

Can the anatomy of abnormal flowers elucidate relationships of the androecial members in the ginger (Zingiberaceae)?

Li Xiumei

Agricultural biological Gene Research Center, Guangdong Academy of Agricultural Sciences

Fan Tian

Guangzhou University

Zou Pu

South China Botanical Garden

Zhang Wenhui

Guangdong Academy of Agricultural Sciences

Wu Xiuju

Guangdong Academy of Agricultural Sciences

Zhang Yixin

Hunan Agricultural University

JINGPING LIAO (✉ liaojp85231555@163.com)

South China Botanical Garden <https://orcid.org/0000-0002-9453-493X>

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Abstract

Background Interpretation of the floral structure of Zingiberaceae has long concentrated on the relationships of the androecial members. It has been suggested that the labellum is composed of two structures rather than three or five, and the glands are not only the epidermis of the ovary but are similar to nectaries.

Results Serial sections were used to observe the vasculature of normal and two-staminate flowers in *Alpinia intermedia* 'shengzhen'. Floral diagrams were drawn to interpret the morphological category of the floral organs and the relationships of the androecial members. Androecial vascular bundles were associated with carpellary dorsal bundles (CDBs) and parietal bundles (PBs) in a Zingiberales phylogeny setting using ancestral state reconstruction. Anatomical observations demonstrate that the fertile stamen(s) incorporate parietal strands both in normal and two-staminate flowers. The three appendages represent the three members of the outer whorl of the androecium while the labellum represents the inner whorl of the androecium in the two-staminate flower. Reconstruction of the origin of the vascular system in the androecium suggests that the outer whorl of androecium receives its vascular supply from the CDBs and the inner whorl of androecium receives from the PBs in both the basal banana group and the more derived ginger clade.

Conclusions The present study adds to a growing body of literature suggesting that anatomy of abnormal flowers may not provide enough evidence for elucidating the relationships of the androecial members. Abnormal flowers are diverse in the Zingiberaceae and may derive from different types of mutations.

Background

Interpretation of the floral structure has long concentrated on the relationships of the androecial members in Zingiberales, which is divided into the Banana Group (Musaceae, Strelitziaceae, Heliconiaceae and Lowiaceae (Orchidantha)) and the Ginger Group (Zingiberaceae, Costaceae, Marantaceae, and Cannaceae) (Kress, 1990, Kress et al., 2001). The androecium is acknowledged to typically consist of two whorls, of which possess three members each. In the Banana Group, one fertile stamen is replaced by a staminode or is completely absent, with five functional stamens remaining in the most of flowers. Except for the staminode of the outer whorl is opposite the sepal in Heliconiaceae family, the staminode of the inner whorl is opposite the petal in the other three families of the Banana Group. In the Ginger Group, by contrast, the flowers are highly modified compared to the idealized monocotyledon; five fertile or more stamens are transformed into petaloid structures, leaving only one (Zingiberaceae, Costaceae) or one-half stamen (Marantaceae, and Cannaceae). This single (or half) fertile stamen is always the same member of the inner androecial whorl. Thus homeosis has played an important role in floral evolution of the ginger group, these sterile stamens share positional homology with stamens in the banana group and other monocots (Kirchoff, 1991, Bartlett and Specht, 2010).

Homeosis is “the assumption by one part [of an organism] of likeness to another part” (Bateson, 1894). Leavitt (Leavitt, 1909) accepted and perfected the term by describing the phenomenon of homologous heteromorphism in many plants. If the degree of likeness is very high, one organ type direct replace with another in a 1: 1 substitution. If, however, the degree of likeness is lower, an organ intermediate between two normally distinct organ categories develops (Sattler, 1988). One can discover morphologies that correspond more or less to some of the homeotic mutants in many plant species (Sattler, 1988). As early as 1760, Caspar Friedrich Wolff believed that different flower organs could be transformed and equal to each other within an idealist morphological perspective. After about 20 years later, a similar theory was proposed by Johann Wolfgang Goethe base on the observation of serial abnormal flowers (From Wikipedia, the free encyclopedia Metamorphosis of Plants). Abnormal flowers provide opportunities for study homeosis from the view of comparative anatomical morphology. There are more or less cases for abnormal flower in Zingiberales. In Musaceae, for example, a decreased number of stamens in the abnormal flowers of the Dwarf Cavendish banana, are the result of the adnation of stamens to the style and the arrested development of some of the stamens which remained dwarf and staminodal (Fahn et al., 1961). In Cannaceae, many of these “abnormal” or “mutant” phenomena have been recognized as homeotic, they represent a biological phenomenon that creates phenotypic and genotypic variation, which is important for evolution. The abnormal flowers have also been reported in Zingiberaceae especially in the genus *Alpinia*: only two loculi in lateral positions in *Hedychium coronarium*, two stamens and one sterile appendage in *Alpinia vittata* (Rao, 1963). Maas (Maas, 1977) found an abnormal flower of *Renealmia goyazensis*, in which the lateral appendages of the labellum were replaced by two fully developed anthers. Song (Song et al., 2003) reported a considerable diversity of developmental switches in the genus *Alpinia*. Analyses of the abnormal flowers allow us to deduce robust rules of homeotic transformation that could potentially be generalized across the Zingiberaceae. Floral anatomy in terms of vasculature for evolutionary relationship was under suspicion for failing to provide clear details and valid lines of evidence (Carlquist, 1969). However, because of the nebulous and reticulate relation between evidence and phylogeny, not only anatomists but all others have yet to establish objective and useful clues that can solve phylogeny without any ambiguity (Periasamy and Swamy, 1978). The existence of vascular connections and the development of primordial facilitate distinguishing a staminodial structure (Decraene and Smets, 2001). Therefore, the significance of anatomical characters was not downgraded. Even today next-generation sequencing technologies provide access to genomic character data but have been insufficient to resolve the phylogenetic relationship among primary lineages of Zingiberales (Barrett et al., 2014).

Staminode evolves from an imperfect, sterile stamen into a regressing or vestigial organ, and further is biased between complete loss and a transformation into a highly specialized structure. In Zingiberaceae, unlike six stamens arranged in two whorls, staminodes fuse in varieties of combinations to form a novel structure—staminodial labellum (Kirchoff, 1991). Its nature is considered to be linked up with the androecium, and its relationship to glands has received contrasting interpretations (Fig. S1). Brown (Brown, 1830) regarded the outer androecial whorl is represented by the labellum and the two lateral staminodes (if exist), while the inner androecial whorl is represented by the posterior stamen and the two

glands. In Payer's (Payer, 1857) perspective, the labellum is a double structure. He interpreted that the outer androecial whorl is represented by the two lateral staminodes, and the anterior member of this whorl is missing. The inner androecial whorl is composed of the posterior stamen and the labellum, the latter is derived by the congenital fusion of two lateral staminodes. Gregory (Gregory, 1936) proposed the labellum as a triple structure, the median portion of the labellum belongs to the outer androecial whorl, while its lateral portions belong to two members of the inner androecial whorl. Thompson (Thompson, 1936) agreed with the "triple structure" by ontogenetic observations but of a different derivation. In his description, the labellum was developed from primordial 8, 11 and 13 of a spiral sequence, and the epigynous glands were called stylodes. However, Payer's "double structure" interpretation on labellum was the most widely accepted later, following Kirchoff's (Kirchoff, 1988, Kirchoff, 1991, Kirchoff, 1997) more detailed ontogenetic work. In the observation, the labellum is generated by connecting the primordia of the two innerpetaloid staminodes of intercalary growth, the outer androecial staminode initiated between the two primordia ceases growth shortly after the labellum initiating.

Here we survey the anatomy on the vasculature of normal flower and abnormal, two-staminate flower in *Alpinia intermedia* 'shengzhen', aim to provide new evidence to evaluate the morphological category of the floral organs. We intend also to evaluate if anatomy of abnormal flowers elucidate relationships among the androecial members in the Zingiberaceae. In addition, we summarized the vascular system in floral structure based on the earlier work in the eight families of Zingiberales. Therefore, the vasculature of normal and abnormal flower may play an important role in morphological character data for assessing floral homologies and phylogenetic relationships.

Results

Floral morphology

Normal flowers are bisexual and zygomorphical, calyx tubular, split on one side, formed by 3 sepals. Corolla tubular with three lobes, 1 adaxial wider than 2 abaxial. Stamen 1, adaxial, with two thecae. Lateral appendages 2, subulate, adnate to base of the abaxial labellum (Fig. 1). Ovary with 3 locules. Style 1. The two-staminate flowers are different in that the fertile stamens are 2, subulate appendages alternated with stamens and labellum are 3, and three petals are opposite to stamens and labellum respectively (Fig. 1). The two types of flowers are found on the same plant.

In the two-staminate flower, the gland is smaller than the usual one, and the labellum is much smaller than that in the normal flower. It inferred that the more the primordia, the more space they occupy on the apical meristem at floral development, thus reducing the space available for subsequent organs to be initiated. Two adaxial petals embrace the abaxial petal. Two adaxial petals are against the two fertile stamens, while the abaxial ones against the labellum. So the position of the two fertile stamens and the labellum are anti-petalous.

Floral vascular bundles of the normal flowers and the two-staminate flowers

Serial transverse sections were taken from the normal and the two-stamen flower buds to study the vascular veins and differentiation of the parts composing the flower (Fig. 2, 3). At the floral axis beneath the ovary, a number of vascular strands distributed peripherally in two rings in the inverted trigonal pedicel. The bundles of the outer ring are arranged peripherally along the pedicel, whereas those in the inner region are irregularly arranged (Fig. 2a).

In the locular region, of the three carpels, one is anterior and the other two are postero-lateral. Hence, of forming the three septa, one septum is posterior and the other two are antero-lateral. The locules with axile placentation appear not simultaneously but in a spiral sequence which is in an anticlockwise way, as reported previously e.g. *Crataeva*, *Alpinia clocrata* and *Caulokaempferia coenobialis*. The three large bundles of the inner ring extend laterally and shift opposite the septa of the ovary as the parietal bundles (PBs) in the ovary wall. Carpellary dorsal bundles (CDBs), alternated with the 3 PBs, are oriented toward the three median of each carpel. The remaining bundles of the inner ring anastomose into the ovary axis, forming the placental bundles (Fig.2b). The three axile cavities fuse to form a central tri-radiate ovary fassula, which continues into the style and becomes the stylar canal. The placental bundles assemble around the ovary fassula before terminating in the base of the ovary prolongation (Fig. 2c, d).

A little higher in the locular region, the CDBs diverge outwards and divide into 2–3 branches (Fig. 2c). The inner branches of the CDBs connect with the PBs to anastomose vascular plexus in the upper part of the ovary locules, while the outer branches afterward enter the sepals and become the midribs of the sepals (Fig. 2e-g). The vascular bundles from the outer ring in the pedicel are irregularly scattered. Those large bundles join the branches of the CDBs and the PBs to produce anastomosing vascular plexus, while the small traces contribute to the sepals after the calyx tube begins to separate (Fig. 2e-g). The inner branches of the CDBs and the PBs become obscure but can be identified from their locations in the plexus.

Below the junction of the calyx tube, two antero-lateral masses of vascular bundles that are derived from the vascular plexus support the glandular emergence of the carpellary tissue (Fig. 2 h). The two abaxial glands are originally attached to the inner column of the style but lie freely at a little higher within the floral tube (Fig. 2i, j). The branches of the CDBs and the PBs can be differentiated in their original location at the point of the plexus termination. There are also small traces left from the plexus, which enter the petals ultimately (Fig. 2h, k, l, m). The outer branches of the CDBs get further apart from the inner ones that would supply the two lateral appendages and the labellum (Fig. 2i, m, n).

The large adaxial (posterior) PB with two strands further divide into five strands, and two abaxial (anterolateral) PBs divide into two strands (Fig. 2i). Thereafter the outer strand of each PB diverges further as the midrib of the petal until the petals are separate from the inner whorls (Fig. 2k-m). All of the four posterior parietal strands subsequently enter the fertile stamen, whereas both of the remaining

abaxial PBs become the lateral strands of the labellum (Fig. 2m-o). The style separates from the outer column of tissues above the ovary prolongation (Fig. 2i). All the floral organs differentiate completely and can be identified (Fig. 2o). Sepals enclose all the other floral parts except the ovary, which being inferior is below it. The outmost, posterior petal embraces the other two small, antero-laterals, while the position of the fertile stamen is anti-petalous. The floral vascular system in the normal flower is summarized in Fig. 4a.

At the pedicel underneath the ovary of the two-staminate flower, a number of vascular strands distributed peripherally in two rings in the inverted elliptical pedicel, the bundles of the outer ring are arranged peripherally along the pedicel, whereas those in the inner region are arranged as a triangle.

The ovary of the two-staminate flower also has three locules with axile placentation as the normal flower, but in a 180°invert way (Fig. 3a). Of the three locules, two are in the abaxial while one is in the adaxial. Three CDBs alternate with 3 PBs, respectively. Hence, the location of the CDBs and the PBs in the two-staminate flower are at the angle 180°to the normal flower. A little higher above the locular region, the abaxial PB divides horizontally to anastomose vascular plexus with inner branches of the CDBs (Fig. 3c-d). Each adaxial PB divides into two bundles which join the plexus shortly afterward (Fig. 3e-f). Some bundles of the outer ring may be connected with the plexus but those would enter the sepals remain unaffected. The anastomosing vascular plexus is formed by the three inner branches of the CDBs and the PBs (Fig. 3f). The outer CDBs gradually go outside, enter the sepals and become the midrib of each sepal (Fig. 3g, h). The inner dorsal bundles stay in the inner whorl until floral organs differentiate.

When the vascular plexus fade away between the top of locular region and the bottom of the ovary prolongation, there is only single glandular tissue at the abaxial side (Fig. 3g). The adaxial and the abaxial PBs divide into two or three and then four strands, bearing small traces (Fig. 3g-i). With the gland disappearing gradually, the outer PBs and the other daughter ones get further apart. Successively, the outer PBs go into the petals, and the other ones arrange in a line (Fig. 3j). The abaxial PBs enter the labellum, while the two adaxial ones enter the two fertile stamens (Fig. 3k, l). The inner CDBs supply to three appendages (Fig. 3g-m). The floral vascular system in the two-staminate flower is summarized in Fig. 4b.

Floral diagrams of the normal and the two-staminate flowers

Floral diagrams, which show the relative position of flower in an inflorescence, floral fusion, orientation, symmetry and structure details, have long been employed by botanists for comparative researches and describing the arrangement of floral organs with great simplicity (2010). Zingiberaceae flowers frequently possess a median-adaxial (MAD) petal which closes to the floral axis and is situated away from bract (Fig. 1, 5a). By contrast, the two-staminate flowers have a median-abaxial (MAB) petal which closes to the bract (Fig. 1, 5b). If the two-staminate flower is considered as a 180° resupination, then its labellum,

the two stamens, and the median appendage correspond to the fertile stamen, the labellum and the absent androecial member in the normal flower, respectively (Fig. 5c). The inner androecial whorl (the antepetalous whorl) consists of labellum and two stamens, and the outer androecial whorl is comprised of three appendages. The outer androecial bundles extend from the CDBs while the inner androecial bundles from the PBs.

Phylogenetic analyses of Zingiberales

100 accessions, representing 41 species within the Zingiberales and 4 species within commelinales, were included in phylogenetic analyses. We detected substitution saturation for sequences before phylogenetic reconstruction under MEGA 6 and DAMBE. For fully resolved sites, plotting rates of transitions (s) and transversions (v) against model-corrected pairwise distances revealed little saturation in MEGA and DAMBE, but for all sites, saturation was detected by the statistical test of Xia in DAMBE (Supplement table 1, Fig. S2). Phylogenetic relationships were better resolved in the combined dataset, comparing with the independent analyses of individual loci. Bayesian inference generated a topology similar to the ML tree, but had higher support in some backbone branches (Fig. S3). In both the ML and BI analyses, phylogenetic relationships were weakly resolved within Musaceae and Heliconiaceae diverging lineage with weak support value. The placement of Heliconiaceae and Musaceae is crucial in resolving relationships at the base of Zingiberales. However, different topologies in previous studies have proved difficult to resolve the earliest divergences among families of Zingiberales ([Barrett et al., 2014](#)). The topology of the phylogenetic trees obtained from the ML analyses in this study was used for ancestral state reconstruction of the floral vascular system.

Trait evolution

We compared the origin of the floral vascular system in androecium to help us understand the nature of the petaloid organ in Zingiberales. Based on the anatomy of more than 20 species that have been studied (Supplement table 2), the androecial vascular was associated with CDBs and PBs (Fig. 6). In the banana group, the inner androecial whorl has the same source, which is derived from the PBs. The outer androecial whorl is derived from the CDBs or accompanying CDBs. The staminode of Heliconiaceae belongs to the outer whorl of androecium supported by the CDBs, and the staminode of the other three families is part of the inner whorl supported by the PB. In the ginger group, the bundles of Zingiberaceae, Costaceae and Marantaceae tend to anastomose vascular plexus before separate into CDBs (carpellary dorsal-cure-outer staminal strands in Marantaceae) and PBs in their original location; the CDBs enter the outer androecial members while the PBs enter the inner androecial members. The androecial whorls are supplied by the CDBs and the PBs simultaneously in Cannaceae.

Discussion

Abnormality is often observed in migratory species of ex situ conservation and in vitro conservation. Nevertheless, we observed two labella of *Amomum.sp* in wild (unpublished data). Multiple male buds phenomenon in banana is a natural but rare occurrence, caused by the branching of inflorescence axis or the differentiation of male flowers into male buds (Chomchalow et al., 2012). Abnormality may occur by interspecific hybridization, tissue culture, chemical treatment, radiation exposure, parasitism and climatic change during acclimatization. The outer staminodes initiate and usually abort during early development in *Canna* species (Miao et al., 2014). In *Alpinia calcarata*, abnormal flowers showed two stamens, a median sterile appendage and a single gland in the postero-lateral positions. The explanation was that one of the antero-lateral glands has become staminiferous, owing to the position of the missing gland is occupied by the additional fertile stamen in the abnormal flower (Raghavan and Venkatasubban, 1941). Therefore the labellum which is a single organ, together with the fertile stamen, forms the outer androecial whorl. Another example of abnormal flower *A. vittata* had two lateral fertile stamens and a median sterile appendage as well as a single gland. The explanation was that the two fertile stamens correspond to the two lateral staminodes, and the median sterile appendage corresponded to the posterior fertile stamen of the normal flower. Then the labellum was believed as the fusion of two antero-lateral staminodes. In this study, the two-staminate flower has two stamens, two lateral appendages, a median sterile appendage and a single gland. So the labellum seems to be composed of three staminodes, as the presence of a third appendage. This result supports Gregory's view the labellum is a triple structure. From the above how many staminodes composing labellum largely depends on the staminode development. In the abnormal flowers of *Alpinia* genus, a tendency for the number of fertile stamens varied from 2, 1 to 0.5 may be the reappearance of the evolutionary history of the stamen in the ginger group and even in Zingiberales (Song et al., 2003). Nevertheless, so far no fossil evidence has indicated that the number of stamen evolves between 6/5 and 1. We need to be aware of that any type or number of organs can occupy any whorl on abnormal flower (Meyerowitz et al., 1989). Many cases of homeosis are based on a phylogenetic hypothesis; environmentally induced homeosis (phenocopying) may occur through similar mechanism as genetically induced homeosis (Sattler, 1988). That means homeosis induced by the environment is also associated with changes of expression of genes. Mutations that cause abnormal flowers have been providing important keys to the nature of the gene products that regulate development. Abnormal flowers are diverse in the Zingiberaceae and may derive from different types of mutations. Recently, mutagenesis of desired traits has been demonstrated in major crops using CRISPR systems targeting whole genomes or gene families. The door is wide open for in vivo directed evolution in eukaryotes (Zhang and Qi, 2019).

The labellum is a major morphological structure in Lowiaceae, Zingiberaceae, Costaceae and Cannaceae, but is not homologous among families. The labellum in Lowiaceae, which is regarded as the conspicuous petal or also receives the bundle of the sixth lost stamen, is different from the labella in the other three families, which are only derived from staminodes (Liao, 1998). In Zingiberaceae, the mid-anterior bundle in the floral tube is regarded as significant in the consideration of the morphological nature of the labellum (Pai, 1965). The mid-anterior bundle which is derived from the vascular plexus, continues for a considerable length in the labellum, or shows an early division into two strands. Both of

the above two cases are recorded in the different *Elettaria* flowers of the same plant, the latter case is also seen in some other species, such as *Kaempferia capoa*, *Curcuma amada*., In this study, the two-staminate flowers show a median apical split whereas the normal ones do not. Pai suggested that when the labellum is emarginate, the mid-anterior bundle quickly divides into two and the bundles run into the two components. There is also a third case that the marginal bundle of the two constitutes instead of mid-anterior bundles, laterally on either side of the mid-anterior line, keep upwards into the two segments of the labellum, e.g. *Zingiber macrostachyum* and *Curcuma decipiens*. The mid-anterior bundle may expediently be interpreted as a composite bundle which is the fusion of the marginal bundles of the two component members of the inner androecial whorl (Pai, 1965). It seems that the development of a mid-anterior bundle and its further performance in the labellum is relevant to the degree of connation of its two components (Kirchoff, 1991). The determination or circumscription of an organ is itself rather equivocal. The labellum and appendages are not one of the four main organ types (sepals, petals, stamens and carpel). Staminodes belonging to an androecial whorl may also become secondarily petaloid in Zingiberales (Baum et al., 2001). The labellum is a petaloid organ that blends characteristics of more petal than stamen and cannot be precisely classified as either. In the two-staminate flower, direct replacement of the fertile stamen with labellum (vice versa) results in a 1:1 substitution. It suggests that the labellum has androecial identity. However, this result does not refute Payer's view that the labellum is derived from the congenital fusion of two lateral staminodes. It is insufficient to resolve the relationships of the androecial members. Abnormal flowers may not provide enough evidence for elucidating complicated structures.

Septal nectaries are universally present in Zingiberales, except in Lowiaceae where they are aborted and in Zingiberaceae and Costaceae where they are highly transformed (Rao, 1954, Kirchoff, 1992). Of Musaceae family, nectaries are limited to the upper part of the ovaries above the locules in female flowers, while in male flowers nectaries entirely occupied the aborted ovaries. Carpel margins fuse incompletely to develop the septal nectaries such as in *Strelitzia* flower and *Heliconia* flower, whereas the carpels fuse so completely that the structure of septal nectaries is replaced by glands in many Zingiberaceous flowers. The glands of Zingiberaceae are typically two in number, and are above the septa on the antero-lateral of the flower. In some, they are basally connate on the anterior side. In rare cases, the antero-lateral glands may extend to the posterior side. The gland on the posterior side became reduced and absent in the evolution of the Zingiberaceous flower (Rao, 1963). The two basal glands were interpreted as staminodes, epidermal appendages or stylodes. Gregory regarded the glands as epidermal appendages of the ovary, and Pai suggested glands are more deeply associated with organs of the ovary by comparative observations (Gregory, 1936, Pai, 1965). In *Arabidopsis*, the nectary is not regulated by ABC functional genes but is independent of any floral organogenesis gene and is position-determined (Baum et al., 2001). It supported the independence of nectary gland development from the specification of floral organ identity.

In monocots, for the common MAD petal initiation, two primary patterns of floral zygomorphy are observed, which differ mainly in the configuration of the androecium (Rudall and Bateman, 2004). In pattern 1, the suppression of the adaxial median stamen is often associated with the presence of a

relatively well-differentiated MAD petal, such as in Musaceae, Lowiaceae and Strelitziaceae. In pattern 2, one or more abaxial stamens are reduced or modified, such as in more derived Heliconiaceae and ginger families. Interestingly, pattern 2 normally occurs in taxa that are embedded within pattern 1 clades such as Zingiberales and pentapetalous flowers (Bukhari et al., 2017, Rudall and Bateman, 2004). The two-staminate flower seems to accord with pattern 1 and pattern 2 on account of suppression in both the adaxial and abaxial stamen. In contrast to the normal flower, variation in the two-staminate flower occurred not only in the number and arrangement of androecium but also in the distribution of petals. It displays putative developmental rotation in floral orientation with the median-abaxial petal.

In normal flower, the bundles of outer ring in the pedicel travel out exhibiting extensive branching during their upward through the flower, the sepals are supplied by these small traces derived from the outer ring and the outer branches of CDBs. The sepals are also supplied by the outer CDBs in the two-staminate flower. The large bundles from the outer ring in the pedicel, along with the branches of CDBs and the PBs, form an anastomosing vascular plexus in the normal flower. In the two-staminate flower, the plexus mainly consist of the branches of CDBs and PBs. The plexus, which contributes to the petals, gland(s), and androecium, is a very important anatomical development and has been universally observed in Zingiberaceae (Pai, 1965). Small traces left from plexus and outer branches of CDBs go into the petals of the normal flower, while outer PBs enter the petals of the two-staminate flower. Comparing to the labellum in normal flower receives abaxial (antero-lateral) PBs and inner branches of CDBs, the smaller labellum in the two-staminate flower only receives abaxial PBs. The fertile stamen(s) incorporate parietal strands both in the normal and two-staminate flower. The lateral appendages are absent in some *Alpinia* genus flowers, and the vasculature of these appendages is not detected. In this study, two lateral appendages of the normal flower are supported by two inner branches of CDBs, three appendages of the two-staminate flower by three ones. Based on the previous studies, the outer androecial whorl receives its vascular supply from the CDBs and the inner androecial whorl from the PBs in both the basal banana group grade and the more derived ginger clade (Liao, 1998, Xie, 2002, Tang et al., 2000, Liao et al., 2001).

Methods

Plant materials

Living materials of *Alpinia intermedia* 'shengzhen' were collected at the Ginger Garden of South China Botanical Garden (SCBG) in Guangzhou, Chinese Academy of Sciences. Mr. Ye Yushi provides the materials and plant identification. Both of the normal flowers with an accession number 20150507 and the abnormal flower that has been isolated without accession number are located in SCBG. The vouchers are preserved in the South China Botanical Garden Herbarium (supplement 3).

Paraffin section

Flowers of young inflorescences were immersed in formalin-acetic acid-alcohol (FAA) and were vacuumed to allow FAA to penetrate into the tissues. The samples were fixed in FAA for at least 48 hours and then preserved in 70% ethanol, as described previously. Briefly, three to five fully developed flowers selected were dehydrated and then embedded in paraffin. Transverse serial sections in the floral parts, ovary prolongation, locular region, and pedicel were cut at 8–12 μm using a microtome and mounted on glass slides before staining with Ehrlich's hematoxylin. Terminology here followed Rao (Rao et al., 1954, Rao and Donde, 1955, Rao and Pai, 1959, Rao and Pai, 1960, Rao and Gupte, 1961, Rao, 1963) and Liao et al (Liao, 1998). Adaxial/abaxial was used to describe the position of floral organs with respect to the inflorescence axis at stages of floral development. The slide specimens are preserved in the Key Laboratory of Plant Resources Conservation and Sustainable Utilization, SCBG.

Reconstruction of the phylogeny of Zingiberales

To obtain the phylogenetic relationships of these taxa for floral vascular trait analysis, we reconstructed phylogenetic trees. Our sampling focused on Zingiberales to collect data with representatives from each of the eight families, in addition to the outgroup. We selected the nuclear ribosomal internal transcribed spacers 1 and 2 (ITS) and the external transcribed spacer (ETS), the plastid markers *trnL-F*, and *trnL-rpl32*, and introns from the putatively single copy nuclear genes calmodulin (CaM), based on recently publications (Iles et al., 2017, Barrett et al., 2014). Nucleotide sequences downloaded from Genbank (<https://www.ncbi.nlm.nih.gov/genbank/>) were aligned using MUSCLE version 3.5 (Edgar, 2004) implemented in BioEdit v.7.0.0 (Hall, 1999), and then adjusted manually. Individual loci trees were evaluated using RAxML but result in poor support for topological relationships due to the absent sequence information of some species.

Best-fit substitution models for each dataset were selected under the Bayesian Information Criterion (BIC) using MEGA6, the model used was the GTR+G for nrITS, K2+G+I for nrETS, T92+G for CAM, T92+I for TRN, GTR+G+I for RPLN. To access potential substitution saturation, pairwise model-corrected distances were calculated rates of transitions and transversions on all nucleotide sides in MEGA6 (Kumar et al., 2008), and exported the data to graph. The pairwise deletion on gaps/missing data treatment yielded more data than complete deletion but the results were similar. Pairwise GTR-corrected distances were also plotted against rates of transitions and transversions for fully resolved sites and all positions combined in DAMBE (Xia et al., 2003). For sequences containing unresolved bases, we dealt with them as "?". If the test statistic is significantly lower than the critical value, then there is no significant saturation; if not, there is substantial saturation or the sequences are poor for phylogenetics. The phylogenetic relationships were conducted for the five markers using both Maximum Likelihood (ML) and Bayesian inferences (BI) by RAxML version 8.2.0 (Stamatakis, 2014) and MrBayes version 3.2.6 (Ronquist et al., 2012), respectively, on the CIPRES Science Gateway (Miller et al., 2010). The ML phylogeny was inferred under the GTRGAMMA model, and support for the branches was estimated from 1,000 bootstrapping replicates under GTRCAT. The Bayesian inferences were performed with Markov chain Monte Carlo (MCMC) running with four chains each for 1,000,000 generations. Trees were sampled every 1000

generations. The specific model for each dataset suggested by MEGA6 was applied to the Bayesian analyses. Convergence of the four MCMC chains was assessed by checking that the average standard deviation of the split frequencies dropped below 0.01. The first 25% of the trees were discarded as burn-in, and the 75% remaining trees were used to reconstruct the majority-rule consensus tree. Finally, a phylogeny was carried out manually using Mesquite version 3.5 ([Maddison and Maddison, 2018](#)). This phylogeny lacking branch length parameter was used for parsimony-based ancestral state reconstruction.

Trait evolution for the androecial whorl

To achieve an understanding of the evolutionary transition in Zingiberales androecial whorl morphology and vascular, we selected three characters and analyzed within the families. We collected information on floral vascular from the literature. Outgroup taxa from orders of commelinid monocot were selected in terms of the previously published phylogenies ([Barrett et al., 2014](#)). Mesquite was used to build the vascular data matrix and optimize characters on the ML tree based on the combined data set. Character traits and their state matrices were coded as follows: Inner androecial whorl receives strands from 0, undetermined; 1, PBs; 2, CDBs and PBs; 3, vascular plexus formed by CDBs and PBs with other branches. The outer androecial whorl is supplied by 0, undetermined; 1, CDBs; 2, CDBs and PBs; 3, vascular plexus formed by CDBs and PBs with other branches.

Conclusion

By comparing the vasculature and anatomy of normal and abnormal flower under the microscope, we can learn about the architecture of the floral organs and tissues, how they are put together, how they relate to each other, and what difference between them. The present study suggests that anatomy of abnormal flowers may not provide enough evidence for elucidating the relationships of the androecial members. Abnormal flowers are diverse in the Zingiberaceae and may derive from different types of mutations. However, it provides data for further research in plant molecular biology, environmental studies and genetics, as well as other fields.

Abbreviations

CDBs: carpellary dorsal bundles

PBs: parietal bundles

MAD: median-adaxial

MAB: median-abaxial

ML: Maximum Likelihood

BI: Bayesian inferences

ITS: internal transcribed spacers

ETS: external transcribed spacer

CaM: calmodulin

Declarations

Ethics approval and consent to participate (Not applicable)

This study is not including human participants, human data, human tissue or animal.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LXM, FT, ZP, LJP conceived the study. LXM and ZP collected plant materials. FT, ZP, ZWH and WXJ performed paraffin section. LXM, FT and ZYX planned, organized, and conducted the sequencing steps and phylogenetic trees. LXM, FT and LJP conducted trait evolution. LXM, LJP drafted the manuscript with everyone's inputs on relevant sections. All authors approved the final manuscript.

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Figures

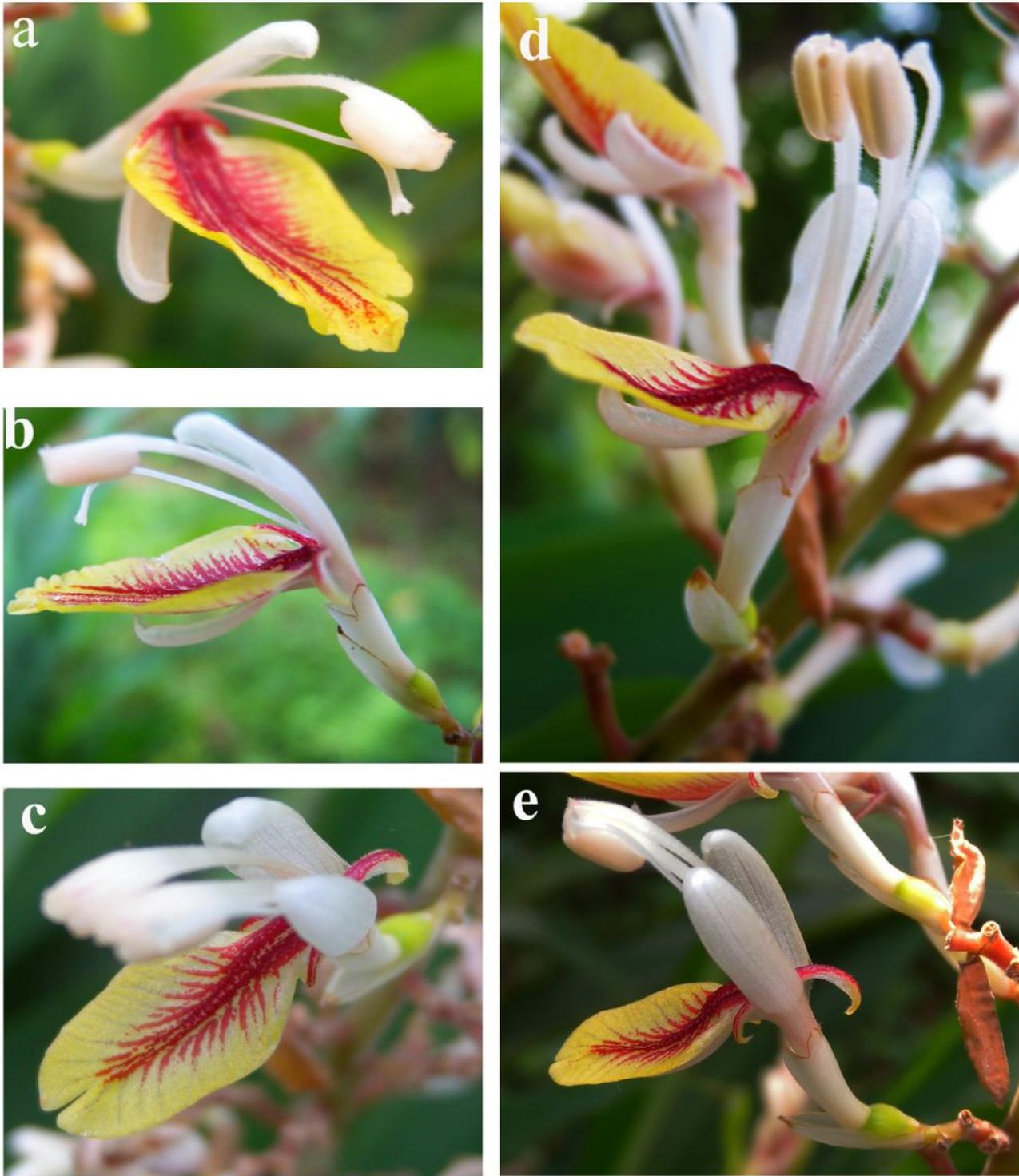


Figure 1

The normal and two staminate flowers. a, appendage; s, sepal; p, petal; st, fertile stamen; lab, staminodial labellum; sty, style.

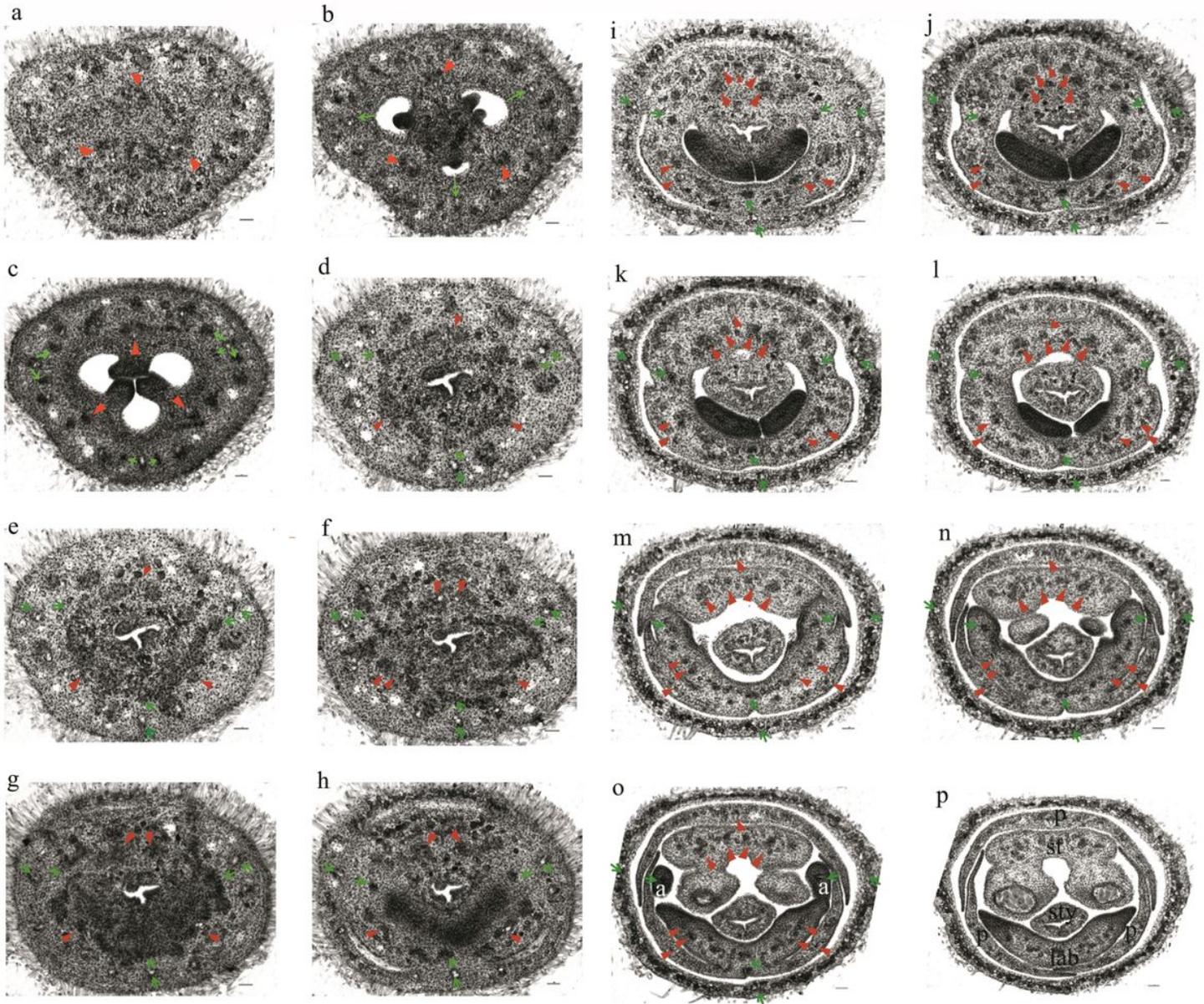


Figure 2

Floral vasculature in the normal flower of *Alpinia intermedia* 'chenshengzhen'. All figures are presented in transverse section with the adaxial side upward. Arrow (↑), CDB; arrowhead (▼), PB. a, Pedicel, displaying an outer ring and an inner region of vascular bundles. b, Sublocular region, showing the ovuliferous zone. c, Locular region, displaying the CDBs and the PBs. d-g, The upper part of the ovary locules, displaying an anastomosing vascular plexus is formed by the CDBs and PBs bearing branches each other, and merging some outer large bundles from the ovary wall. h, Below the junction of the calyx tube, showing the calyx tube beginning to differentiate before the anastomosing vascular plexus fading out. i-k, The floral tube, showing the gland emergence and petals beginning to separate. m-p, A fully differentiated floral organs, showing the development of petals, labellum, fertile stamen and two appendages. Bars = 100 μm.

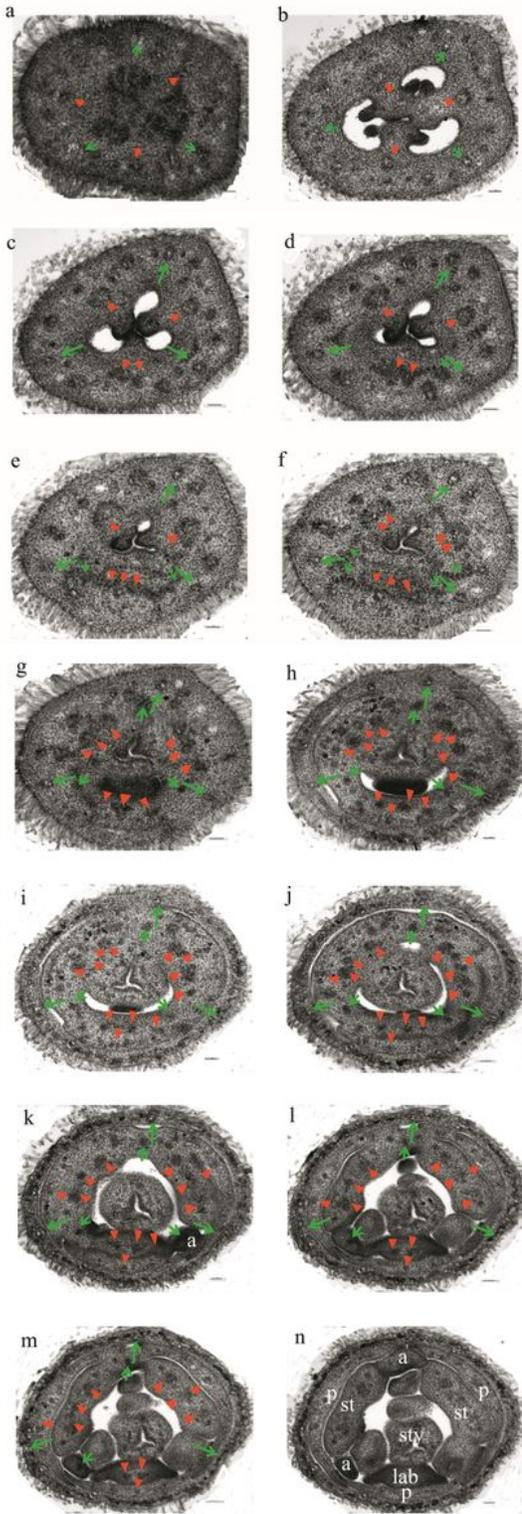


Figure 3

Floral vasculature in the two-staminate flower of *Alpinia intermedia* 'chenshengzhen'. All figures are presented in transverse section with the adaxial side upward. Arrow (↑), CDB; arrowhead (▼), PB. a, Sublocular region, showing initiating CDBs and PBs. b, Locular region, showing invert axile placentation bearing ovules. c-d, A little higher above the locular region, showing an extension of the bundles. e-f, The upper part of the ovary locules, displaying the anastomosing vascular plexus being formed by the CDBs

and PBs bearing branches each other, with some outer large bundles from the ovary wall also joining. g, Below the junction of the calyx tube, displaying the calyx tube beginning to differentiate before the anastomosing vascular plexus fading out. h-j, The floral tube, showing the gland emergence and petals starting to separate. k-n, Fully differentiated floral organs, showing petals, labellum, two fertile stamens and three appendages. Bars = 100 μ m.

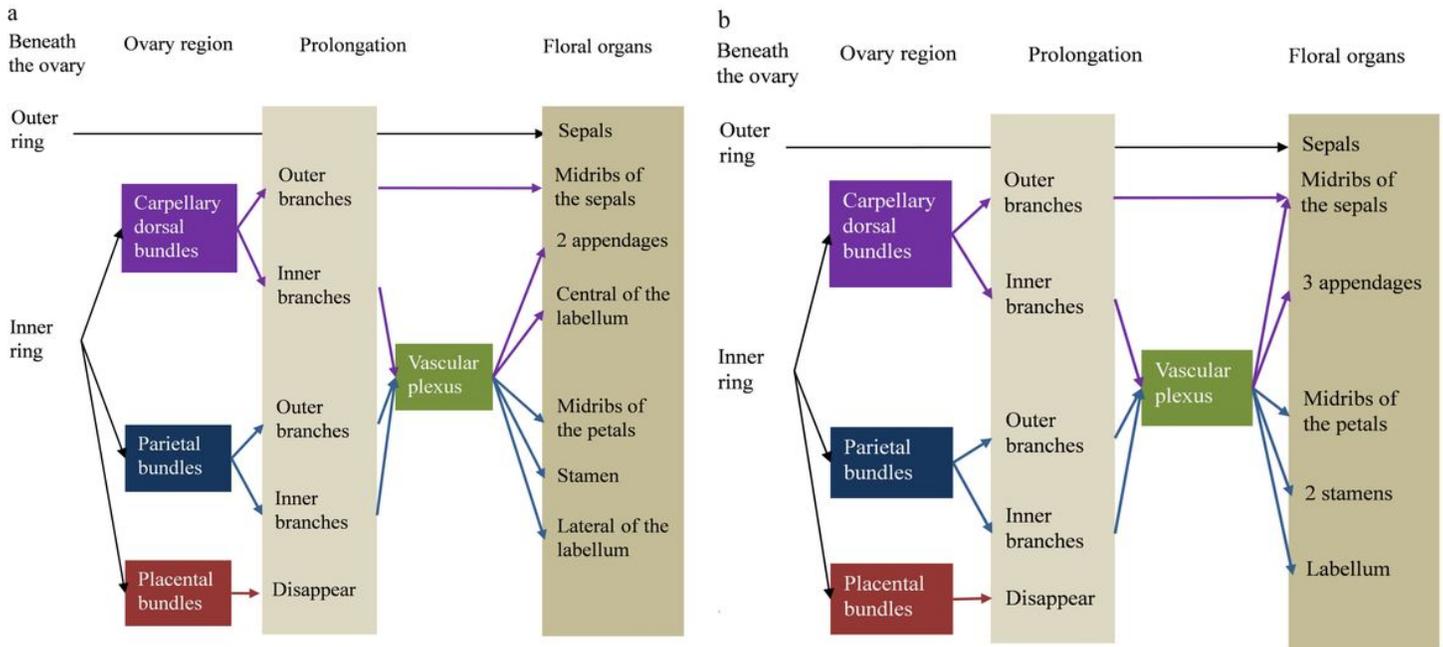


Figure 4

Summary of the floral vascular system in the normal flower (a) and the two-staminate flower (b). The CDBs and PBs in pedicel were distributed from the locular region where anastomose vascular plexus, to floral organs.

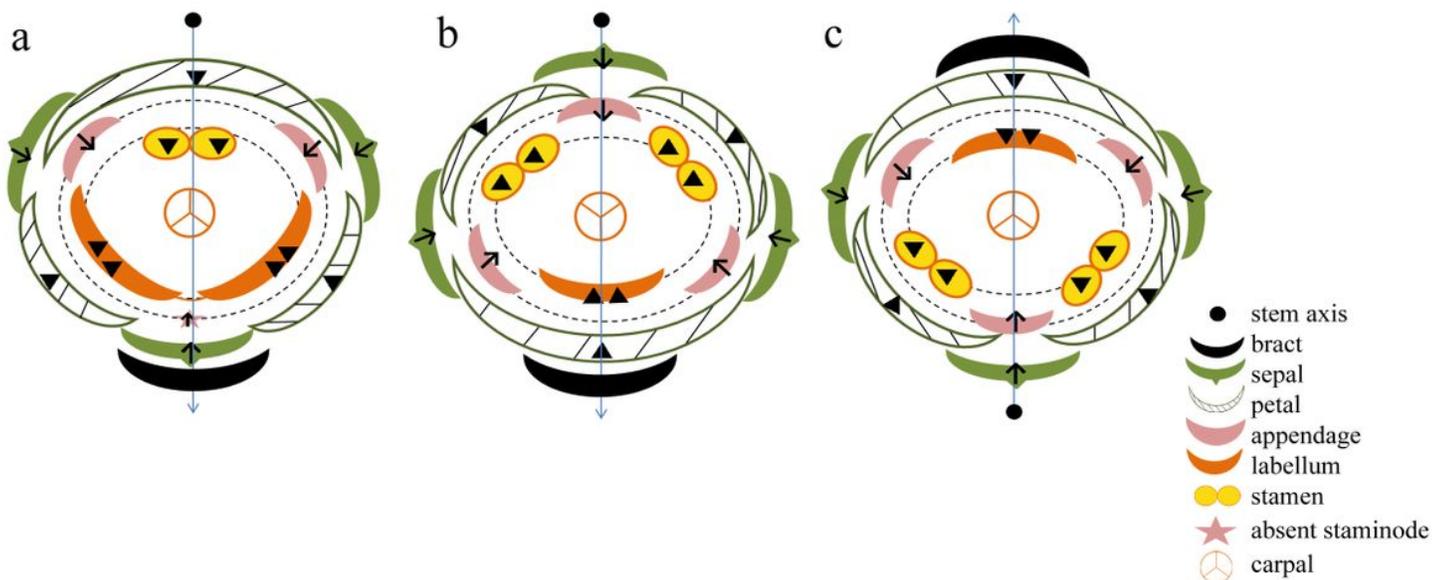


Figure 5

Comparison patterns of the floral organ in the normal flower and the two-staminate flower. a, floral diagram of the normal flower is based on observations by Kirchoff et al. (Kirchoff, 1983, Kirchoff, 1988, Kirchoff, 1991, Kirchoff et al., 2009), Kress (Kress, 1990), Bartlett and Specht (Bartlett and Specht, 2010). b, the two-staminate flower. c, the two-staminate flower with resupination. Lines with arrows indicate the planes of floral symmetry. Dotted circles indicate the inner and outer androecial whorls.

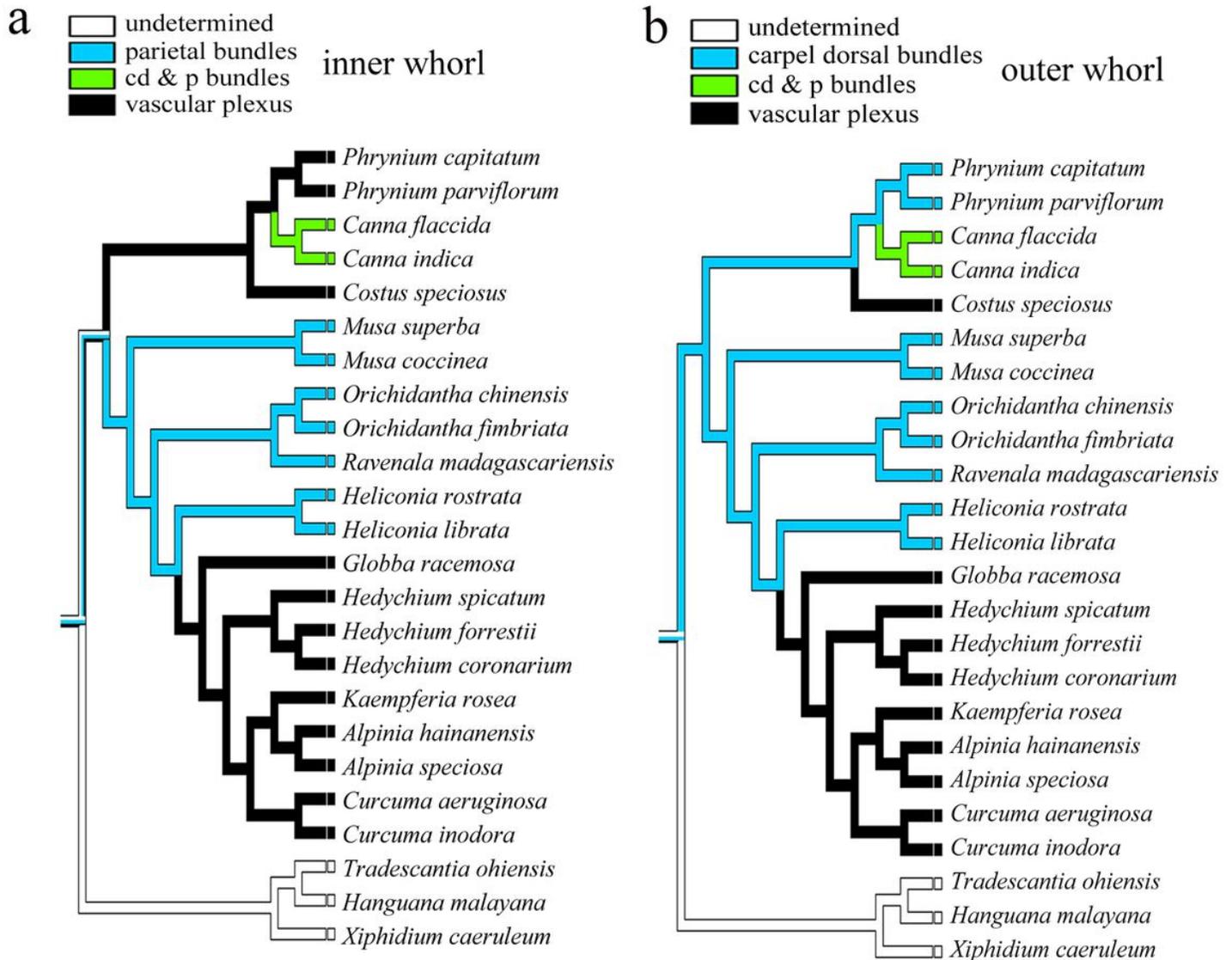


Figure 6

Ancestral character state reconstructions in the commelinids monocots. a, inner androecial whorl; b, outer androecial member

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