Evolution and development of flowers, fruits and inflorescences of Phalaenopsis and other orchid species
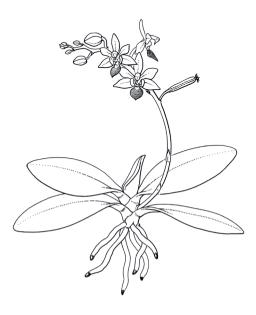
Pramanik, D.

Citation

Pramanik, D. (2023, September 13). *Evolution and development of flowers, fruits and inflorescences of Phalaenopsis and other orchid species.*

Version:	Publisher's Version
License:	[Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)
Downloaded from:	

Chapter 7

Synthesis

Dewi Pramanik[1,2,3]

1 Evolutionary Ecology group, Naturalis Biodiversity Center, Leiden, The Netherlands

2 Institute of Biology Leiden, Leiden University, The Netherlands

3 National Research and Innovation Agency (BRIN), Central Jakarta, Indonesia

Studies on orchid flowers, inflorescences and fruits have mainly focused on documenting the vast macro-morphological variation that exists in this diverse plant family, rather than placing this variation in an evolutionary and developmental context. When I started my PhD thesis, the floral diversity within the economically important pot plant genus *Phalaenopsis*, for instance, was well studied. Research on genes that control development of floral organs has been carried out in species such as *Phalaenopsis equestris.* (Pan et al., 2014; Tsai et al., 2005, 2004), *P. amabilis* (Hsu et al., 2015), *P. aphrodite* ssp. formosana (Pan et al., 2014), and *P. pulcherrima* (Huang et al., 2016). These studies revealed the important role of MADS box in the development of floral organs in *Phalaenopsis* through the synergy of multiple protein complexes that duplicated during orchid evolution. However, these studies are limited to the first and second floral whorls. How the diversity in shape and size of floral organs in the third whorl, related to pollination in different species, evolved over time was still a mystery, as were the genetic mechanisms driving this evolution.

Similarly, it was known that there is variation in fruit macro-morphology within the orchid family, but this variation had never been examined in a phylogenetic context nor studied from an anatomical or evo-devo perspective. This thesis aims to fill some of these knowledge gaps in order to gain a deeper understanding of how orchid morphology could have become so diverse.

7.1 EVOLUTION AND DEVELOPMENT OF ORCHID FLOWERS

The evolution of orchids over millions of years resulted in a high diversity of shapes and sizes of orchid flowers. Duplication and subfunctionalization of MADS box and other transcription factors, such as MYBs, and TCPs, are known to play an essential role in shaping diversity of orchid floral organs. Gene expression studies were done with different orchid species. These explained how a combination of B-class genes, together with other MADS box genes, involved in the flower identity program, results in distinctly shaped sepals, petals and a labellum. However, not much is known about the function of these genes in forming the inner whorl floral structures of orchids such as the callus, stelidia, and mentum, which have an important role in pollination. Thus, my major research question was: what is the role of MADS box and other genes in regulating the evolution of floral structures related to pollination? To better understand the role of MADS box, MYB and TCP genes in the evolution of these floral structures, I studied the expression of these genes in floral buds, callus, stelidia and mentum of two *Phalaenopsis* species, *Phalaenopsis equestris* and *P. pulcherima*, which have a differently shaped callus, stelidia

and mentum. I also constructed gene lineage trees for MADS box, TCP and MYB genes to discover how many copies of these genes are present in the genome of *Phalaenopsis*. I then combined gene expression data with SEM and 3D micro-CT scans to investigate the ontogeny of these structures (**chapter 3**). I found differential expression of MADS box *AP3/PI*-like, *AGL6*-like and *SEP*-like, and *MYB DIV*-like gene copies in the callus, stelidia and mentum of the two species of *Phalaenopsis* investigated. Dirks-Mulder et al. (2017) previously discovered that vascular bundles running to the stelidia and callus on the labellum of *Erycina pusilla* originated in the same position as the stamens in close relatives of orchids. I was the first to detect similar vascular bundle patterns in the stelidia and callus of *Phalaenopsis*. The vascular bundles detected by me in the mentum of *Phalaenopsis* showed that this highly specialized organ is of mixed sepaloid-petaloid-staminodial origin, a completely new discovery.

This discovery calls for further investigation to test different hypotheses of the floral evolutionary modularity in *Phalaenopsis*. Floral modularity is the concept of viewing floral organs as an independent cluster from other clusters within an organism (Armbruster et al., 2014). Modularity is believed to promote morphological evolution. Extending our research to investigate the floral modularity hypothesis could provide more insight into whether the evolution of diversity in the shape and size of *Phalaenopsis* floral organs and structures is influenced by modularity, and which modularity theory best explains this variation. Four modularity hypotheses have been proposed. These can be summarized as the efficiency, attraction, and developmental model proposed by Diggle (2014) and the developmental-genetic evo-devo model proposed by Mondragón-Palomino and Theissen (2009). In a recent study of orchid flower modularity, Artuso et al. (2022) determined developmental genetics as the best-fitting modularity theory for *Malagasy Bulbophyllum* out of these four hypotheses. In the study of Artuso et al. (2022), the flowers were divided into four modules: M1= sepal, M2= petal, M3= labellum+ mentum, and M4= column-part. According to these authors, the result of these modulations is in line with current developmental-genetic models of the orchid flower, which propose that the petal, sepal, labellum, and column development are controlled by differential expression of B class and other MADS box genes. I found that these MADS box genes, together with other developmental genes, are also involved in development of the mentum and stelidia in *Phalaenopsis*, which are both part of the column sensu Artuso et al. (2022) (**chapter 3**). These authors also postulated that the specific "labellum+column-foot" modulation might be related to the pollination process. In *Malagasy Bulbophyllum*, the labellum connects to a mentum. The labellum is used by pollinators as a landing platform, and the mentum acts as a hinge, slamming an insect walking over the labellum against the upper part of the gynostemium

to ensure the removal and/or deposition of pollinia. The hypothesis that treats "labellum-mentum" as a single module may also be applied to *P. pulcherrima*. Firstly, I detected similar MADS box B class gene expression in this module. Secondly, the labellum and mentum are initiated later than the sepals and petals during floral ontogeny. Furthermore, they share petal-derived vascular bundles. In addition, the mentum also contains sepal and stamen-derived vascular bundles.

Other highly specialized orchid floral structures to investigate include for instance the stipe, a strap-shaped organ that connects the pollinia to the viscidium, which is often sticky for attachment to visiting insects. The evolutionary origin of the stipe is not clear. Some postulate that it is derived from the stigma, while others claim a staminoid origin (Freudenstein and Rasmussen, 1996). By including the orchid stipe in a modulation hypothesis, the evolutionary origin of this structure could be further investigated. 3D micro-CT scanning and scanning electron microscopy (SEM), combined with transcriptomics analysis, are suitable approaches to increase our understanding of the homology of the stipe. If derived from the stigma, I would expect to observe carpel-derived vascular bundles and the expression of MADS box *AG* and *SEP* developmental genes. If derived from the stamen, on the other hand, I would expect to find staminode derived vascular bundles and the expression of MADS box *AP3, AG,* and *SEP* genes.

Another interesting floral structure to be investigated is the rostellum. The rostellum is a protruding part of the gynostemium that separates pollinia from the stigma, and prevents self-pollination. The rostellum is generally believed to be a modification of the median stigma lobe (Gamisch et al., 2013). Based on this hypothesis, I expect MADS box C and A/E developmental genes to control the development of the rostellum. Information on the developmental genes involved in rostellum formation may be applied for breeding of commercial orchid crops, such as *Vanilla planifolia* and *V. pompona*.

In South and Meso-American rainforests, the flowers of the aromatic pods of *V. planifolia* are pollinated by *Euglossine* bees (Lozano Rodríguez et al., 2022), while *V. pompona* is pollinated by *Eulama cingulate* via floral fragrance rewards and food deception (Watteyn et al., 2022). In commercial production sites, however, wild pollinators cannot survive. To produce *Vanilla* pods, *Vanilla* flowers are exclusively hand-pollinated (Arditti et al., 2009). This method is time-consuming, labour-intensive, and requires special skills (Wurz et al., 2021). A breeding program to modify *Vanilla* flowers into selfers could save time. One of the approaches to obtain such a selfer is by modification of genes related to rostellum development. By silencing the developmental genes of the rostellum, it could be narrowed or completely removed, allowing pollinia to reach the stigma of the same flower, resulting in a self-pollinated *Vanilla* cultivar.

Chapter 7

7.2 EVOLUTION AND DEVELOPMENT OF ORCHID FRUITS

Despite the advanced study of orchid floral development, little attention has been paid to orchid fruit morphology and development. This is due to the limited available resources in both herbaria and greenhouses, the lack of knowledge of the structure and physiology of orchid fruits (Rasmussen and Johansen, 2006), and the common misconception that fruits of orchids have little morphological and structural variation (Cribb, 1999). To fill this knowledge gap, an in-depth study of fruit morphology, anatomy and evolution was needed to better understand the adaptive evolution of orchid fruits. Therefore, in **chapter 4**, I explored the development of orchid fruit dehiscence using ancestral character reconstruction analysis (ASR) by conducting fruit anatomy surveys and mapping fruit traits on a molecular phylogeny to better understand the evolutionary relationship between fruit traits.

My first important finding was that fruit orientation, fruit dehiscence, and lignification of the dehiscence zone (DZ) are evolutionary informative characters. Fruit orientation coevolved with lignified DZs and fruit dehiscence at the base and apex. The orientation of fruits during dehiscence may have an important role in ensuring wider seed dispersal, as has been shown in *Campanulaceae* s. str. (Y. Niu et al., 2016). An erect fruit with an upright dehiscence position can disperse seeds over a larger distance than pendant fruits (Y. Niu et al., 2016). *P. equestris* with pendant fruits, valves fused at the base and apex during dehiscence and a single opening slit, may have less chance to disperse seeds over long distances. To increase the possibility of seed dispersal over a greater distance, the opening slits of *P. equestris* fruits face different directions during dehiscence to ensure that seeds can disperse in as many directions as possible (**chapter 5**). Seed dispersal vectors are also correlated with fruit orientation (Y. Niu et al., 2016). To better comprehend the relationship between fruit characters and seed dispersal, more pedant and non-dehiscence fruits need to be included in follow up studies. Such studies may reveal that some species have multiple strategies for seed dispersal, as was recently found for *V. planifoli*a and *V. pompona*. Seeds from open fruits are collected and dispersed by *Euglossine* and *Meliponine* bees while mature indehiscent fruits of *V. planifolia* and *V. pompona* drop to the ground. Terrestrial rodents and marsupials consume these fruits and disperse the seeds after ingestion (Karremans et al., 2023).

My second finding was that erect orchid fruits co-evolved with a more extended ripening period and non-lignified DZs. In our sampling, fruits with extended ripening periods and non-lignified DZs were mainly found in epiphytic orchids, whereas lignified DZs and shorter fruit maturation were primarily found in terrestrial orchids (see S4.1 Table-Traits scoring, **chapter 4**). Ecology has been mentioned as an important driver for evolution of

seed dispersal traits. Fan et al. (2020) found that the seed air space, a proxy for dispersal, is correlated with the ecological habit of orchids. Terrestrial orchids produce seeds that are more restricted in their ability to disperse, whereas epiphytic orchids have a significantly larger seed air space, resulting in dispersal over larger distances.

Combining seed and fruits characters in follow-up studies may give a broader view of how selection pressure is affecting the morphology of fruits and seeds and whether there are correlations between fruit and seed characters with ecological conditions and seed dispersal vectors. To answer these questions, it is necessary to investigate more orchid species using advanced technologies such as 3D CT micro-scanning, SEM, and morphometric analysis combined with field observations of seed dispersal vectors. Newly collected data should be placed in a phylogenetic context using ASR, which may shed more light on the evolution of these traits.

My third important finding was that lignified DZs in orchid fruits evolved multiple times from non-lignified ones. This result implies that a non-lignified DZ is the ancestral character. The deposition of lignin in orchid fruits varied greatly among species. For some orchids, lignified cells were only found in vascular bundles and the endocarp layer of the valves, such as in the epiphytic species *E. pusilla* and *P. equestris*. The non-lignified DZs of *E. pusilla* fruits contain lipid layers. In contrast, in terrestrial orchids such as *Cynorkis fastigiata*, fruit DZs become completely lignified. Different developmental genes may control the development and dehiscence mechanism in different types of DZs in orchids.

An evo-devo study offruit development of *E. pusilla* was initiated by Dirks-Mulder et al. (2019). The authors used entire fruit tissues, including valves and ovules, to observe fruit-related gene expression using a library of *A. thaliana* fruit developmental genes as a reference. To narrow down the latter study, I used fruit valve tissues 1 week after pollination (WAP) and 3 WAP of *E. pusilla* to investigate the expression patterns of genes related to fruit development, fruit dehiscence and lipid biosynthesis. First, I found that three *FUL* copies were lowly expressed, in both one and 3 WAP fruits, while *EpMADS22/AG2* and *EpSEP3* were similarly expressed in fruit valve tissues of 1 and 3 WAP. It has been reported in both *E. pusilla* and *A. thaliana* fruits that *FUL* genes interact with *AG/SHP* during early fruit development. The different results could be explained by a specific regulator that is active during valve development of *E. pusilla*. In this case, the interaction of *EpMADS22/AG2* and *EpSEP3* may play a crucial role in valve development and growth of *E. pusilla* fruits. Another important finding was that *EpMADS24/EpB$_{sister}$* (*EpBs*) was significantly expressed in 3 WAP fruits of *E. pusilla*. Previously, it was reported for *P. equestris* fruits that *PeMADS28*, an ortholog of *EpBs*, is expressed in the ovules at 32 and 48 days after pollination, synchronized with integument development (C.-Y. Shen et al.,

2021). The significant expression of *EpBs* in 3 WAP fruit valve tissues might indicate a possible novel function of this gene in the formation of orchid DZs and a possible similar function with its ortholog in *A. thaliana* that controls fruit cell expansion. Second, one ortholog gene that governs DZs formation in *A. thaliana*, *RPL*, was strongly expressed in 1 WAP *E. pusilla* fruits and its expression was decreased in 3 WAP fruit valves. Third, I also discovered expression of lipid biosynthesis genes in *E. pusilla* fruit valves, which may be related to the production of lipid content to support early fruit valve growth. These genes probably also contribute to later DZ development in 3 WAP *E. pusilla* fruits. The inverse expression of these lipid genes with *IND/HEC3* and *ALC/SPT* genes, that re gulate lignified cell formation in *A. thaliana*, suggests that the current *A. thaliana* fruit dehiscence model should be adapted for orchids. Whether the correlations between gene expression patterns found in lipified fruits are causal to the absence of lignification, is still unclear. Future research should focus on answering the following question: is gene inhibition responsible for the shift between lignification and lipification of orchid fruits? Expanding the sampling of gene expression studies with fruits with different variations of lignified and lipified DZs, such as those of *Epipactis helleborine* and *Cynorkis fastigiat*a, could be a first step.

It is also essential to zoom in on the cellular level. *E. pusilla* DZs consist of two cell layers with the number of cells ranging from 60-120 and a size of circa 0.05 mm^2 (Dirks-Mulder et al., 2019). This small size makes it difficult to isolate particular cells within a small area. Laser microdissection (Kivivirta et al., 2019) was applied by me to isolate DZ cells from *E. pusilla* fruits. However, it was not possible to obtain sufficiently pure RNA, possibly due to the high levels of phenolic compounds. As an alternative, I pro pose to grow protoplast cultures from orchid DZ samples. Combining protoplast samples with single-cell isolation with long-read sequencing will reveal higher-quality cell-specific transcripts. Such an approach (see **chapter 2**) is expected to retrieve full-length transcripts (Wang et al., 2019). Once transcripts relevant to specific cell development are discovered, functional validation is required, for example by virus-induced gene silencing (VIGS) (Hou et al., 2023). The proposed approaches could help develop a model for regulatory networks determining orchid fruit DZ morphology.

7.3 RESUPINATION OF ORCHID ROOTS AND FRUITS

Resupination, a 180 degree twist of structures during plant development, brings the basal part of an organ to the top and the apical part to the bottom. It is considered a "trademark" characteristic of the Orchidaceae (Arditti, 2002; Ernst and Arditti, 1994; Fischer et al.,

2007; Nyman et al., 1984). Resupination was first reported in a drawing dating back to 1550, made by the Swiss naturalist Conrad Gesner (1516-1565) [see Arditti (2002)]. The majority of studies on orchid resupination focus on the flower. Less is known about the resupination of other orchid organs, such as leaves, inflorescences, and fruits (Ames, 1938) . Epiphytic orchids have aerial roots that serve multiple functions, including physical support, water absorption, nutrient uptake (Pridgeon, 1987) and photosynthesis in some leafless epiphyte orchids (Suetsugu et al., 2023). Resupination of orchid aerial roots may be associated with maximizing the absorption of light, water, and nutrients. Resupination of leaves may be influenced by light, transpiration and particular physiological phases. For example, in *Nepenthes*, leaves resupinate when they reach the adult stage and convert their tips into pitchers (Hill, 1939; Schwallier et al., 2020). Flowers resupinate to attract pollinators, as a resupinated labellum serves as a landing position for pollinators (see chapter 3). Fruits resupinate to maximize seed dispersal.

The majority of orchid resupination research focused on anatomical and morphological aspects. None of the orchid resupination studies involved the evolution and development of resupination genes. The difficulties in determining the precise time of resupination may be the most important bottleneck for studying evo-devo of orchid resupination. To improve our understanding of the precise timing of resupination and to answer several questions regarding the developmental stages during which resupination occurs, I built a bioinformatics pipeline to generate high-resolution 3D time-lapse videos to record resupination in weekly CT scans of roots and flowers of *E. pusilla* (**chapter 5**). In addition, I also captured the resupination of *P. equestris* fruits with 2D photographs made weekly.

The results from the time-lapse video showed that aerial roots of *E. pusilla* twist and resupinate multiple times from early development onwards, also in opposing directions. The final orientation of a root tip was found to be independent of its starting position. Aerial roots grow against gravity, above the medium and hang in the air. Similar to resupination of flowers, resupination of aerial root tips could be caused by gravitropism. It is widely accepted that gravitropism influences root development in *A. thaliana*. Gravity stimulates the lateral auxin gradients between the lower and upper halves of a root, which are first seen in the lateral cap and then extend into the elongation zone (Ottenschläger et al., 2003). From there, the root cap, a major gravity-sensing organ, changes the polarity of the lateral auxin transport (Su et al., 2017). During the onset of sensing, a gravity-induced lateral auxin gradient is generated across the cap to reach the responding zone (Swarup et al., 2005). After onset of gravity sensing, differential cell elongation between the upper and lower sides of the elongation zone causes a curvature of the root (Ottenschläger et al., 2003; Su et al., 2017). Differential cell growth due to asymmetric distribution of the

Chapter 7

growth regulator auxin in response to gravity was also found in the resupinated pedicel of *Aranda* Kooi Choo and a *Dendrobium* hybrid (Ernst and Arditti, 1994). However, whether auxin and gravitropism affect resupination in *E. pusilla* roots requires further empirical testing.

The fruits of *E. pusilla* twisted 45 degrees eight to 9 WAP. Resupination of fruits of *P. equestris* started a week before dehiscence and ended a week after dehiscence. Orchid fruit resupination had an independent direction and degree of torsion from the initial to the final position. I found major differences in the resupination time between *E. pusilla* and *P. equestris* fruits. I propose a number of hypotheses to explain this finding. Firstly, differences in resupination time may be caused by the variation in fruit maturation and dehiscence times. Fruits of *E. pusilla* matured at 6 to 11 WAP and dehiscence occurred at 12 to 14 WAP, whereas fruits of *P. equestris* matured at 9 WAP and dehiscence occurred from 20 to 24 WAP (see **chapter 5**). Fruit twists of *E. pusilla* may be caused by differential cell elongation in the fruit pedicel due to rapid expansion of the ovary 8 to 9 WAP. Auxin fluxes are probably responsible for this. On the other hand, fruits twisted and resupinated only just before and after dehiscence in *P. equestris*. This could be caused by a similar mechanism as the fruit valve coiling of *A. thaliana* during dehiscence. In *A. thaliana*, silique coiling is caused by differential drying of the inner and outer layers which generates tension that is released by the loss of adhesion between the cells of the DZ (Vaughn et al., 2011). The variation in timing and direction of resupination in orchid fruits hypothetically suggests that resupination is triggered by an environmental factor such as gravitropism which is regulated by complex molecular and biochemical events affecting the cell structure and water turgor. These hypotheses need to be confirmed by empirical testing.

A simple test to assess whether gravitropism induces resupination of orchid aerial roots can be carried out by removing the root cap and observing whether root resupination still occurs. Mass spectrophotometry can be used to measure the levels of plant hormones, particularly auxin, in resupinated or twisted tissues for comparison with non-resupinated tissues. Differential cell growth in resupinated tissues could be visualized using histology. Whole transcriptome analysis of resupinated and non-resupinated tissue could be done to pinpoint genes related to gravitropism, hormone production, and fruit dehiscence to confirm the involvement of these genes in resupination. Once more information on resupination-related genes in orchids has been obtained, in situ hybridization and VIGS could be used to confirm interaction between proteins and discover more about the function of these genes in specific fruit and root tissues.

7.4 EVOLUTION AND DEVELOPMENT OF ORCHID INFLORES-CENCES

Floral architecture, color, and shape are essential traits of the commercial orchid genus *Phalaenopsis* for the global floricultural market, both for cut flowers as well as pot plants. An important commercial characteristic is the orientation of the inflorescence. The inflorescence of *Phalaenopsis* consists of a peduncle, raceme, pedicels, and flowers. Being a monopodial orchid, the inflorescence in *Phalaenopsis* develops from a single vegetative shoot. It can be pendant, arching, sub-erect, or erect (Fig. 6.1, **chapter 6**) to ensure flowers and fruits are presented in the most optimal way for pollination, water and nutrition uptake, and seed dispersal. *Phalaenopsis* species and hybrids with erect inflorescences are suitable parents for a breeding program to produce new hybrids with a strong and erect inflorescence orientation. Existing hybrids produce so many flowers that their inflorescences need a clip and stick for support during development. Replacing these clips and sticks during plant growth needs to be done manually. Creating *Phalaenopsi*s hybrids with erect and strong inflorescences that no longer need support from a clip and stick during their development, can reduce maintenance and labor costs.

Lignin is known to have an important role in plant growth and provides mechanical strength (Liu et al., 2018). Lignin content has been an important trait in the development of new varieties with strong inflorescence in peony (Zhao et al., 2020) and lodging resistance in maize (Guo et al., 2021). However, the relationship between lignin content and inflorescence orientation in *Phalaenopsis* was unknown, as were topological and evolutionary trends in lignification patterns and orientations. To answer these questions, I investigated six natural species and 17 horticultural hybrids of *Phalaenopsis*. I surveyed the degree of lignification of six different positions across the peduncle and rachis on the basal, middle, and apical parts of inflorescences of four *Phalaenopsis* hybrids. I calculated the variation of lignin among these samples to understand whether this character is heritable and could be passed on to the next generation. Furthermore, I placed lignification in a phylogenetic context using ASR (**chapter 6**)

Lignin deposition in the inflorescence of *Phalaenopsis* is concentrated in sclerenchyma cells and fiber caps. Sclerenchyma cells are situated in the hypodermis layer, while fiber caps are located in the upper vascular bundles (see Fig. 6.5, **chapter 6**). I discovered that the amount of lignin among *Phalaenopsis* species and hybrids varies, with erect inflorescences having the highest degree of lignification. Topologically, the highest degree of lignification was found at the basal parts of the peduncle. In contrast, the lowest degrees were found at the apical parts of the rachis, corresponding with the age of the tissues inves-

Chapter 7

tigated. ASR suggested that closely related species appear to have the same inflorescence orientation and degree of lignification. An erect and lignified peduncle and rachis are ancestral characters in *Phalaenopsis*. Heritability analysis indicated that the lignification trait is highly heritable, meaning that the genotype predominates over the environmental effect.

I recommend extending the sampling to more *Phalaenopsis* species to determine which ecological environments favor lignification and other inflorescence orientation traits. Only then will it be possible to assess whether these characters are conserved phylogenetically throughout the genus *Phalaenopsis*, or whether environmental factors are key drivers of the diversity in inflorescence orientation and lignification within the genus.

My results cannot explain the organization, spatial arrangement, network structure and phytochemical content of lignified tissue. Understanding these aspects would provide a more complete picture of how lignin affects the orientation of an inflorescence. It is also important information for follow-up studies on genes associated with depositing a specific type of lignin in a particular location. The peduncle should be examined from fully developed inflorescences with 40-50% of flowers open. I recommend to choose species and varieties with high lignin content and an erect inflorescence, such as *Phalaenopsis viridis* and *P.* "Purple Gem" and compare these with *P. javanica* or *P. violacea var.* "Alba", of which the peduncle is much less lignified and pendant.

To improve the view of the specimen and virtually expose a plane in any direction (Brodersen, 2013), a 3D CT scan could show the organization of lignified tissue from the basal part of the peduncle to the top of the rachis. Such 3D scans could be dissected to produce 2D plane images, as has been done for flowers of *Phalaenopsis* (See Figs. 3.11 and 3.12).

The phytochemistry of the inflorescence could also be investigated to provide additional information on the chemical composition of the lignified tissue at different inflorescence positions and orientations. Laser ablation direct analysis in real-time imaging-mass spectrometry (LADI-MS) has been used to identify a wide variety of molecules (Fowble et al., 2018, 2017) including lignin in woody plants (Deklerck et al., 2022).

Future comparative transcriptome investigations of inflorescences will be required to uncover genes involved in lignification and orientation to elucidate the genetic basis of these traits. The results of 3D CT scans and LADI will be essential to specify the location and type of lignin in an inflorescence sample prior to RNA extraction. Specification of lignin-related compounds and tissues is important to determine candidate genes that may be associated with a particular tissue, to avoid having to deal with hundreds of lignification-related genes during data processing.

My PhD thesis on the evolution and development of orchid floral organs, fruit dehiscence, resupination, inflorescence orientation, and lignification has provided new insights into the origin, development, genetic basis, and evolution of these traits. Future studies along the lines suggested above will help to answer more questions about the origin of the fascinating diversity of orchid morphology.