# Evolution and development of flowers, fruits and inflorescences of Phalaenopsis and other orchid species

Pramanik, D.

# Chapter 1

## General introduction

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## 1.1 BRIEF INTRODUCTION ON FLOWERS AND ORCHIDS

Angiosperms, or flowering plants, arose between 140 and 250 million years ago during the early Cretaceous (Beaulieu et al., 2015; Foster et al., 2017; Magallón et al., 2015). The angiosperm flower is diverse, but it typically consists of perianth organs (sepals, petals) surrounding the reproductive organs (stamens and carpels). Reconstruction of the ancestral character states of angiosperms revealed that the perianth and stamens were organized in whorls as opposed to spirals (Sauquet et al., 2017) (Fig. 1.1A-B). Two possible evolutionary pathways that led to spatial re-organization of the perianth from spiral into whorls in angiosperms are a reduction by loss of complete whorls (Hoekstra et al., 2018; Mitchell and Diggle, 2005; Sauquet et al., 2017) or reduction by merging or integration of floral organs concurrent with an increase in the number of organs per whorl (Armbruster et al., 2014, 2009; Sauquet et al., 2017) (Fig. 1.1A-F). Reduced floral whorls promoted organ synorganization, resulting in increased spatial and functional complexity (Endress, 2011a, 2011b). Synorganization of angiosperm flowers resulted in spectacular species radiations (Endress, 2016).

## 1.2 EVO-DEVO STUDIES IN ORCHIDS

Evolutionary developmental biology (evo-devo) investigates changes in developmental pathways and processes that occur during the evolution of a particular lineage through comparative analysis (Moczek et al., 2015). Variations in the expression of regulatory genes contribute to morphological innovations (Prud'homme et al., 2007). A mutation of a regulatory gene enables the alteration of protein structures, which in its turn alters protein-protein interaction, these changes will eventually cause the adaptation of plant forms into new ones (Specht and Howarth, 2015).

The frequent occurrence of gene duplications, which play a crucial role in developing morphological and functional variety, is a significant factor driving the evolution of plant transcription factors (Teichmann and Babu 2004). Gene duplication can result from local events such as tandem duplication (Jander and Barth, 2007), transposable elements (Lisch, 2013), or more significant genomic events such as the duplication of chromosomal regions or the duplication of entire chromosomes, often known as whole genome duplication (WGD) (Soltis and Soltis, 2009). Duplicated genes are referred to as paralogs, denoting their homology. Paralog genes can persist in the genome by being involved in transcriptional regulation, signal transduction, and development through sub-functionalization or neofunctionalization (Aagaard et al., 2006). Sub-functionalization occurs when

an ancestral function is shared among duplicate copies, whereas neo-functionalization occurs when one paralogue can acquire a new function (Lynch and Force, 2000). There are two WGD events in the orchid genome, the first is an ancient WGD in the common ancestor of monocots (Jiao et al., 2014; Ming et al., 2015; Zhang et al., 2021). The more recent WGD represents an independent event, specific to the Orchidaceae lineage (Zhang et al., 2021). These duplication events led to functional redundancy or sub-functional differentiation of many genes (Cai et al., 2015; Zhang et al., 2021).

A thorough comparative analysis of the functional diversity of gene families has been conducted for several orchid model species. According to Chang et al. (2016), selecting a plant as a model necessitates the evaluation of three critical aspects. The first is economic importance; the second is intrinsic laboratory properties, such as tiny size, ease of culture, fecundity, and quick generation time; and the third is susceptibility to genetic manipulations, small genome size, and the ability to change gene activity. *Phalaenopsis equestris* is a common orchid utilized as a model plant because it is economically important and serves as a parental line for many hybrids, particularly those used to generate the multiflora variety types (Hsu et al., 2021). *P. equestris* comprises three varieties and hybrids of various flower colors and sizes (Hsu et al., 2021).

The complete genome (see Table 1.1) (Cai et al., 2015) and transcriptome assembly data of *P. equestris* (Klepikova et al., 2021; S. C. Niu et al., 2016) have been published and are available in an online database (Chao et al., 2017; Hsiao et al., 2021; Lee et al., 2018). Various transcriptomics studies on *P. equestris* have been conducted. In addition to *P. equestris*, *Erycina pusilla* is an emerging orchid model. It is an epiphytic orchid that can be grown and bred in tissue culture and has a relatively short life cycle (less than half a year) (Bolaños-Villegas et al., 2021; Dirks-Mulder et al., 2017; Yeh et al., 2017). The following section will briefly discuss the evolution and development of genes elucidated so far that are responsible for orchid flower, fruit, and inflorescence diversity.

### 1.2.1 Evo-devo studies of orchid floral organs

The morphology of orchid flowers is very diverse, with a typical flower consisting of two whorls of petaloid organs surrounding the reproductive organs (Figs. 1.1E and 1.1F). The reproductive organ is specialized with a compound structure formed by the fusion of male and female organs, called the gynostemium (Dressler, 1993; Rudall and Bateman, 2002). Variation of a flower in the orchid family is present as different shapes and sizes of the sepals and petals gynostemium, anthers, type and number of pollinia, and the presence or absence of highly specialized additional floral structures such as a callus, mentum and

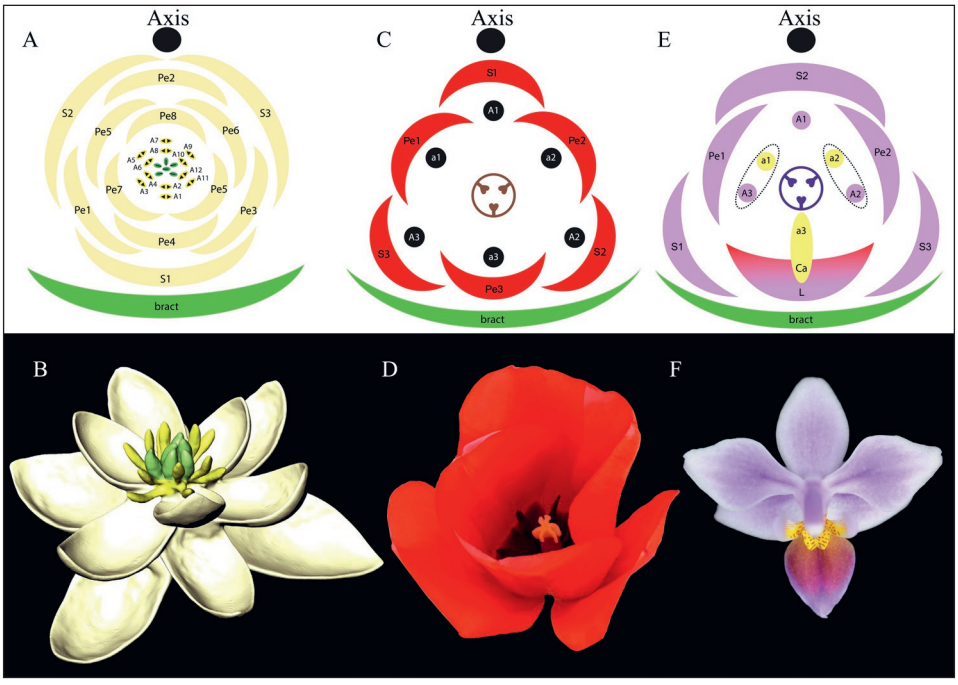


**Figure 1.1. Diagrams representing a cross section with accompanying frontal view of different flowers.** (**A-B**) Early angiosperm flower as reconstructed by Sauquet et al. (2017). (**C-D**) Flower of *Tulipa* representing early monocots. (**E-F**) Flower of *Phalaenopsis equestris* representing Epidendroid orchids. *Abbreviations: A/a=anther; Ca=calllus; L=labellum; Pe=petal; S=sepal.*

stelidia. Callus refers to a specialized structure found on the lip or labellum of an orchid flower. The callus often acts as a landing platform for pollinators, providing them with a stable surface to land on while they access the flower's reproductive organs (Carmona-Díaz and García-Franco, 2009). While stelidia are elongated projections or appendages found on the lip or labellum of orchid flowers (Kurzweil and Kocyan, 2002). Stelidia serves several functions, including guiding pollinators towards the flower's reproductive structures, increasing visibility and attractiveness to specific pollinators, or facilitating the transfer of pollen. Lastly, the mentum is a distinct structure located at the base of the gynostemium, labellum, and lateral sides of the lateral sepals (Dressler, 1990). The mentum functions as a hinge to strike an insect that traverses the labellum, directing it towards the upper portion of the gynostemium, ensuring that the pollinia are precisely removed and/or deposited at a particular position on both the pollinator and the orchid flower. The development of these structures in orchids is thought to be results from coevolution between the plants and their pollinators. However, the evolution and development of all these orchid flower structures is far from known yet.

**Table 1.1 Whole orchid genomes sequenced to date.**

| Family | Genus | Species | Chromosome number | Sequencing technology | Genome size | Number of protein coding genes | Reference |
|---|---|---|---|---|---|---|---|
| Apostasioideae | *Apostasia* | *A. shenzhenica* | 2N=2X=68 | Illumina HiSeq 2000 | 349 Mb | 21,841 | Guo et al. 2017 |
| Apostasioideae | *Apostasia* | *A. ramifera* | na | Illumina HiSeq 2001 | 0.37 Gb | 22,841 | Zhang et al. 2021 |
| Epidendroideae | *Dendrobium* | *D. catenatum* | 2N=2X=38 | Illumina HiSeq 2000 | 1.11 Gb | 28,910 | Zhang et al. 2016 |
| Epidendroideae | *Dendrobium* | *D. officinale* | 2N=2X=38 | Illumina HiSeq and PacBio | 1.35 Gb | 35,367 | Yang et al 2015 |
| Epidendroideae | *Gastrodia* | *G. elata* | 2N=2X=36 | Illumina HiSeq and PacBio | 1.18 Gb | 18,969 | Yuan et al. 2018 |
| Epidendroideae | *Phalaenopsis* | *P. equestris* | 2N=2X=38 | Illumina HiSeq 2000 | 1.6 Gb | 29,431 | Cai et al. 2014 |
| Epidendroideae | *Phalaenopsis* | *P. Brother Spring Dancer 'KHM190'* | 2N=2X=38 | Illumina HiSeq 2000 | 3.45 Gb | 41,153 | Huang et al 2017 |
| Epidendroideae | *Phalaenopsis* | *P. aphrodite* | 2N=2X=38 | Illumina HiSeq 2000 | 1.2 Gb | 28,902 | Chao et al. 2018 |
| Vanilladeoideae | *Vanilla* | *V. planifolia* | 2N=4X=24 | NextSeq | 2.26 Gb | na | Hu et al. 2019 |
| Epidendroideae | *Cymbidium* | *C. sinensis* | 2N=2X=40 | Illumina HiSeq 2000 | 3.52 Gb | 29,638 | Yang et al. 2021 |
| Epidendroideae | *Cymbidium* | *C. ensifolium* | 2N=2X=40 | Illumina HiSeq 2500/PacBio Sequel Platform | 3.56 Gb | 29,073 | Ai et al. 2021 |

*Abbreviations: Gb= Gigabase; Mb= Megabase.*

The genetic basis of orchid flowers can be explained with several models. Coen and Meyerowitz (1991) first proposed the ABC model, a theory regarding floral organ identity. This model was derived from genetic analyses of *Antirrhinum majus* and *Arabidopsis thaliana*. According to the ABC model, distinct organ identities are determined by a unique combination of homeotic 'A,' 'B,' and 'C' gene activities within each floral whorl encoded by MADS-domain transcription factors. Then the ABCDE model was proposed to complete the original ABC model. In the ABCDE model, the MADS box gene *APETALA1* provides the A class function (*APETALA1/AP1*), *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) provide the B class function, *AGAMOUS* (*AG*) provides the C class function, *SEEDSTICK* (*STK*) provides the D class function, and *SEPALLATA* (*SEP*) provides the E class function (Bowman et al., 2012). Interaction of MADS box protein complexes controls the development of floral whorls. Class A+B+E control the formation of sepals, A+B+E control the development of petals, B+C+E control the development of stamens, C+E determine the carpels, and D+E control the development of ovules (Bowman et al., 2012). The ABCDE model was subsequently applied to *A. thaliana* floral development and evolution to create the "floral quartet model" (Theißen and Saedler, 2001). According to this model, identity of the sepals, petals, stamens and carpels is determined by four combinations of floral homeotic proteins known as MADS box proteins: *AG, AP1, AP3, PI,* and *SEP.* These models were further expanded to explain orchid floral development with the combined action of four A*P3*-like genes and one *PI*-like gene (B- class genes) in regulating the formation of petal and stamen identity (Mondragón-Palomino and Theissen, 2008; Mondragón-Palomino et al., 2009) (Fig. 1.2A). In addition to the "orchid code" model, the Homeotic Orchid Tepal "HOT" model was proposed by highlighting the role of four *AP3* genes (B-class) and *GLO*-like/*PISTILATA* gene. These ortholog *AP3* lineages play different roles in regulating *P. equestris* perianth organ identities, particularly in distinguishing petal and labellum development programs (Pan et al., 2011) (Fig. 1.2B). In a more elaborated model, Hsu et al. (2015) proposed the "Perianth code mode" or "P-code" model demonstrating antagonism between two protein complexes containing different copies of *AP3/PI/AGL6* in determining perianth complex development while *OPI* serves as a base for perianth identity (Fig. 1.2C). This model has been validated in *Oncidium* Gower Ramsey, *P. equestris* and other hybrid species. According to this model, the sepal-petal complex (SP) promotes sepal/petal identity. The lip complex (L) determines labellum formation (Hsu et al., 2015) (Fig. 1.2C). Dirks-Mulder et al. (2017) discovered more about the MADS-box genes involved in sepal differentiation in *E. pusilla*, which they summarized in the "Oncidiinae model".

The existing models for the development of orchid flowers cannot explain the evolution and development of callus, stelidia, mentum and other specialized orchid floral structures. The evolution of the labellum is a significant factor in the derivation of bilateral symmetry of orchid flowers. The MYB transcription factors *DIVARICATA* (*DIV*), *DIV*-and-*RAD-Interacting-Factor* (*DRIF*), and the small interfering peptide *RADIALIS* (*RAD*), together with TCP family genes *CYCLOIDEA* and *DICHOTOMA* (*DICH*), play a crucial role in establishing floral dorsoventral asymmetry in *Anthirrhinum majus* (Costa et al., 2005; Galego and Almeida, 2002; Raimundo et al., 2013). The role of these genes in shaping floral symmetry is currently being expanded to better understand the zygomorphic symmetry of orchid flowers. Phylogenetic and genomic studies have identified the copy number of MYB genes involved in floral symmetry of *Orchis italica*, including eight copies of *DIV*, four copies of *RAD*, and two copies of *DRIF* (Valoroso et al., 2017). Expression studies discovered that *DIV, DRIF*, and *RAD* proteins had higher expression levels in the labellum than in the other perianth parts (Valoroso et al., 2019). Similar gene expression patterns were also seen in peloric orchid flowers (*Phalaenopsis* Joy Fairy Tale), where labellum-like structures substitute the lateral inner tepals (Valoroso et al., 2019).

### 1.2.2 Evo-devo studies of orchid fruits

After pollination, the flower wilts, and the fertilized ovary develops into a fruit (Arditti, 1992). The three formerly fused carpels form six valves, three of which are fertile and bear a placenta with ovules, while the remaining three are sterile (Rasmussen and Johansen, 2006). Orchid fruits are generally classified as capsules, berries (Kocyan and Endress, 2001), or pods (Karremans et al., 2023; Pridgeon et al., 1999). Capsule fruit is typically dry and dehiscent, meaning it splits open when mature to release the seeds. Once the capsules split open, tiny orchid seeds are dispersed into the surrounding environment, employing various strategies to enhance seed dispersal and increase the chances of establishing new individuals in different habitats. Most orchid seeds are dispersed by wind (Arditti and Ghani, 2000). Orchid seeds have large internal air spaces that allow them to float in the air column (Arditti and Ghani, 2000), facilitating their movement through the air. When released, they can be carried by even gentle air currents, aided by structures like feathery hairs (Fan et al., 2020). Pod-type fruits tend to remain intact until they are ripe. In contrast to dust-like seeds with fragile, thin seed coats (McCormick et al., 2012), fleshy indehiscent pods with thick seed coats can withstand endozoochory ingestion (Jordano, 1995). The fruit pods may possess attractive colors, shapes, or scents that entice particular

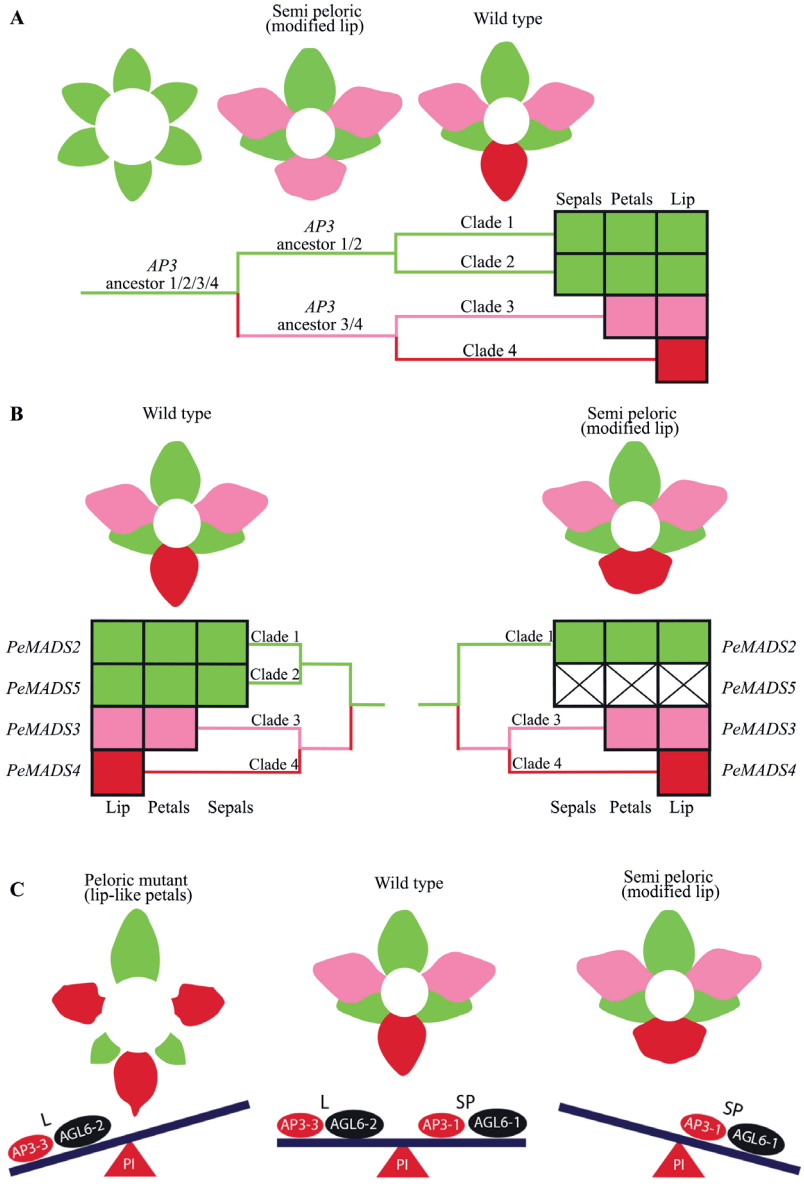


**Figure 1.2. Different models of floral organ development in orchids.** (**A**) Orchid code, (**B**) HOT model, (**C**) Perianth code model. *Color codes: light green= sepals; pink= petals; red= lip. Abbreviations: AP3= APETALA3; AGL6= AGAMOUS-LIKE6; PI=Pistillata; L=lip complex; SP=sepal complex.*

animals. For these orchids, seed dispersal often relies on external agents, such as mammals (Karremans et al., 2023; Pansarin & Suetsugu, 2022), birds (Karremans et al., 2023; Suetsugu et al., 2015; Zhang et al., 2021), or insects (Lozano Rodríguez et al., 2022; Suetsugu, 2018a, 2018b, 2020). These animals aid in seed dispersal by consuming the fruit or transporting the seeds on their bodies or through their feces. As an illustration, seeds of dehiscence fruits of *Vanilla* fruits are collected and dispersed by *Euglossine* and *Meliponine* bees. On the other hand, in species like *V. planifolia* and *V. pompona,* rodents and marsupials act as dispersal agents for fully developed, unopened fruit pods that fall to the ground (Karremans et al., 2023).

Fruit studies in orchids have mostly been done on post-pollination ovule development (Mayer et al., 2021; Niimoto and Sagawa, 1962; Sagawa and Israel, 1964; Yeung, 2017) and fruit characters related to seed dispersal (Pansarin and Suetsugu, 2022; Suetsugu et al., 2015; Suetsugu, 2018a, 2020). However, evolution and development of ovules, capsules, and seeds is less reported. Especially interaction among different genes and the regulatory connectors on ovule and fruit developmental programs still needs further study. These studies also do not provide detailed information on fruit dehiscence.

### 1.2.3 Tracking orchid resupination

Plants can perceive and respond to the environment surroundings through observable motions to optimize their survival, development, and reproduction. There are two types of movement: tropic and nastic (Darwin and Darwin, 2009). The tropic movement is a growing movement in plants in which the direction of the external stimulus is a determining factor. In contrast, the nastic movement is a non-growth movement where the external stimulus is not determinant. Tropic movement includes hydrotropism (Jaffe et al., 1985), phototropism (Iino, 2001; Whippo and Hangarter, 2006), chemotropism (Izzo et al., 2019), thigmotropism (Simmons et al. 1995; Moulton et al. 2020), and geotropism (Wilkins, 1966). The type of movement toward these stimuli includes bending and twisting. Bending occurs when there is uneven growth on two sides of an organ (Friml et al., 2002). On the other hand, twisting refers to the rotation of a cross-section around the central axis. Stimulus-induced twisting has been referred to using various terms, such as resupination (Dines and Bell, 1994; Harley et al., 2017) and torsion (Borchers et al. 2018). When twisting occurs over an angle of more than 180 degrees this is called resupination.

Resupination in orchid flowers mostly occurs just before anthesis, but in some orchids, the flowers de-resupinate following pollination. The bud will make a 180 degree turn so that the labellum faces downwards to facilitate pollination, as the labellum serves

as a landing platform for pollinators (Nyman et al., 1984). It is assumed that resupination evolved after the flower became zygomorphic (Mondragón-Palomino and Theissen, 2009). This scenario raises questions about the molecular mechanisms linking zygomorphy with resupination.

Besides orchid flowers, other structures can also rotate during development, altering the angle at which a root, leaf, or fruit is oriented compared with the horizon. Due to this rotation, roots, leaves, flowers, and fruits are placed in optimal positions for capturing sufficient sunlight, water droplets, the attention of pollinating insects, or gusts of wind. Fruits in orchids can for instance resupinate to face different directions to maximize seed dispersal. Leaves in orchids resupinate to optimize sunlight uptake by rotating the leaves into such angles that both sides of the leaf area are exposed to sunlight equally. Roots of *E. pusilla* resupinate to optimally absorb sunlight, nutrients, and water from its environment. The exact moments and direction in which an orchid root, leaf, or flower rotates differs and are still poorly known.

### 1.2.4 Orchid inflorescence orientation and lignification

The inflorescence of monopodial orchids develops from an axillary bud adjacent to the vegetative bud. The inflorescence is branched, and flowers develop in succession or simultaneously (Freudenstein and Rasmussen, 1999). Inflorescences are a vital structure to support flowers and fruits. In *Phalaenopsis*, inflorescence strength may be associated with its orientation and degree of lignification.

Lignin is one of the major components of plant cell walls. It is formed by the direct deposition of polyphenolic compounds present in secondary cell walls of various cell types, notably sclerenchyma fibers, and sclereids are scattered across many different tissues and organs (Barros et al., 2015). To a lesser extent, lignin is also a major component of the cell wall in xylem cells that promote long-distance transport of water and minerals in inflorescences (Schuetz et al., 2014). As a complex phenolic polymer, lignin also increases plant cell wall rigidity, enhances hydrophobic characteristics, and facilitates mineral transport across a plant's vascular bundles (Liu et al., 2018).

The inflorescence stem orientation is one of the important breeding traits in *Phalaenopsis*. Breeders put efforts into producing a sufficiently strong and flexible inflorescence stem of *Phalaenopsis* orchids with an upright or erect orientation. Different inflorescence stem orientations in response to light have been defined in orchids, i.e., erect, sub-erect, arching, and pendant (Christenson, 2009). It is not yet known whether orientation and topology are phylogenetically correlated.

## 1.3 AIMS, RESEARCH QUESTIONS AND THESIS OUTLINE

This thesis focuses on increasing knowledge on the evolutionary development of orchid flower structures, fruit dehiscence zones, resupination of roots and fruits, and inflorescence orientation and lignification.

The aims outlined above were met by answering the following research questions.

What is the evolutionary origin of callus, stelidia and mentum, three highly specialized structures of *Phalaenopsis* flowers that are crucial for pollination by bees?

Which types of dehiscence zones exist in orchid fruits, what type is ancestral and what type is derived, and which genes are differentially expressed in fruits with lignified versus lipified dehiscence zones?

When does resupination of orchid roots and fruits start and when does it end? Are direction of twisting and torsion predictable and comparable among different species?

Does the degree of lignification of the inflorescence of *Phalaenopsis* follow any topological and phylogenetic pattern to further explore in future genetic precision breeding?

To answer these questions, I performed transcriptome analyses (**chapters 2, 3** and **4**), combined micro-macro morphological (3D CT scan, LM, and SEM), molecular (RNA-seq, RT-PCR, and qPCR), and phylogenetic approaches (**chapters 3, 4 and 6**), produced 3D-CT scans and photographs and incorporated these in a bioinformatics pipeline (c**hapter 5**).

The coherence among the different chapters of this thesis is schematically presented in Fig. 1.3. In **chapter 1**, evolution of flowers, fruits and inflorescences of angiosperms in general is briefly described. This chapter also highlights the need for more knowledge about orchids, one of the most diverse groups of angiosperms. In **chapter 2,** I describe transcriptomics as a tool to increase knowledge for lesser studied angiosperms such as orchids. Evolution and development of three highly specialized floral structures involved in pollination of *Phalaenopsis* flowers is studied in **chapter 3** to answer the first research question. **Chapter 4** presents a comparative study on evolution and development of orchid fruit dehiscence zone formation to answer the second research question. In **chapter 5**, bending and twisting of developing orchid roots and fruits, also called resupination, is studied to answer the third research question. **Chapter 6** presents an analysis of different lignification patterns of inflorescences in the orchid genus *Phalaenopsis* to answer the last research question. The outcomes of all separate chapters are synthesized and discussed in **chapter 7**.

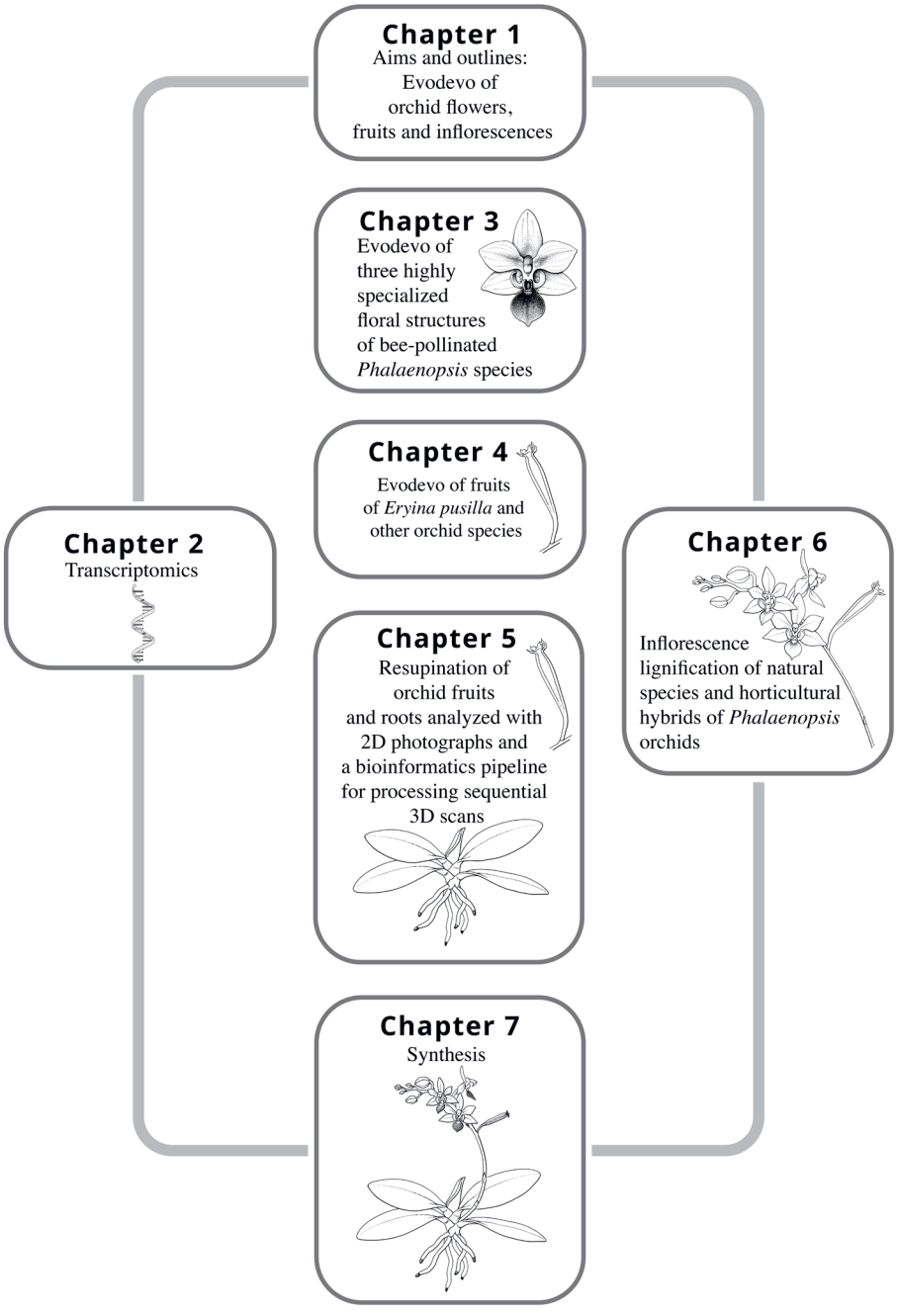


**Figure 1.3 Schematic diagram of the coherence among the different chapters of this thesis.**