* (Annonaceae), with observations on the interval between anthesis and fruit formation. *Phytom-Annales Rei Botanicae* 51:315–327.

Chatrou LW, Firie MD, Erkens RHH, Couvreur TLP, Neubig KM, Abbot JR, Mois JB, Maas JW, Saunders RMK, Chase MW. 2012. A new subfamily and tribal classification of the pantropical flowering plant family Annonaceae informed by molecular phylogenetics. *Botanical Journal of the Linnean Society* 169:5–40.

Dafni A. 1992. Pollination ecology: a practical approach. Oxford: Oxford University Press.

Deroin T. 1989. Definition and phylogenetic significance of floral cortical systems: the case of Annonaceae. *Comptes Rendus de la Académie des Sciences Serie III* 308:71–75.

Deroin T. 1999. Functional impact of the vascular architecture of flowers in Annonaceae and Magnoliaceae, and its bearing on the interpretation of the magnoliaceous gnepoecyme. *Systematics and Geography of Plants* 68:213–224.

Doyle JA, Bygrave P, Le Thomas A. 2000. Implications of molecular data for pollen evolution in Annonaceae. In: Harley MM, Morton CM, Blackmore S, eds. *Pollen and spores: morphology and biology*. Kew: Royal Botanic Gardens, 259–284.

Doyle JA, Endress PK. 2010. Integrating early Cretaceous fossils into the phylogeny of living angiosperms: Magnoliidae and eudicots. *Journal of Systematics and Evolution* 48:1–35.

Doyle JA, Le Thomas A. 1994. Cladistic analysis and pollen evolution in Annonaceae. *Acta Botanica Gallica* 141:149–170.

Doyle JA, Le Thomas A. 1996. Phylogenetic analysis and character evolution in Annonaceae. *Bulletin du Muséum’ d’Histoire Naturelle, Paris, B* 18:279–334.

Endress PK. 1980. Floral structure and relationships of *Hortonia* (Monimiaceae). *Plant Systematics and Evolution* 133:199–221.

Endress PK. 1984. The role of inner staminodes in the floral display of some relic Magnoliaceae. *Plant Systematics and Evolution* 146:269–282.

Endress PK, Armstrong JE. 2011. Floral development and floral phyllotaxis in Anaxagorea (Annonaceae). *Annals of Botany* 108:835–845.

Endress PK, Hufford LD. 1989. The diversity of stamen structures and dehiscence patterns among Magnoliidae. *Botanical Journal of the Linnean Society* 100:45–85.

Endress PK, Lorenz DH. 2004. Heterodichogamy of a novel type in *Hernandia* (Hernandiaceae) and its structural basis. *International Journal of Plant Sciences* 165:753–763.

Gottsberger G. 2016. The reproductive biology of the early-divergent genus *Anaxagorea* (Annonaceae), and its significance for the evolutionary development of the family. *Acta Botanica Brasilica* 30:313–325.

Gottsberger G, Schrauwen J, Linskens HF. 1984. Amino acids and sugars in nectar, and their putative evolutionary significance. *Plant Systematics and Evolution* 145:55–77.

Guo X, Tang CC, Thomas DC, Couvreur TLP, Saunders RMK. 2017. A megaphylogeny of the Annonaceae: taxonomic placement of five enigmatic genera and support for a new tribe, Phoenicantheae. *Scientific Reports* 7:7323.

Guo X, Thomas DC, Saunders RMK. 2018. Organ homologies and perianth evolution in the Dasymaschalon Alliance (Annonaceae): inner petal loss and its functional consequences. *Frontiers in Plant Science* 9:174.

Haydak MH. 1970. Honey bee nutrition. *Annual Review of Entomology* 15:143–156.

Hong-Qi Z, Croues AF, Linskens HF. 1982. *Apidologie* 9:731–1982.

Johnson DM, Murray NA. 2018. A revision of *Xylopia* L. (Annonaceae): the species of Tropical Africa. *Phytotaxa* 97:1–252.

Kessler PFA. 1988. Revision der Gattung Orophea Blume (Annonaceae). *Blumea* 33:1–80.

Lau JYY, Pang CC, Ramsden L, Saunders RMK. 2017. Stigmatic exudate in the Annonaceae: pollinator reward, pollen germination medium or extragynoecial compitum? *Journal of Integrative Plant Biology* 59:881–891.

Linskens HF, Schrauwen J. 1969. The release of amino acids from germinating pollen. *Acta Botanica Neerlandica* 18:605–614.

Maas PJM, Westra LYT. 1984. Studies in Annonaceae. I. A monograph of the genus *Anaxagorea* A. St. Hil. Part 1. *Botanische Jahrbücher für Systematik* 105:73–134.

Maas PJM, Westra LYT. 1985. Studies in Annonaceae. II. A monograph of the genus *Anaxagorea* A. St. Hil. Part 2. *Botanische Jahrbücher für Systematik* 105:145–204.

Maas PJM, Westra LYT, Koek-Noorman J. 1986. Studies in Annonaceae. V. Additional notes on *Anaxagorea* A. St. Hil. *Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen* 89:75–82.

Maas-van de Kamer H. 1993. *Floral biology of Anaxagorea dolichocarpa*, and some notes on flower biology in other Annonaceae. *Annals of Botany* Newsletter 9:19–24.

Nicolson SW, Thornburg RW. 2007. Nectar chemistry. In: Nicolson SW, Nepi M, Pacini E, eds. *Nectaries and nectar*. Dordrecht: Springer, 215–264.

Rohwer JG. 2009. The timing of nectar secretion in staminal and staminodial glands in *Lauraceae*. *Apidologie* 40:155–166.

Ronse De Craene LF. 2007. Are petals sterile stamens or bracts? The origin and evolution of petals in the core eudicots. *Annals of Botany* 100:621–630.

Ronse De Craene LF, Smets EF. 1990. The floral development of *Popovia whitei* (Annonaceae). *Nordic Journal of Botany* 10:411–420.
Ronse De Craene LP, Smets EF. 2001. Staminodes: their morphological and evolutionary significance. The Botanical Review 67:351–402.

Saunders RM. 2010. Floral evolution in the Annonaceae: hypotheses of homeotic mutations and functional convergence. Biological Reviews of the Cambridge Philosophical Society 85:571–591.

Sauquet H, Doyle JA, Scharaschkin T, Borsch T, Hilu KW, Chatrou LW, Le Thomas A. 2003. Phylogenetic analysis of Magnoliidae and Myristicaceae based on multiple data sets: implications for character evolution. Botanical Journal of the Linnean Society 142:125–186.

Scharaschkin T, Doyle JA. 2005. Phylogeny and biogeography of Anaxagorea (Annonaceae) using morphology and non-coding chloroplast sequence data. Systematic Botany 30:712–735.

Scharaschkin T, Doyle JA. 2006. Character evolution in Anaxagorea (Annonaceae). American Journal of Botany 93:36–54.

Schodde R. 1969. A monograph of the family Atherospermataceae. PhD Thesis, University of Adelaide, Adelaide, Australia.

Schrauwen JAM, Linskens HF. 1974. Influence of the extraction conditions on the recovery of free amino acids in plant material. Acta Botanica Neerlandica 23:42–47.

Staedler YM, Endress PK. 2009. Diversity and lability in floral phyllotaxis in the pluricarpellate families of core Laurales (Gomortegaceae, Atherospermataceae, Siparunaceae, Monimiaceae). International Journal of Plant Sciences 170:522–550.

Staedler YM, Weston PH, Endress PK. 2007. Floral phyllotaxis and floral architecture in Calycanthaceae (Laurales). International Journal of Plant Sciences 168:285–306.

Staedler YM, Weston PH, Endress PK. 2009. Comparative gynoecium structure and development in Calycanthaceae (Laurales). International Journal of Plant Sciences 170:21–41.

Su YC, Smith GJ, Saunders RM. 2008. Phylogeny of the basal angiosperm genus Pseuduvaria (Annonaceae) inferred from five chloroplast DNA regions, with interpretation of morphological character evolution. Molecular Phylogenetics and Evolution 48:188–206.

Teichert H, Dotterl S, Gottsberger G. 2011. Heterodichogamy and nitidulid beetle pollination in Anaxagorea prinoides, an early divergent Annonaceae. Plant Systematics and Evolution 291:25–33.

Van Heusden E. 1992. Flowers of Annonaceae: morphology, classification, and evolution. Blumea 7:1–218.

Webber AC. 1996. Biologia floral, polinização e aspectos fenológicos de algumas Annonaceae na Amazônia Central. PhD Thesis, Instituto Nacional de Pesquisas da Amazônia e Fundação Universidade do Amazonas, Brazil.

Webber AC. 2002. Floral biology and pollination of some neotropical Annonaceae. Annonaceae Newsletter 13:18–21.

Xue B, Saunders RM. 2013. Reassessing morphological homologies in the early-divergent angiosperm Fenerivia (Annonaceae) based on floral vascular anatomy: significance for interpreting putative homeotic mutations. PLoS One 8:e81923.