# Evolution and development of orchid flowers and fruits

Dirks, A.

# Chapter 1

## General introduction

## Evolution of flowering plants

Flowering plants or angiosperms dominate the terrestrial flora. With more than 350,000 species they make up about 90% of all living plant species. This was not always the case, as flowering plant first evolved 150 million years ago (mya) from non-flowering plants (Soltis *et al*., 2008;Doyle, 2012;Brown and Smith, 2018;Magallon *et al*., 2019). The non-flowering and thus more ancient group of living seed plants, commonly known as gymnosperms contain just over 1000 species. The gymnosperms possess male and female cones, while the angiosperms evolved several key innovations such as the flowers containing stamens (male reproductive organs) and carpels (female reproductive organs with ovules) surrounded by sepals and petals.

Understanding the mode and mechanisms of angiosperm evolution is a central challenge of the field of plant evolutionary developmental biology (evo-devo). Explanation of the apparently ''sudden'' origin ~150 mya and the early diversification of the angiosperms, as revealed by fossils, proved to be difficult because fossils with reproductive structures intermediate between ancestral gymnosperms and angiosperms are lacking. The sudden appearance of angiosperms in the fossil record puzzled many scientists including Charles Darwin. In 1879 he wrote a letter to Joseph Hooker, the director of the Royal Botanic Gardens in Kew, UK, stating that ''The rapid development, as far as we can judge, of all the higher plants within recent geological time is an abominable mystery" (Darwin *et al*., 1903). Molecular dating methods suggest that flowering plants are much more ancient (Magallon, 2010;Magallon *et al*., 2019), estimating the origin of crown group angiosperms between 140 and 130 mya (Crane *et al*., 1995;Smith *et al*., 2010;Doyle, 2012;2014) but the current discrepancy between the oldest fossils and age estimates of molecular clock analyses is probably caused by the fact that the fossil record is far from complete.

Sauquet *et al*. (2017) made a reconstruction of the earliest common ancestor of flowering plants by modeling the distribution of floral organs present in modern angiosperms on their phylogeny. These authors concluded that the ancestral flower, like most modern flowers, was bisexual, had multiple whorls of petal-like organs arranged in concentric circles similar to a modern *Magnolia* flower. This ancestral flower went through a series of simplifications, in which organs were reduced or merged until it settled on an optimal and stable morphology. Once this basal morphology had evolved, it further diversified in selected clades into for instance bilateral symmetry. The latter is believed to optimize efficiency of interaction with pollinators (Sauquet *et al*., 2017). It is not known which animals might have eaten or pollinated the presumed ancestral flowers. Studies from fossilized dinosaur dung from 100.5-113.0 mya show that these animals ate angiosperms (Vajda *et al*., 2016).

Angiosperm flowers exhibit an enormous diversity, but usually contain four floral organs: sepals and petals - forming the perianth - stamens and carpels. The evolution of reward systems for animal-pollinated flowers allowed for species diversifications in many different clades by a high variety of pollinating animals and other vectors. Within the angiosperms, orchids are one of the most species-rich

families. They are very diverse in terms of floral shape, size and color. Reproductive isolation of many orchid species occurred through highly specialized interactions with pollinators. Unique floral organs, such as modified sepals and petals, a callus on a modified median petal (the lip) and a gynostemium with wing-like structures (stelidia), allow efficient pollen transfer via specific body parts of pollinators. Orchid flowers are composed of five whorls of three parts each, including two perianth whorls (sepals and petals), two staminal whorls (gynostemium and stelidia) and one carpel whorl (**Figure 1**).

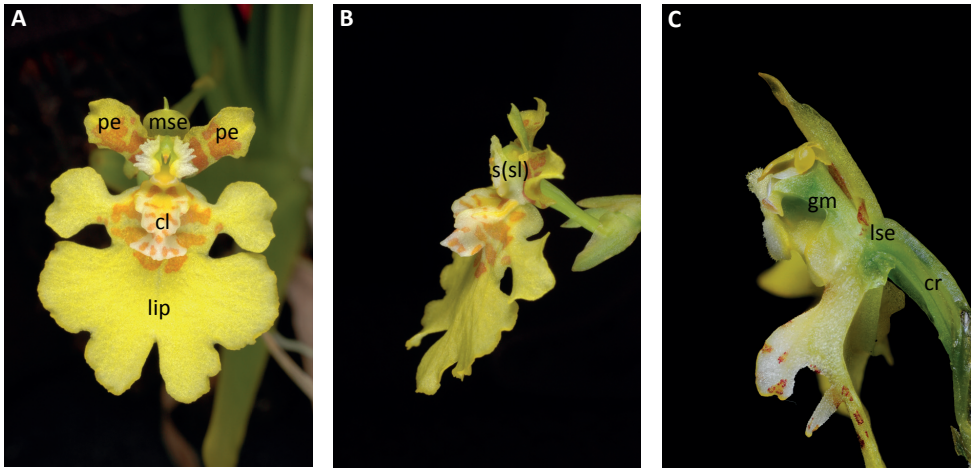


**Figure 1. Flower of the orchid species *Erycina pusilla*. (A)** Frontal view. **(B)** Lateral view. **(C)** Transversal section through the flower (one petal and part of the lip were removed). cl = callus; mse = median sepal; lse = lateral sepal; pe = petal; gm=gynostemium; cr = carpel; s(sl) = stelidium (photos by Joel McNeal (A,B) and Jean Claessens (C)).

"Why are orchids so diverse?" is a question that scientists have been wondering about for many centuries. Charles Darwin for instance wrote an entire book about the various contrivances by which orchids are fertilized by insects (Darwin, 1862). To explain the abominable mystery of the origin and evolution of orchids, similar to the angiosperms in general, fossil surveys and molecular clock analyses were carried out (**Figure 2**). Ramirez *et al*. (2007) dated a fossil orchid pollinarium, carried by a worker bee preserved in Dominican amber from 15-20 mya, and concluded that the most recent ancestor of extant orchids lived 76-84 mya (**Figure 2 C-D)** . Another fossilized orchid pollinarium, this time carried by a fungal gnat preserved in Baltic amber from 45-55 mya (Poinar and Rasmussen, 2017), further confirmed this estimate of the origin of the orchids. Radiation of orchids began around 73 mya (Magallon *et al*., 2019). Multiple hypotheses exist about the main drivers behind the high diversity of modern orchid species. These include: (i) the evolution of highly specific pollination interactions, in which pollinia are deposited on very specific body parts of a few species of pollinators only, (ii) symbiotic associations with species-specific groups of mycorrhizal fungi (important for germination and seedling development), (iii) colonization of different epiphytic habitats and (iiii)

the development of multiple types of photosynthesis including Crassulacean Acid Metabolism (Gravendeel *et al*., 2004;Silvera *et al*., 2009;Givnish *et al*., 2015). All these factors likely contributed to the high diversity of orchids observed today.

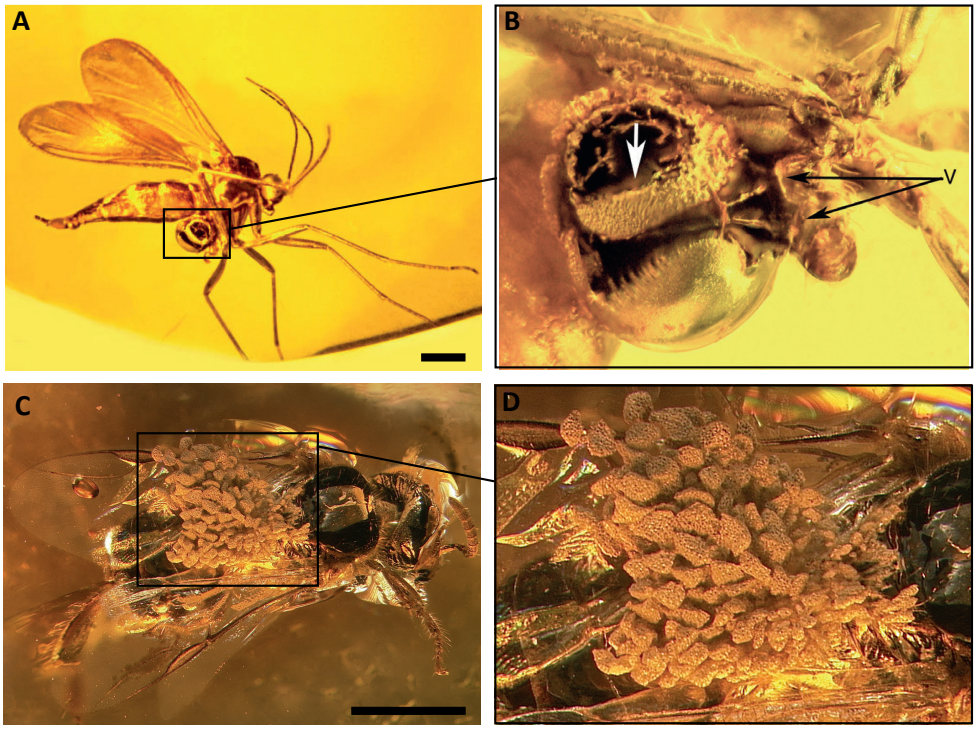


**Figure 2**. **Undisputed fossils of orchid flowers. (A-B)** *Succinanthera baltica* (Poinar and Rasmussen, 2017). **(C-D)** *Meliorchis carribea* (Ramirez *et al*., 2007).White arrow = pollinium. V = viscidia. Scale bars: 1 mm.

## Floral organ identity genes

Moyroud *et al*. (2017) eventually solved part of the puzzle of how angiosperm flowers evolved by studying the gymnosperm *Welwitschia mirablis*, a gymnosperm species with separate male and female reproductive structures organized in cones. These authors studied the genetic circuits that control the development of *Welwitschia* reproductive units and compared these circuits to those active in the cones. They discovered that the same developmental genes play not only a central role in the development of flowers but also in the cones of this gymnosperm species. A similar genetic cascade was found in angiosperms and two other gymnosperm genera: *Pinus* and *Picea*, indicating that this cascade was inherited from their last common ancestor. The results of this study show that flowers did not appear all of a sudden but that the developmental genes involved were probably already present, being inherited and reused during plant evolution. I will now summarize what we currently know about the most

important developmental genes driving angiosperm and orchid flower evolution.

The majority of the genes involved in floral organ identity belong to the family of MADS-box transcription factors. Their different interactions, resulting in the different floral organs, was first explained by the "ABC" model in *Arabidopsis thaliana* and *Antirrhinum majus* (Coen and Meyerowitz, 1991). According to this model, the development of sepals, petals, stamens and carpels are specified by class A, B and C MADS-box genes. Mutations in each class exhibit homeotic transformations of organ identity in two adjacent floral whorls: A class mutants have sepals transformed into carpels and petals into stamens; B class mutants have petals transformed into sepals and stamens into carpels; and C class mutants have stamens transformed into petals and carpels transformed into sepals. In Arabidopsis, genes corresponding to the three classes have been well characterized, *APETALA1 (AP1)* and *APETALA2 (AP2)* represent the A class, *APETALA3 (AP3)* and *PISTILLATA (PI)* the B class and *AGAMOUS (AG)* the C class. Later the ABC model was extended by D class MADS-box genes *SHATTERPROOF (SHP)* and *SEEDSTICK (STK)*, involved in ovule development (Angenent and Colombo, 1996), and E class MADS-box genes *SEPALLATA (SEP)* (Theissen, 2001), needed for petal, stamen and carpel development. MADS is actually an acronym derived from the initials of four loci: MCMI of the yeast *Saccharomyces cerevisiae*, AG of Arabidopsis, DEF of Antirrhinum and SRF of humans (*Homo sapiens*). MADS-box genes can be found in all eukaryotes and while the human genome contains only a few of these genes, most angiosperm genomes contain more than a hundred. Martinez-Castilla and Alvarez-Buylla (2003) recovered 104 MADS-box genes from the Arabidopsis genome, which can be divided in M-type and MIKC-type based on their protein domain structure (**Figure 3**). The M-type proteins only have the MADS-domain in common and are involved in seed and female gametophyte development (Masiero *et al*., 2011), while the MIKC-type genes share four conserved domains (Alvarez-Buylla *et al*., 2000;Henschel *et al*., 2002;Nam *et al*., 2004). The MADS (M) domain contains around 60 amino acids and is involved in DNA binding and protein dimerization. The intervening (I) and keratin-like (K) domains are critical for dimerization and tetramerization with other MADS-domain proteins. The C-terminal (C) region contains short, highly conserved clade specific motifs and is involved in the formation of higher-order protein complexes (Riechmann *et al*., 1996;Honma and Goto, 2001;Smaczniak *et al*., 2012).

Tetrameric protein complexes of MIKC-type proteins, according to the Floral Quartet Model, specify the identity of different floral organs. The different quartets probably function as transcription factors of the DNA of the target genes. By activating or repressing these genes, the quartets control the development of the floral organs. For example, AP3, PI, AG and SEP MIKC-type proteins form a quartet that controls the development of stamens and AP3, PI, AP1 and SEP proteins form a quartet that controls the development of petals (Theissen and Saedler, 2001;Smaczniak *et al*., 2012).

Duplications, followed by sub-functionalization, have been suggested to lead to several homologous and paralogous lineages in different plant groups outside the core eudicots. For example the B-class genes, which are

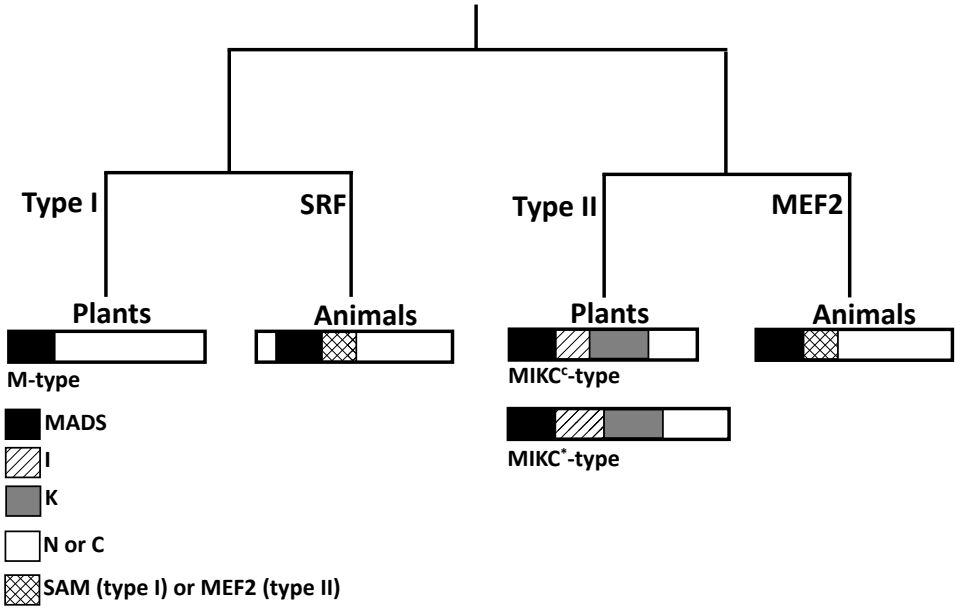


**Figure 3. Domain structures of type I and II MADS-box genes in plants and animals.** Adapted from (Nam *et al*., 2004).

highly conserved in members of the core eudicots, including the model species Arabidopsis, Antirrhinum and Petunia (Angenent *et al*., 1992;Jack *et al*., 1992;Kramer, 1998) were subjected to several duplication events during plant evolution. Before the origin of the angiosperms, gene duplications not only created the *AP3* and *PI* lineages, which are present in all angiosperms, but occurred also in the other MADS-box gene classes (Litt and Irish, 2003;Kramer *et al*., 2004). Duplications, followed by sub-functionalization, are suggested to have led to several homologous and paralogous lineages in different plant groups outside the core eudicots. Duplications of an ancestral MADS-box B-class gene were found to be responsible for the creation of euAP3 and TM6 lineages in core eudicots and B-sister lineages in the gymnosperm *Gnetum gnemon* (Becker *et al*., 2002;Gioppato and Dornelas, 2018) and monocots (Chang *et al*., 2010;Yang *et al*., 2012).

In the orchid lineage, additional duplications have occurred in the B-class lineage, resulting in more (sub-functionalized) genes that may be in part responsible for the enormous flower diversity in the orchid family. The class B MADS-box genes are central to the specification of petal and stamen identity. Most eudicots have two B-class genes, *AP3/DEF-like* and *PI/GLO-like*. However, gene duplication events in orchids have generated several paralogs, in particular of AP3-like genes. In case of *AP3*, a duplication event first gave rise to the AP3A and AP3B clades. Further duplication resulted in four sub-clades, namely AP3B1 (clade 1), AP3B2 (clade 2), AP3A1 (clade 3) and AP3A2 (clade 4). These four sub-clades are found in different orchid subfamilies. In addition to the ABCDE model, the "Orchid Code" and "Homeotic Orchid Tepal (HOT) model" were proposed, both describing the expression of AP3

genes during orchid perianth formation. According to these models, B-class genes are expressed in floral whorl two (petals), following the ABCDE model, but extended to whorl 1 (sepals) when the morphology of the sepals and petals is more or less similar, with expression of a lip-specific copy restricted to this organ (Mondragon-Palomino and Theissen, 2008;Pan *et al*., 2011).

## *Erycina pusilla* as emergent model system for orchid evo-devo research

Arabidopsis (Brassicaceae, Eudicots) is by far the most popular flowering plant model system and widely studied already for over a century. It was the first species of which the genome was fully sequenced because this is relatively small (The Arabidopsis Genome, 2000). Discoveries in this species have proven to be widely applicable to many other plant species. However, based on our knowledge of Arabidopsis only not all plant developmental processes can be understood. The flowers of the monocot orchid species for instance, are very different from the Arabidopsis flowers. Several modifications had to be made to the ABCDE model in the form of the "Orchid code" and "HOT" models as discussed above, and new models discussed and proposed in this PhD thesis.

During a visit in 2006 to Elena Kramer of Harvard University in the United States, my co-promoter Barbara Gravendeel decided to develop a model system for evo-devo research on orchids. Various orchid species were carefully considered for this. What makes a plant a good plant model system? Based on the Field Guide to Plant Model Systems by Chang *et al*. (2016), different properties should be taken into account. For laboratory use, small sized plants, which are easy to culture, with a short generation time and high fecundity are preferential. The capability of self-fertilization for maintenance, being susceptibility to genetic manipulations such as crossing and mutagenesis by for instance UV-irradiation, chemicals, a small-sized diploid genome (preferably fully sequenced and annotated) and the ability to manipulate gene function are more intrinsic properties of a plant species to make it suitable as model system. When laboratory procedures are standardized for e.g. gene transformation, more research institutions will use the model and develop community properties, such as stock centers with genetic strains, reporter gene constructs but also databases with gene annotations, sequences which can be downloaded, and bio-informatics tools (e.g. Blast, gene search, GO and KEGG pathways, chromosome map tools).

Most orchids have long life cycles (~3–5 years), large chromosome numbers and complex genomes, which make functional studies difficult. The final choice therefore fell on the meso-American twig epiphytic species *Erycina pusilla* (Epidendroideae, Oncidiinae), which is easy to maintain and propagate *in vitro*. This species has a low diploid chromosome number (2n, n=6); a relatively small sized genome (1C = 1.5 pg), a short juvenile phase (less than a year from seed to flowering stage) and can complete its life cycle *in vitro* (Chase *et al*., 2005;Felix and

Guerra, 2012;Dirks-Mulder *et al*., 2017). An on-line transcriptome database for *E. pusilla* is available (Chao *et al*., 2017), as well as a protocol for transformation with Agrobacterium, although the efficiency is still low and published only once (Lee *et al*., 2015). Several labs in the world are now using this emergent orchid model, for instance to study MADS-box gene evolution (Lin *et al*., 2016) and the genetic basis of crassulacean acid metabolism (Heyduk *et al*., 2018). In my PhD project, I tried to answer the question 'Why are orchids so diverse?' by carrying out evo-devo research with *E. pusilla*. Below, I will summarize the main results.

## Aim and outline of this PhD thesis

The goal of this PhD project is to gain more insight into the evolutionary development of orchid flowers and fruits by studying the emergent orchid model plant *E. pusilla* with a combination of micro- and macromorphological, molecular and phylogenetic techniques. Research on orchid flowers is described in **chapters 2 and 3.** In **chapter 2** the formation of the lip, a highly modified petal present in most orchid flowers, is described (Gravendeel and Dirks-Mulder, 2015) in line with work from Hsu *et al.* (2015), who proposed the "P-code" model, we found that two different developmental gene complexes are involved in either sepal/petal or lip formation. In **chapter 3** (Dirks-Mulder *et al*., 2017) the evolutionary origin is investigated of three other highly specialized orchid floral organs of *E. pusilla*: the median petaloid sepal, the callus on the lip, and the stelidia along the gynostemium. We discovered that these organs are derived from a sepal, a stamen that gained petal identity, and stamens that became staminodes, respectively. The "Oncidiinae" model was proposed, explaining the duplications, diversifying selection and changes in spatial expression of different MADS-box genes that shaped the sepals, petals and lip, enabling the rewardless flowers of *E. pusilla* to mimic an unrelated rewarding flower for pollinator attraction.

Once an orchid flower is pollinated, the inferior ovary, which is composed of three carpels, develops into a fruit. In **chapter 4** (Dirks-Mulder *et al*., 2019) this process is described in detail for *E. pusilla* from pollination of the flower up to dehiscence of the capsule. Morphological analyses were also carried out on fruits of two other orchid species: *Cynorkis fastigiata* and *Epipactis helleborine* to find further support for the "split carpel" model as proposed by Rasmussen and Johansen (2006). The fruit associated MADS-box genes and proteins together with other dehiscence-related genes were analyzed for *E. pusilla* in order to propose a first "orchid fruit developmental protein and gene network" model.

To further study fruit development of *E. pusilla*, in **chapter 5** (unpublished results) transcriptome analyses are presented that were obtained from different developmental phases as defined in **chapter 4**. The final step in fruit development is the shattering of the seeds, which in most cases are dispersed by wind after the fruit dehisces. Lignification of the fruit valves is generally known to be important for fruit dehiscence but is this also the case for orchid fruits? Also in **chapter 5** this topic

was further studied by analyzing the anatomy of ripe fruits of a total of 41 orchid species from all over the world and investigating possible correlations between fruit valve lignification patterns, life form, growth strategy, ecology, fruit orientation, dehiscence type, number of valves and slits, and phylogenetic relationships. In **chapter 6** the results from the preceding chapters are summarized and discussed and implications for future research and implementation of *E. pusilla* as a plant model system are provided.

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