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„The various contrivances by which pollen–ovule ratios are influenced in orchids“

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## Abstract

Plants are stationary organisms that must rely on external agents for mating. Plant male and female reproductive investment depends on the efficiency and behaviour of the external agent in transferring pollen. In the highly diverse flowering plant family Orchidaceae, tightly aggregated pollen masses in two (or more) units allow very efficient transfer of male gametophytes. The high efficiency of pollen transfer allows orchids to have very low ratios of male to female investment (pollen to ovule ratios, P/Os). Food or sexual deception of pollinators is very common in orchids and pollinators learn to avoid them after few visits: the first flowers to open are often visited, but the subsequent ones tend not to be. Does this behavioural difference reflect in intra-individual reproductive investment?

In order to answer this question, we developed a new set of methods to precisely quantify the notably hard to acquire pollen and ovule counts in orchids. Flowers from different positions on the inflorescences (bottom, middle, top) were collected and infiltrated with phosphotungstic acid for Computed Tomography (CT). Since contrasting agents for CT tend to selectively enrich in ovules and pollen, they could be isolated from their surrounding tissues by image processing in order to be counted. Image processing and counting was performed with the programs Amira and Fiji/ ImageJ.

The method we developed allows for single pollen grains (not tetrads) to be counted manually as well as automatically. Automatic counting of ovules could not be achieved, but manual counting is possible. The risk of ovule loss was minimal since closed ovaries could be scanned.

In this study, no P/O difference between rewarding and deceptive species could be detected. However, clear differences between flowers within the same inflorescence could be demonstrated. In all taxa studied, the number of ovules gradually decreases from the bottom to the top of the inflorescence, whereas the number of pollen grains fluctuates without recognisable pattern around its mean. The P/O was thus highest in the upper regions of the inflorescences whereas the lowest values could usually be found in the lower regions. Between different plants, bigger variations in ovule as well as in pollen grain

numbers could be detected, which also affected the P/O. Ovule free domains on a placenta or placenta reductions ("placental anomalies") were commonly observed and decreased ovule numbers. Given the observed intra-individual and intraspecific variation, we suggest that a single P/O value for a whole orchid species (or populations) is therefore incorrect.

This method finally introduces computer-based volumetric analyses of computed tomographic data in the field of reproductive investment in plants (counting of pollen and ovules). Since hard- and software continuously improve, this study introduces a promising method for future works. Although sampling has to be improved (especially more rewarding species are needed) for a better comparison between deceptive and rewarding species, consistent patterns could be observed. Bottom flowers appear to function as the main seed producers, whereas top flowers possibly mainly act as pollen donors and ovule reserves in case of herbivory. Larger placental anomalies additionally often reduce the number of ovules in the most likely unpollinated top flowers.

Adaptations in ovule and pollen grain numbers are possible ways to optimize the resources that are available to the plant and thus improve a plant's fitness. The computed tomography-based method presented in this thesis provides a robust way of P/O measurements even in the numerous and minute pollen grains and ovules of orchids. The possibilities of the method we have developed include the measurement of phenotypical traits and reproductive investment on the same material, thereby opening entirely new alleys in the study of reproductive investment in plants.

*Key-words: Orchidaceae, orchids, pollen to ovule ratio, micro-CT, deceptive pollination, pollination reward, nectar, flower position, counting technique, pollen number, ovule number*

## Zusammenfassung (German)

Pflanzen sind ortsgebunden, was sie bezüglich ihrer Bestäubung von äußeren (biotischen oder abiotischen) Faktoren abhängig macht. Die Effizienz des Pollentransfers hat dabei direkten Einfluss darauf, wie viele Ressourcen eine Pflanze in die Fortpflanzung investieren muss. In der enorm diversen Pflanzenfamilie der Orchidaceen ermöglichen zwei (oder auch mehr) dicht gepackte Pollenpakete (Pollinien) eine sehr effiziente Pollenübertragung, was sich in einem geringen Pollen-Samenanlagen-Verhältnis (P/O) niederschlägt. Täuschblüten (Nektartäuschblumen, Sexualtäuschblumen etc.) sind überaus häufig in Orchideen und deren Blütenbesucher lernen oft nach nur wenigen Besuchen, diese zu meiden: Jene Blüten, die sich zu allererst öffnen, werden daher tendenziell häufiger besucht als jene Blüten, die sich erst später zu öffnen beginnen. Hat das einen Einfluss darauf, auf welche Weise die Ressourcen, die für die Reproduktion aufgewendet werden, innerhalb eines Pflanzenindividuums aufgeteilt werden?

Um diese Frage zu beantworten, haben wir neue Methoden entwickelt, damit die bisher schwer zählbaren Pollen und Samenanlagen möglichst exakt bestimmt werden können. Blüten von unterschiedlichen Bereichen innerhalb eines Blütenstandes (oben, mittig, unten) wurden gesammelt und mit Wolframphosphorsäure infiltriert, um ein Scannen mittels eines Computertomografen (CT) möglich zu machen. Wolframphosphorsäure bewirkt eine unterschiedlich starke Kontrastierung verschiedener Pflanzengewebe, wobei Pollen und Samenanlagen ganz besonders stark kontrastiert werden. Spezielle Bildverarbeitungsprogramme machen es möglich, Pollen und Samenanlagen vom restlichen Gewebe zu trennen, was ein Zählen ermöglicht. Dabei wurden die beiden Bildverarbeitungsprogramme Amira und Fiji/ ImageJ verwendet.

Die hier vorgestellte Methode macht es möglich, einzelne Pollenkörner (und nicht etwa Pollentetraden) zu isolieren und automatisch (oder auch manuell) zu zählen. Ein automatisches Zählen von Samenanlagen konnte nicht bewerkstelligt werden, manuelles Zählen ist jedoch möglich. Das Risiko, dass Samenanlagen verloren gehen könnten, ist mit

dieser Methode minimal, da gesamte und ungeöffnete Fruchtknoten gescannt werden können.

In dieser Studie konnten keine Unterschiede zwischen belohnenden und nicht-belohnenden Arten festgestellt werden. Es konnten jedoch klare Unterschiede innerhalb einer Sprossachse beobachtet werden. In allen untersuchten Individuen nahm die Anzahl der Samenanlagen von der Basis zur Spitze graduell ab. Die Pollenzahl hingegen scheint ohne erkennbares Muster um den Mittelwert zu schwanken. Das P/O war daher in den oberen Regionen des Blütenstandes am größten und in den unteren am kleinsten. Auch zwischen einzelnen Individuen konnten größere Unterschiede in Pollen- und Samenanlagenzahl festgestellt werden, die sich wiederum auf das P/O auswirken. Samenanlagenfreie Regionen auf Plazenten oder ganze Plazentareduktionen („plazentale Anomalien“) konnten häufig beobachtet werden und hatten eine mehr oder weniger gravierende Reduktion der Samenanlagen zur Folge. Aufgrund der großen P/O-Schwankungen ist es daher wenig zielführend, wie bisher einen einzelnen Wert anzugeben, der das P/O einer ganzen Art (oder Population) repräsentieren soll.

Die hier vorgestellte Methode schafft eine längt überfällige Einbindung computerunterstützter Verfahren, um reproduktive Investitionen (Pollen- und Samenproduktion) zu quantifizieren. Nachdem Hardware und Software beständig weiterentwickelt werden, birgt diese Methode enormes Zukunftspotential. Obwohl die Stichprobenzahl Erweiterungsbedarf aufweist (vor allem was belohnende Arten betrifft), um belohnende und nicht-belohnende Arten besser miteinander vergleichen zu können, konnten trotzdem allgemeine Trends festgestellt werden. Blüten an der Basis fungieren als die hauptsächlichen Samenproduzenten, wohingegen die oberen Blüten wahrscheinlich vorrangig für eine Pollenabgabe zuständig sind und deren Samenanlagen als Reserve im Falle von Fraß der unteren Blüten fungieren. Größere plazentale Anomalien können die Anzahl der Samenanlagen in den meist ohnehin nicht bestäubten oberen Blüten weiter reduzieren.

Anpassungen bei der Pollen- und Samenanlagenzahl sind mögliche Wege, um die für die Pflanze zugänglichen Ressourcen bestmöglich einzusetzen und so die Fitness einer Pflanze zu erhöhen. Die computerunterstützte Methode mittels CT liefert dabei eine solide Möglichkeit, das P/O von Pflanzen zu bestimmen, selbst wenn diese, wie im Fall der Orchideen, enorm vielzählige und winzige Pollen und Samenanlagen besitzen. Es können mit dieser Methode nicht nur reproduktive Investitionen gemessen werden, sondern mit demselben Pflanzenmaterial können zusätzlich auch phänotypische Merkmale untersucht werden. Es können damit vollkommen neue Wege in der Untersuchung reproduktiver Investitionen beschritten werden.

*Schlüsselwörter: Orchidaceae, Orchideen, Verhältnis von Pollen zu Samenanlagen, Micro-CT, Täuschblumen, Nektar, Belohnung, Blütenposition, Zählmethode, Pollenzahl, Anzahl der Samenanlagen*

## Introduction

In sexually reproducing organisms, conflicts for resources between genders to maximize individual fitness are omnipresent (Barrett and Hough 2013; Steer and Burns 2008; Lloyd and Webb 1977; Freeman, Klikoff, and Harper 1976), but nowhere more acute than in hermaphroditic organisms in which the two genders have to draw from the resource pool of the same individual (Jong, Shmida, and Thuijsman 2008).

In the overwhelmingly hermaphroditic flowering plants, pollen to ovule ratios (P/Os) are a measure of reproductive investment well-correlated with breeding system and pollination (Cruden 1977). P/O values range from below 10 (usually in cleistogamous species; Cruden 1977), to up to more than a million (usually in wind pollinated plants; Tomlinson, Primack, and Bunt 1979; Pohl 1937, p. 458; Ramirez and Seres 1994). Plants with pollen aggregated in pollinia seem to form an exception by having lower P/Os than observed in other plants with the same breeding system (Cruden 1977; Wyatt, Broyles, and Lipow 2000; Lehnebach and Riveros 2003; Nazarov and Gerlach 1997). Cruden (1977) stated that a P/O value of around 5800 is typical of allogamous plants. However, in allogamous, pollinia-bearing species of *Asclepias* he determined P/O values of around 10. Such a low P/O value is, according to Cruden (1977), usually characteristic of cleistogamous or obligate autogamous plants. Lower P/O values in pollinia bearing plants may be explained by increased pollen transfer efficiency (Cruden 1977).

Variation in P/Os within a population, can be quantified via the phenotypic gender ($G_p$) (Lloyd and Bawa 1984).

$$G_p = \frac{o_i}{o_i + p_i * E} \quad (1)$$

The $G_p$ quantifies how male or how female a flower is compared to the average within a population. Value ranges from 0 to 1: a value of 1 indicates a strictly female flower, a value of 0 a strictly male flower, and a value close to 0.5 indicates a flower with the same P/O than the average in this population (Lloyd and Bawa 1984; Barret and Harder 2006).

(1) $G_p = phenotypic\ gender, o_i = \#\ ovules,\ p_i = \#\ pollen\ grains,\ E = average\ ovule\ to\ pollen\ ratio\ within\ a\ population$

Unlike the P/O, the $G_p$ is not a fixed value, but dependent on the population's average ovule to pollen ratio (E).

Several components that influence the P/O were already observed, such as the type of pollinator (Neiland and Wilcock 1995; Erbar and Langlotz 2005), the method of pollen delivery (scraping vs. contact method) (Nazarov and Gerlach 1997), the way how pollen grains are aggregated (Nazarov and Gerlach 1997; Cruden 1977; Johnson and Edwards 2000), and the position of a flower within an inflorescence (Zeng et al. 2009). The impact of reward or deceit has -to our knowledge- not been investigated to date.

Presence or absence of reward leads to different behaviour in pollinators: in rewarding flowers, the pollinators tend to visit the open flowers of an inflorescence regularly in order to harvest its rewards (van der Cingel 1995, p. 42). In deceptive flowers however, pollinators tend to learn to avoid the deceptive flowers and visit only the first flowers to open, when the pollinator is still naive (Jersáková and Kindlmann 1998; van der Cingel 1995, p. 40). Consequently, in deceptive plants, only the first flowers to open tend to bear fruits (Jersáková and Kindlmann 1998), whereas in rewarding plants, the fruit set is usually higher and fruits are spread across the inflorescence (Neiland and Wilcock 1998). Within inflorescences of deceptive plants, the flowers from different positions fulfil different functions: the first flowers to open bear fruits and export pollen, whereas the later flowers rarely bear fruits and possibly mostly export pollen since naive pollinators are rare. If only one pollinator visits several top flowers of an inflorescence, it will remove the pollinia, but most likely will not pollinate a flower with those pollinia during its visit due to delayed pollinaria bending; a hygrometric movement of depression (Darwin 1886, p. 12 et seqq. & p. 270 et seqq. ; Johnson and Nilsson 1999; Johnson and Edwards 2000; Cozzolino and Widmer 2005). In rewarding flowers the differences in function should be smaller due to higher and similar visitation frequencies of all flowers of an inflorescence. Are differences in pollinator behaviour reflected in differences in reproductive investment within inflorescences? In a first scenario, the P/Os in deceptive flowers are so strongly constrained by the flower´s bauplan that there are no adaptation to the differences in behaviour of the

pollinators (similar intra-inflorescence variation of P/Os in rewarding and deceptive inflorescence). In a second scenario, the differences in floral visits are enough to lead to adaptation, and the first flowers to open (usually the lower flowers) tend to be significantly more female than the later flowers. This would reflect in lower P/Os (and a higher $G_p$) in the lowermost flowers of deceptive species. In order to test these hypotheses, the study of a group with variation between pollinator reward and deception at the lowest taxonomic level is required, so that bauplan differences can be factored out.

Approximately one third of the world's orchids are thought to offer no nectar-reward (Porsch 1909; Ackerman 1986; van der Pijl and Dodson 1966, p. 22). Although other forms of reward (such as food-hairs, resin like structures, oils etc.) can be present (Porsch 1909; van der Pijl and Dodson 1966, pp. 21-26; Davies and Stpiczyńska 2009 and references therein), deception is the most common system in those nectarless orchids (van der Pijl and Dodson 1966). It is thus not surprising that also within the European orchids, many are deceptive (Claessens and Kleynen 2011). The phylogenetic relationships and pollination biology of Orchidinae, the tribe to which most European orchids belong, are very well understood (Inda, Pimentel, and Chase 2012; Claessens and Kleynen 2011; van der Cingel 1995), and several genera (*Dactylorhiza*, *Anacamptis* and eventually *Orchis*) comprise deceptive and rewarding species (Claessens and Kleynen 2011 and references therein). Pollinator specialization is often high in Orchidinae, possibly owing to their unique floral morphology: pollen is transferred aggregated in many tight packages (massulae) loosely aggregated together in two larger packages (pollinia) (Claessens and Kleynen 2011; Johnson and Edwards 2000; Freudenstein and Rasmussen 1997). Pollinia can comprise from around 5000 (Nazarov and Gerlach 1997) up to several million pollen grains (Schill, Dannenbaum, and Neyer 1992). Such high numbers are usually found in orchids which do not possess sectile, but hard pollinia (Nazarov and Gerlach 1997; Johnson and Edwards 2000; Schill, Dannenbaum, and Neyer 1992; Zavada 1990). The pollinia are transferred to the pollinator as units and are, in the case of Orchidinae with its sectile pollinia, deposited in groups of massulae. The ovary in orchids comprises large numbers of ovules: from less than a thousand (Nazarov and Gerlach 1997) up to several

million per capsule (Arditti and Ghani 2000 and references therein), all of which can be pollinated by a single pollination event.

Within an inflorescence, flowers of Orchidinae usually open acropetally, *i.e.* from bottom to top (with some exceptions such as *Orchis simia*) (Griebl 2013), and flower size decreases from the bottom to the top of the inflorescences (Bateman and Rudall 2006; Vallius 2000). Orchidinae are an ideal group to study P/O and $G_p$ variations in rewarding and deceptive inflorescences, due to (1) presence of deceptive and rewarding species in the same genera (factoring out bauplan changes), (2) large number of ovule and pollen grains (high potential for variability and pressure for reduction), (3) known pollination systems, (4) pollinator specialization (simple systems), and (5) known phylogenetic relationships.

However, pollen and ovule counts tend to be difficult in Orchidinae due to the dense aggregation of pollen grains in massulae and pollinia and the high numbers of pollen grains as well as of ovules. Counting seeds is a proxy for ovule number, but reliability is uncertain because there is no way to know if all ovules have been fertilized. New, reliable methods to count pollen and ovules are therefore needed. It has been shown that X-Ray contrasting agents, especially phosphotungstic acid (PTA), semi-selectively accumulate in pollen and ovules of flowers, possibly due to the higher protein content of these tissues relative to their surroundings (Staedler, Masson, and Schönenberger 2013). More X-Ray absorption translates into darker areas, but as it is customary to display scan data in negative, the strongly X-Ray absorbing tissues appear brighter in the 3D model of the sample. It is possible to separate the brighter tissues from their surroundings by selecting only the brightest pixels of the 3D model (grayscale thresholding). Obtaining the volume of such a selection is straightforward, as is pollen grain or ovule number once the average volume of a pollen grain or an ovule is known.

This study aims at (1) establishing a new method for pollen and ovule counting, and (2) uncover if the differences of pollinator behaviour in rewarding vs. deceptive plants lead to different patterns of gender investment at the individual level.

# Materials and methods

## Choice of study group

Our main goal was to investigate the differences in reproductive investment within inflorescences between rewarding and deceptive orchids. We chose to work on the subtribe Orchidinae of Orchidoideae, in which species have sectile pollinia, *i.e.*, pollinia that are comprised of several smaller sub-units (massulae) that are released as units on the stigmas during floral visits (Claessens and Kleynen 2011, p. 26). We hypothesize that the release in subunits relaxes P/O constraints and leads to a higher variability than in species with hard, entire pollinia such as those usually found in Epidendroideae (Dressler 1979; Johnson and Edwards 2000). We also investigated P/Os in *Epipactis palustris* (tribe Neottieae, Epidendroideae), which possesses loosely aggregated "mealy" pollinia (Bernardos et al. 2006; Claessens and Kleynen 2011, p. 26).

In order to obtain comparable results, we aimed at collecting rewarding and deceptive species of the same genus or of closely related genera out of the subtribe Orchidinae (Inda, Pimentel, and Chase 2012). Only Austrian orchids were collected because (1) Austria harbours enough species to address our scientific questions; (2) the collected species experience more or less similar climatic conditions and possible climatic influences were thereby minimized; (3) all orchids are under protection, and collecting in various different states or countries requires various permissions which makes collecting more complicated. Even in Austria it is necessary to get a permit in each state where collection is to take place. (4) Finally, this is a diploma thesis in the course of an Austrian teacher training; the focus on regional species was therefore advisable and appropriate.

Although several genera in Austria comprise deceptive and rewarding species, obtaining rewarding species turned out to be difficult. *Orchis anthropophora* is the only possibly rewarding representative of its genus that can be found in Austria; however, the species has only been observed in two localities within Austria (Fischer, Oswald, and Adler 2008, p. 1063) and is difficult to obtain. Thus only in the genus *Dactylorhiza* rewarding and deceptive species could be obtained.

## Materials

**Table 1:** Overview of all collected species

| Species | Collection locality | Collected flowers; collection date | Collected fruits; collection date | Rewarding/ deceptive | Pollinators |
|---|---|---|---|---|---|
| *Anacamptis morio* | Lobau, Lower Austria | 12; May 13th 2013 | 10; May 29th 2013 | deceptive | Hymenoptera |
| *Anacamptis pyramidalis* | Lilienfeld, Lower Austria | 20; June 20th 2013 | 10; July 4th 2013 | deceptive | Lepidoptera, (Hymenoptera) |
| *Dactylorhiza incarnata* | Inzing, Tyrol | 20; June 5th 2013 | - | deceptive | Hymenoptera |
| *Dactylorhiza fuchsii* | Lilienfeld, Lower Austria | 20; June 13th 2013 | 10; June 26th 2013 | deceptive | Coleoptera; also: Diptera, Hymenoptera |
| *Dactylorhiza majalis* | Mooshuben, Styria | 20; June 5th 2013 | - | deceptive | Hymenoptera |
| *Dactylorhiza viridis* | Rax, Lower Austria | 18; June 19th 2013 | - | rewarding | Coleoptera, Hymenoptera |
| *Epipactis palustris* | Botanical Garden Vienna, Vienna | 30; June 26th 2013 | 10; July 24th 2013 | rewarding | Diptera, Hymenoptera, Coleoptera |
| *Gymnadenia conopsea* | Lilienfeld, Lower Austria | 20; June 13th 2013 | - | rewarding | Lepidoptera, (Hymenoptera) |
| *Orchis militaris* | Lobau, Lower Austria | 12; May 13th 2013 | 10; June 29th 2013 | deceptive | Hymenoptera |

Sampling was conducted in May-July 2013 depending on the flowering time of each species. Since the permit of the Lower Austrian government granted collecting of no more than 20 flowers and 10 fruits per species, the collection possibilities were restricted, and the selection of plants had to be carefully considered. If possible, the maximum amount of permitted flowers and fruits was collected.

*Anacamptis morio* and *Orchis militaris* were collected on a meadow in the Lobau in mid-May (see table 1). Both species grew and flowered at the same time and place; fruits were collected 16 days later. The second collection locality, where several species could be found, was a meadow close to a forest in Lilienfeld. *Dactylorhiza fuchsii* and *Gymnadenia conopsea* were collected at this locality in mid-June, whereas *Anacamptis pyramidalis* was collected one week later; fruits were collected around two weeks later (fruits of *D. fuchsii* were

collected after 13 days and fruits of *A. pyramidalis* after 14 days). However, no fruits of *G. conopsea* were collected since it was not considered as a main study object at that time. *Dactylorhiza viridis* was collected on a meadow on the Rax Mountain in Lower Austria at an altitude of ca. 1600m. The collection was performed in mid-June; no fruits were collected. One of the analyzed species, *Epipactis palustris*, grew in the Botanical Garden of the University of Vienna and was therefore collected at that locality at the end of June. *Dactylorhiza incarnata*, collected in Inzing in Tyrol, and *Dactylorhiza majalis*, collected in Mooshuben in Styria were collected by Dr. Ovidiu Paun from the University of Vienna. Both were collected in the beginning of June.

## Methods

### *Collection method*

The flowers were removed from the selected plants with razor blades and/ or tweezers. Inflorescences with a larger number of flowers were selected in order to be able to collect flowers from the bottom, the middle and the top of the inflorescence; well opened flowers including pollinia (or at least well developed buds) were collected on the whole inflorescence. Due to the short stalk of many collected orchids, special attention was required upon flower removal in order not to damage the inflorescences. After removal, the flowers were immediately fixed in formalin-acetic acid-alcohol (FAA).

After flower removal, a bottom, middle and top flower of each previously selected plant was cross-pollinated by hand. The labellum of pollinated flowers was removed as a marker for later identification. In order to avoid any disturbances caused by possible flower visitors, each plant was caged. The cages consisted of a metal grid with a mesh size of around 2x2 mm. The grid was connected and closed by metal wires or staples. Each cage was attached to the ground by three 15 cm long nails.

Around two weeks after pollination, 10 out of the 12 pollinated flowers were removed from the plant in the same way as the virgin flowers. The developing fruits were immediately fixed in FAA as well.

### *Laboratory*

The sampled flowers were transferred to FAA+1% PTA (phosphotungstic acid) as soon as possible and deaerated for 20-30 minutes with a water-jet vacuum pump. In each sample FAA+PTA was changed at least twice before scanning. Mounting was performed in a 200µl pipette tip (Standard UNIVERSAL (Art. No.: B002.1), Carl Roth GmbH+Co KG) as described in Staedler et al. (2013) with the following differences: (1) before mounting the bottom end of the pipette tip was cut off. This allowed us to enter the tip with a preparation needle and to drag down the samples to optimize the used space without displacing or breaking pollinia. (2) The samples were not washed with 70% EtHO before scanning, but immediately mounted in FAA+PTA. In order to further optimize the available space and to prevent the formation of air bubbles, the petals and sepals of the samples were removed with tweezers and dissecting scissors before mounting.

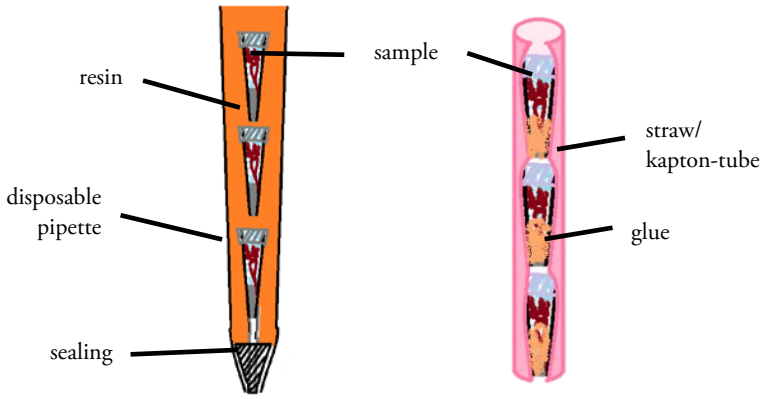



**Figure 1:** The left graphic shows the batching method with resin in a disposable pipette, the right graphic illustrates the batching method with a straw (or a similar plastic tube)

The individually mounted flowers could then be batched; either in disposable pipettes or in a slit plastic tube (see figure 1). Both batching methods allowed no more than three samples to be placed in one batch. In the first scans the samples were batched in modified 3.2ml plastic disposable pipettes (Art. No.: EA65.1, Carl Roth GmbH+Co KG). Both ends of the disposable pipettes were cut. The top was cut so that the samples could be placed inside. The bottom end was cut so that it could be fixed to a sample holder; a 20mm long aluminum tube with a diameter of 7mm. The bottom end of the disposable pipette was sealed with remains of the cut parts of the pipette and by the bottom sample (see figure 1).

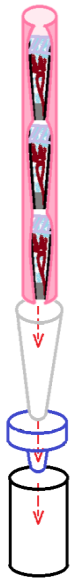


**Figure 2:** Batched samples with adapter and sample holder

In order to further stabilize the samples inside the disposable pipettes, the latter were filled with resin (GT type, Kurt Wolf & Co KG). The pipettes were then glued (UHU PLUS®, UHU GmbH & Co) into a sample holder, which was glued to a sample table. *E. palustris* and *O. militaris* were batched according to this method. Later samples were placed in a longitudinally slit plastic tube; either kapton-tubes (Ø: ca. 3mm) or drinking straws (CleanPac Trinkhalme, Müller Ltd. & Co. KG, Ø: ca. 3mm) were used. The tension produced by the shape of the straw stabilized the samples; they were additionally stabilized by gluing them to the straw. The batch was then placed in an in-house sample holder and fixed with glue. Since the sample holder was made for 1ml syringe tubes (Omnifix-F, Braun Medical Inc.), the bottom part of a syringe tube was used as an adaptor (see figure 2). If necessary a part of a cut pipette tip was used as further adapter between straw and syringe tube part (see figure 2). Glue was added between all the abovementioned parts to stabilize them. In *A. pyramidalis*, kapton-tubes were used instead of drinking straws. Due to the considerably higher price of kapton and its lack of significant advantages compared to simple drinking straws, most of the samples were batched in drinking straws.

### *Scanning*

An XRadia MicroXCT-200 system was used to perform scanning. The scanning conditions varied between different scans. Low resolution scans were performed with a 4x objective. High-resolution scans were performed with 10x and 20x objectives (see table of scanning conditions in the appendix). Low-resolution scans were usually performed in batches; high-resolution scans were performed either on batched samples or on singly mounted samples.

### *3D Reconstruction*

The software XMReconstructor 8.1.6599 (XRadia Inc.) was used to reconstruct 3D models of the scanned samples. In order to have repeatable grayscale values from one scan to another, byte scaling and CT scaling were used. Byte scaling is a procedure in which

minimum and maximum grayscale values are set for the whole reconstructed scan volume (Xradia, Inc. 2010). Byte scaling was used for the reconstruction of scans of gynoecia. CT scaling is a procedure by which grayscale values are scaled to the values of two reference materials (Candell 2009); in our studies, air and a solution of 1% PTA in FAA were used. CT scaling requires scanning of a dummy (or phantom) filled with the reference material and the presence of air on both sides of the sample during the whole scan. For that reason a pipette tip was filled with FAA+PTA, sealed at the bottom end with paraffin wax, shortened at the top and sealed with parafilm. For one dummy scan to be used on sample scans, the following parameters have to remain constant: voltage, objective type, beam hardening coefficient, and source filter (Candell 2009). Calibration was performed as described in the manual (Candell 2009). The density of FAA+PTA was measured 7 times by using 4x a 100ml and 3x a 50ml measuring cylinder and an arithmetic mean of 93.7g/100ml was determined and used for calibration. Pollinia were usually reconstructed with CT scaling.

The first samples scanned were reconstructed in TXM format (default setting of the reconstructor program), but most of the scans were later reconstructed in DICOM format. Reconstruction in DICOM format provides series of pictures (virtual sections) that can be directly imported in the data analysis software, whereas reconstruction in TXM format requires additional exporting in order to receive series of single pictures. XMController 8.1.6599 was used for exporting the TXM-data into picture stacks in TIFF format.

### *Data analysis*

The series of pictures were imported into the data analysis software: AMIRA 5.4.1 (Build 006-Se11b; Konrad-Zuse Zentrum Berlin (ZIB) & Visage Imaging Inc.), and Fiji (Schindelin et al. 2012), a distribution of ImageJ (Rasband 2014). Three dimensional visualization and manual counting of ovules and pollen was carried out in Amira. For automatic pollen counting, stacks were first loaded and edited (mostly cropped) in Amira, then exported as a 3D TIFF file. This file was then opened with Fiji; single pollen grains were then separated via “3D Iterative Thresholding”(Ollion et al. 2013), and counted with

the “3D Object Counter” (Bolte and Cordelières 2006). Pollen and ovule data were summarized and visualized in Microsoft Excel (Microsoft office Standard 2010).

### *Significance test*

All statistical analysis were performed using R (version 3.1.1, R Core Team 2014).

The influence of position on the number of ovules, as well as the comparison of *D. viridis* and *D. fuchsii* were tested with a non-parametric analysis of variance (npANOVA) using the function adonis() from the package “vegan” (Oksanen et al. 2013). We first generated a distance matrix with the function vegdist() from the vegan package using the Euclidean distance index and then performed the npANOVA using 10000 permutations (see Anderson 2001).

For linear-regression analysis the function lm() from the package “stats” was used.

# Results

## A new method

### *Fixation and infiltration – the problem of pollinium fragility*

The pollinia of Orchidoideae are either weakly aggregated in single tetrads that are simply connected with elastoviscin in "mealy pollinia" (as in *Epipactis*), or more tightly aggregated in massulae in sectile pollinia (as in Orchidinae; Claessens and Kleynen 2011, pp. 26, 91). Both of the latter types of pollinia are fragile, and can disaggregate in tetrads (mealy pollinia) or in massulae (sectile pollinia), the mealy pollinia being the most fragile, especially during flowering (Tałałaj and Brzosko 2008). Pollinia disaggregation occurred during collection, infiltration, and mounting.

### *Different mounting approaches*

As abovementioned, procedures for sample mounting and batching were constantly refined and adapted to material during this study. We first mounted the flowers in 50ml cylindrical hinged-lid containers (Art. No.: 6068, Semadeni AG) stabilized by pillow foam (Dacron, Comforel) in a FAA-PTA atmosphere. Due to the large amount of samples this mounting strategy proved suboptimal. Furthermore the diameter of the analyzed samples was smaller than 10mm; the recommended size for this mounting technique (Staedler, Masson, and Schönenberger 2013). Batching flowers in 50 and 90ml cylindrical hinged-lid containers (Art. No.: 6068 & 6069, Semadeni AG), respectively, in order to prevent the samples from possible mounting-damage and to make the scanning process more efficient was also attempted; however this method increased the risk of damaging the pollinia. Furthermore, the samples could not be stabilized adequately, which increased the risk of movement during the scan. Samples were orientated horizontally in order to mount several flowers on top of each other. This orientation did not allow scanning the whole gynoecium of a flower with 4x magnification. Thus, this method was only used once in the course of a testing procedure.

Scanned samples were mounted and batched as described in the materials and methods part. Although mounting was performed with great caution, loose massulae as well as a loss

of massulae or destroyed pollinia were detected in several samples. This indicates that mounting is a critical step that needs further improvement. Removal of the petals and sepals seemed to additionally increase the disturbances acting on the pollinia resulting in greater damage. In the present study the protective effect of the surrounding leaves could be shown in unopened buds; in opened flowers it is just hypothesized, but not tested.

Batching in the modified disposable pipettes made it possible to obtain scans with minor movement. However once the pipette is filled with resin, it is very difficult to unmount the samples without damage. Batching in plastic tubes (kapton or drinking straws) turned out to be a well-suited batching method since the batch is straight, stable, extremely space saving and the samples can easily be removed. By using drinking straws instead of kapton tubes the batching method is additionally very cheap. Disadvantages of the drinking straws could not be detected.

### *Scanning*

High resolution scans (20x, 10x magnification) and low resolution scans (4x magnification) were performed. Since ovules could be counted on low resolution scans without difficulties, no high resolution scans were performed on gynoecia. However, this was not possible with pollen grains. Thus, for each species studied, one pollinium was scanned in high and low resolution; the remainder were scanned only at low resolution. As mentioned before, low resolution scans are less time consuming. One low resolution scan of a pollinium required approximately 3-5 hours whereas a high resolution scan required 20h and more. The radioactive source was weakening during the course study; a new radioactive source can reduce scanning times by a factor of 2 or more (personal observation). In order to optimize time, we counted the pollen grain number in the high resolution scan, transferred the result to the low resolution scan and determined the average pollen volume at a certain threshold level. It is necessary that the sample has the exact same orientation in the high and low resolution scan (especially if the high resolution scan cannot capture a whole pollinium). If the sample does not have the same orientation in the high and low resolution scans determining equivalent sections in Amira becomes very complex (see chapter “Manual

counting of pollen grains"). Low and high resolution scanning should therefore be performed one after another, and with the least possible changes.

### *Scaling methods*

Two different types of scaling methods were used: byte scaling and CT scaling. Scans have to be performed under the exact same scanning conditions if comparable grayscale values are aimed at via byte scaling (Xradia, Inc. 2010). Since byte scaling could not solve grayscale repeatability within and among pollinia, a different approach, CT scaling, was tried. For reconstructing gynoecia, byte scaling was used for most samples and is highly recommended, especially if stitching is intended.

CT scaling requires, as abovementioned, calibration to two substances (in this study air and FAA+PTA). The determination of the average grayscale values of FAA+PTA turned out to be difficult since grayscale values varied strongly not only within a virtual section image, but also among them. This made calibration sometimes problematic.

Furthermore, even CT scaling could not provide comparable grayscale values of pollen grains. The problem was that the grayscale values were inconsistent not only between different pollinia but also within one single pollinium. Hence it was difficult in the downstream analyses to find a threshold that would fit for all pollen grains in all scans of one species. A possible explanation of those grayscale differences could be, that FAA+PTA was not absorbed equally with and between samples, thereby leading to different grayscale values in different domains or scans.

### *Manual counting of pollen grains*

When scanning is conducted with a 10x objective or higher it is possible to count single pollen grains in Amira by hand. For manual counting, filtering is highly recommended; digital filters in Amira are most convenient and thus recommended (see chapter "Different filtering"). As abovementioned, the pollinia that were scanned at high resolution were also scanned at low resolution so that the average pollen volume at a certain threshold level in low resolution scans can be determined. To do so, it is necessary that the low and the high resolution scan show equivalent domains of the same sample in Amira. In order to achieve

that, both scans were opened in Amira in two different windows, the two data sets were compared in the "Segmentation Editor", and equivalent sections were determined and then loaded into one Amira window.

Due to the very large amount of pollen grains that are usually found in orchids, it is virtually impossible to count all the pollen grains of one pollinium. Darwin already mentioned pollen numbers of more than 100000 in two species of the genus *Orchis* (Darwin 1886); modern authors calculated in several European orchid species 100000 pollen grains and more per flower (Nazarov and Gerlach 1997). Extrapolations are necessary in order to determine the approximate number of pollen grains. It is therefore advisable to count pollen in a few smaller domains and then extrapolate these counts to obtain the total number of pollen grain. Therefore the high resolution data has to be filtered and duplicated in Amira. The duplicated version can then be cut to the requested size with the tool "Crop Editor" so that only a small, countable domain remains.

Counting itself was conducted in the "Segmentation Editor". Smaller domains appeared to be visualized in better quality by the "Segmentation Editor" (see figure 3). It is therefore recommended not to cut too large domains (see figure 3). The "3D Volume Rendering" has to be activated to produce a 3D picture in the "Segmentation Editor". In the 3D model, all the pollen grains can be selected with the "Magic Wand" tool. The 3D threshold has to be set within a proper range and the option "All slices" has to be selected in order to be able to select the whole volume. Selected parts can be assigned to different materials in the "Segmentation Editor". Assigning different parts to different "materials" in Amira is necessary for being able to count properly. It is advisable to create a "new material" in which counted pollen grains can be added; a material for temporarily storage of smaller pollen grain groups may be helpful. Different materials can be displayed or hidden in the 3D visualization of the Segmentation Editor which is necessary for manual counting. Using the option "Same material only" can prevent already counted material to be selected again.

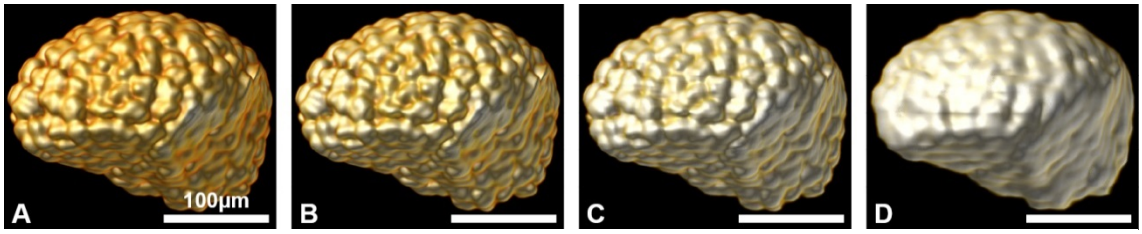



**Figure 3:** Massulae of *A. pyramidalis* with different crop box dimensions. (A) massula cropped to a minimum, (B) edge lengths of the crop box doubled, (C) edge lengths quintupled, (D) edge lengths decupled. Scale bars = 100µm

Once a whole pollen section is selected, the bottom value of the 3D grayscale threshold can be increased slowly. By doing so, single pollen grains or smaller pollen grain groups drop out of the selection. Those single grains or smaller groups can now be counted, selected and added to the designated material for counted pollen grains. Before adding the selected pollen grains to the new material, it is advisable to use the tool "Grow Selection" several times so that not only the previously chosen threshold is added to the designated material but also parts of the selected pollen grains that lay outside this threshold. This prevents already counted pollen grain artifacts to remain in the primary material. It can be helpful to assign groups of pollen grains to a temporary material in order ease the counting process. By hiding all other materials but the temporary one, a better view on the single pollen grains is possible. After counting the whole temporary material it can be selected by clicking "Select material" and can be added to the material for counted pollen grain.

Usually, incomplete pollen grains can be found on the edges of the cropped pollen section. In this study it was tried to estimate, which and how many incomplete pollen grains may have the same volume as half a grain or a complete one. They were then selected all together and counted as such.

The whole counting procedure can be repeated with several domains. In this study 2-3 domains were counted per high resolution pollinium; usually one from each the bottom, the middle and the top of a pollinium. If bigger differences in threshold levels exist within a pollinium, a different criterion for domains selection can be used. By determining the total volume of the pollen mass, the average pollen grain size can be calculated by simple division. For pollen grain selection an appropriate threshold which is not only suitable for the domain, but also for the whole high resolution scan needs to be chosen. There are two

different ways to calculate the average pollen grain size. Either the pollen grain volume of all three domains is divided by the number of all counted pollen grains or the average of the domains' average volumes is calculated. The second calculation method is advantageous if noticeable differences in contrast between the domains exist and the domains are rather unequal in pollen grain numbers. In this study, the second averaging method was used. It always has to be taken into account that the calculated average represents the average pollen grain volume at a certain threshold and does not necessarily represent the actual average pollen grain volume.

Once the average pollen grain volume (at a certain threshold) is determined, the number of pollen grains can be calculated by selecting all the pollen grains of the high resolution scan with the previously used threshold and adding it to a new material. The volume of the material can be determined by using the "Material Statistics"-tool in Amira and the total pollen grain number can be calculated by division.

### *Automatic counting of pollen grains (in Fiji/ ImageJ)*

"3D Iterative Thresholding" was used for separating single pollen grains to make them countable with the "3D Object Counter". The "3D Iterative Thresholding" tool requires large amounts of the Computer's RAM, especially if the domain's size is large. A certain data size or a certain amount of objects to be investigated limits the tools usage. Even a computer with 96 GB RAM reached its limits easily for such calculations, and the iterative thresholding could not be performed. Reducing the size of the data by cropping the domain of interest could solve this issue.

In order to reduce size and possible noise that might cause errors during the counting process the domain of interest was first optimized in Amira. Cropping the domain was performed as described above. In the "Segmentation Editor", a threshold was chosen which only included pollen, but not the surrounding area. The whole pollen selection was then added to a new material. Filtering may be applied first to make this separation easier.

In the “Object Pool” the operation “Arithmetic” can be applied on the dataset in order to only export the data which is included in a certain material. Therefore, it is necessary to additionally link the “Arithmetic”-operation with the layer of the associated data. The command “(b==x)*a”[2] completes the intended separation and the received data can be saved separately. In this study the “3D Tiff”-format was used. This data can be opened with Fiji/ ImageJ.

The following steps have to be performed in Fiji/ ImageJ in the given order:

1. Brightness/Contrast-adjustment: With this step, noise can be further reduced.
2. Changing the format from 16BIT to 8BIT.
3. Filtering of the data set. (A combination of the filters “median 3D”/”mean 3D” and “Gaussian Blur 3D” provided good results.) (see figure 4)
4. 3D Iterative thresholding (The settings may be optimized. This, however, was never necessary during this study; default settings provided good results. Further filtering may or may not be chosen.)
5. Changing the format again from 16BIT to 8BIT.
6. Threshold-adjustment
7. Run the tool “3D object counter” (This tool was used two times in a row; once with activated and once with deactivated “exclude objects on edges”-option. By taking the mean of those two values, the number of pollen grains was calculated.)
8. Saving the dataset before starting the iterative thresholding process and before running the “3D object counter” (each as a different tiff-file) is recommended.

For more detailed instructions see my personal notes (in German) attached in the appendix.

[2] b = Input B should be the designated layer, a = Input A: should be the data set, x = Bundle number of the requested material of the layer (the bundle number can be checked in the “Parameter Editor”)

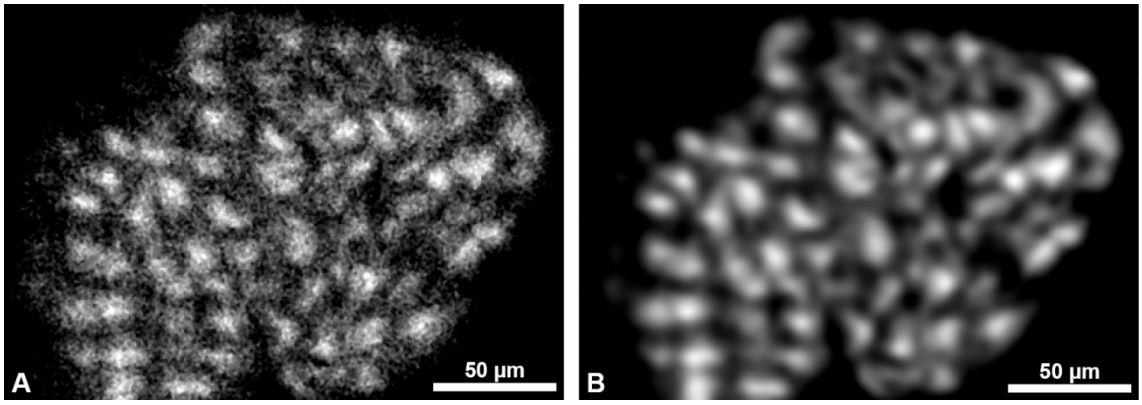



**Figure 4:** 2D section of pollen grains of *D. majalis*: (A) unfiltered, (B) filtered with Fiji/ ImageJ. Scale bar = 50µm

Before counting it is advisable to check whether all pollen grains could be captured with the "3D Iterative Thresholding" tool. This can be done in Amira by simply loading the file before "3D Iterative Thresholding" was applied and the final output file on which the "3D Object Counter" was conducted. In the "Object Pool" two different Volren (Direct Volume Rendering) can be chosen for the two data sets. By simply moving the Volren's threshold-interval of the pre-iterative-thresholding file further to the right (minimum and maximum borders of the interval are thus increased simultaneously), it can be determined, if some pollen grains were not captured with the "3D Iterative Thresholding" tool. Detecting uncaptured pollen grains is easy; detection of noise or other non-pollen particles within a pollinium or detection of pollen grain fusion or splitting is more difficult. The determination of the average pollen grain volume and the calculation of the total pollen grain number can be performed as mentioned above.

### *Comparison manual vs. automatic counting*

Using Fiji/ ImageJ makes it possible to count larger pollen domains with comparatively little effort. Domains containing up to 1800 pollen grains could be counted. This, however, was at the limits of what is possible due to RAM limitations. If the domain is too large, the iterative-thresholding process not only requires more time, but also makes the computer less stable since there is a risk of reaching the RAM limitations. Furthermore, when Fiji/ ImageJ reaches its RAM limits, the counting process has to be canceled and much time is wasted. It is therefore advisable to count smaller, but more numerous domains.

The advantage of using automatic counting is not only the minimization of possible human errors, but also the reduction in the amount of time to be invested. A comparison of manual and automatic counting showed, that the results are very similar. In the top domain of the high resolution scan of *D. viridis* the difference between manual and automatic counting was 0-1 pollen grains. Manual counting provided pollen grain numbers of 224 and 225; automatic counting provided a pollen grain number of 224. The quality of this domain was excellent so that single pollen grains could be detected and separated easily with the naked eye. Furthermore, only one lateral surface of the domain contained cut pollen grains. In a second comparison in which a more central part of a pollinium of *D. incarnata* was counted manually as well as automatically, the outcome was different. All six lateral surfaces of the cube-shaped domain contained cut pollen grains. This made the manual counting process more difficult since the assessment of which amount of the pollen grain's volume is present, can be problematic. However, the difference between manual and automatic counting was less than 4% (388 pollen grain with manual counting vs. 402.5 pollen grains with Fiji/ ImageJ). The term "difference" is more suitable than the term "error" since it is difficult to assess which method is more accurate.

### *Transfer from high to low resolution scans*

Once the number of pollen grains in the high resolution scan is determined, the result needs to be transferred to the corresponding low resolution scan. It is therefore necessary that the low and the high resolution scans show the exact same domain of the pollinium or pollinia. The average pollen grain volume can be determined as described for the high resolution scan before, since the pollen grain number of the low resolution domain is also known. This average pollen grain volume at a certain threshold can then be used to calculate the average number of pollen grains in all low resolution scans of the same population. It was therefore assumed that the average pollen grain size is approximately equal within one species (or at least within one population).

### *Transfer to other low resolution scans*

As mentioned above it was not possible to achieve one threshold that provided comparable grayscale values for all low resolution scans of one species. In order to transfer the estimated average pollen grain volume (at a certain threshold), it is necessary that in each and every scan the same proportions of one pollen grain are selected. To achieve that the low resolution scan of the sample which was also scanned in high resolution was used as a reference. An equivalent threshold was determined three times at three different areas of the other low resolution scans by constantly comparing them with the reference sample. This comparison was conducted in the "Segmentation Editor". The average of the determined threshold was calculated and used as threshold to calculate the total pollen grain volume of the corresponding sample.

### *Manual counting of ovules*

Usually, it was not possible to scan the whole gynoecium at once with the 4x objective. In order to reduce the number of necessary scans stitching was not performed with XMReconstructor since automatic stitching requires overlapping scanning domains of >20% (Zeiss application engineer, personal communication). Additionally, stitching can also be performed in Amira, either automatically (which requires overlapping domains) or manually (which requires only one overlapping 2D section). When two scans are stitched it is highly advisable, that the minimum and maximum grayscale ranges of both scans are equal. This can be accomplished by using a scaling method (preferably byte scaling) during the reconstruction process.

The overlapping domain of two scans was often reduced to a minimum so that most of the analyzed gynoecia could be fully captured by using not more than two scans. The overlapping domains were then identified in the "Segmentation Editor" in Amira. A common point within the overlapping domain could be determined and thereby the domains present in the two scans could be removed (either by cropping or by importing the overlapping domains only once).

Counting ovules could not be automated and was therefore conducted manually in Amira. For counting ovules, no high resolution scan was needed, and scans performed with a 4x objective were sufficient. This was especially time saving, since the ovule size was highly variable between different flowers, depending on the developmental stage of the gynoecium. Due to highly variable ovule size, a transferable average ovule volume could not be determined and high resolution scans would have been necessary for each gynoecium (if counting in low resolution was not possible). Hence, the average ovule size had to be determined in each and every scan. In a section of 50-100 pictures (depending on the size and number of ovules) the number of ovules was determined. If too many ovules are present in one section counting becomes considerably more time consuming. In order to reduce the time consumed by manual counting, it was tried to keep the number of ovules in a reasonable level by changing the number of pictures of an analyzed section. Sections were usually taken from mid-region of a scan and contained in average around 140 ovules.

In order to ease the counting process, each placenta and its corresponding ovules were selected and added to different materials in the "Segmentation Editor". The separation of the placentae and its corresponding ovules was conducted by using the "Lasso" tool in several pictures and by connecting the selections with the command "Interpolation". Depending on the inner structure of a gynoecium, this process can be more or less difficult (see figure 26). It should be checked if all corresponding ovules are included in the selection and that no ovules from other placentae are within the selection. It is advisable to prohibit any further changes within a well selected material by using the "Lock" function in the "Segmentation Editor". The tricarpellate gynoecia with the parietal placentation (Arditti 1992, p. 118 et seq. ; Claessens and Kleynen 2011, p. 17; Simpson 2011, p. 176 et seq. ; personal observation) were usually separated into three parts which were assigned to three different materials. In some samples, anomalies could be detected so that parts or entire placentae were missing (see chapter "Placental anomalies"). By hiding all materials except the one on which counting should be conducted, the counting process could be eased due to a better overview.

The sections of gynoecia also contained partly cut ovules on the top as well as on the bottom edge. The partially cut ovules were counted as full ovules on the bottom edge and were therefore neglected on the top. Especially small parts were also neglected on the bottom edge and almost complete ovules on the top edge were also taken into account and counted as whole.

In order to increase precision, more sections within one scan can be counted or the number of pictures within one section can be increased. However during this study it was experienced that >300 ovules within one section make counting dramatically more time consuming due to a decrease of clarity. It is therefore recommended to rather count more different sections.

The total ovule volume within one section can be determined by adjusting a proper threshold, selecting it with the "Magic Wand" tool, adding it to a new material and checking the material statistics. By simple division the average ovule volume of one scanned domain can be calculated. This average ovule volume was then used to calculate the total number of ovules within one scan.

### *Manual counting of massulae*

In orchid species that possess sectile pollinia, several loose massulae are usually deposited on the stigma rather than whole pollinia (Nazarov and Gerlach 1997; Claessens and Kleynen 2011, p. 26). As mentioned before the pollen grains within one massula are usually tightly packed and well interconnected. The connection between massulae is weaker, which causes massulae to detach from a pollinium rather than single pollen grains or single pollen tetrads (Nazarov and Gerlach 1997). It was thus a point of interest if counting massulae could additionally be performed on the already produced low resolution data. An automatic counting procedure similar to the counting procedure of single pollen grains could not be developed, but manual counting was possible. The 3D software "Amira" was used to count the number of massulae.

In order to do so, the pollinia were separated from the surrounding tissue (by selecting them with the "Magic Wand" tool) and exported to a different data set with the command "Arithmetic" as described above. Due to the fact that most European orchids possess two pollinia which are again subdivided into two halves (Claessens and Kleynen 2011, p. 24), it was tried to not only separate the pollinia from each other and add them to different materials, but to also additionally separate each pollinia in its two halves (see figure 5 (A)). Separation was conducted in the same way as placenta-separation was performed (see chapter "Manual counting of ovules").

By hiding one half of the pollinium it is possible to look at the massulae from all angles (see figure 5 (B)). Counting was performed several times on each material. Changing the threshold level can help to distinguish if a structure consists of one or more massulae.

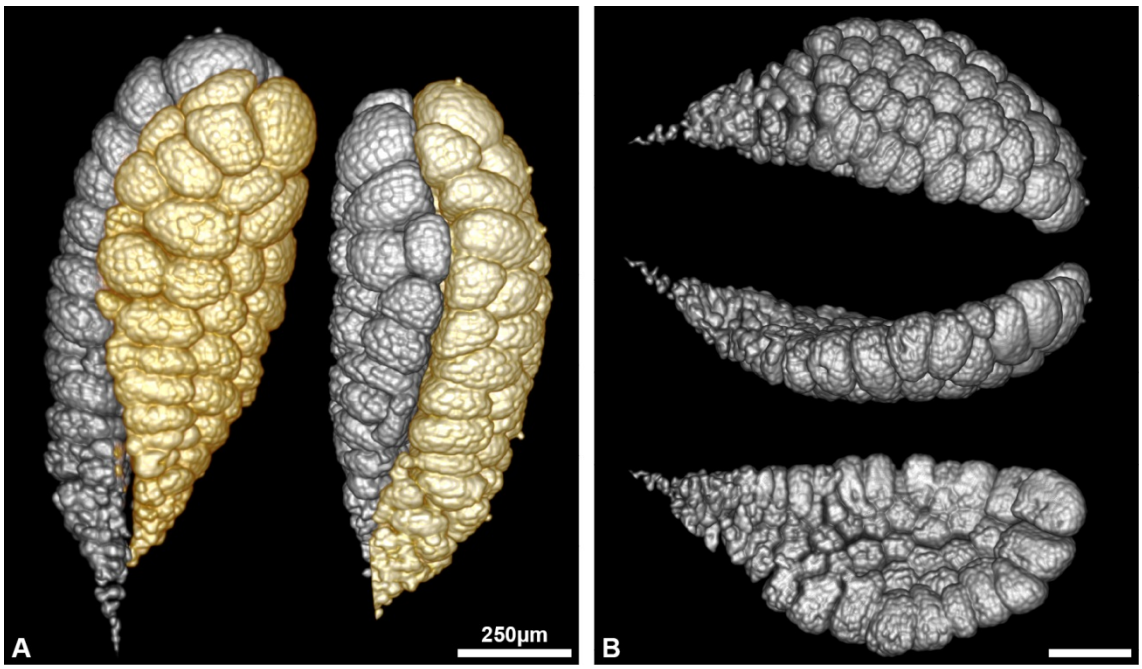



**Figure 5:** *A. pyramidalis:* (A) The two pollinia of a bottom flower each separated into its two halves, (B) One half of a pollinium from different angles. Scale bars = 250µm

### *Different filtering*

Filtering was tested with three different programs: XMController, Amira and Fiji/ ImageJ. Although filtering in all three programs provided good results (see figure 6), filtering with Amira is recommended since it is fast, efficient and the result can be checked in 3D as well as in 2D immediately. The different filters are difficult to compare, since they require very

different filtering times. In addition, the computers' performances (hardware as well as software) differed greatly (see table 3). The "median 3D" (kernel size 6x6x6) filter in Fiji/ ImageJ for instance can be recommended only for small data sets: filtering 56MB of data already required about 5 minutes. In comparison: the filter "Gaussian smoothing" (3D, kernel size 9x9x9) in Amira required only about one minute for 15GB of data and the filter "Noise reduction median" (3D, kernel size 3x3x3) required 3 minutes. The filter "median" (2D, kernel size 6x6) in Fiji/ ImageJ on the other hand could also be performed within 6 minutes on a 15GB data set.

**Table 2:** Different filtering methods of a high resolution scan of *O. militaris* pollinia

| No filtering | Filtering with XMReconstructor | Filtering with Fiji/ ImageJ | Filtering with Amira |
|---|---|---|---|
| - | Median filter (3D, kernel size 7x7x7) | Median filter (2D, radius: 6 pixels)<br>Gaussian blur (3D, sigma values: 2x2x2) | 2x Noise reduction median (3D, kernel size 3x3x3)<br>Gaussian smoothing (3D, kernel size 12x12x12) |
| - | Filtering time: 21h | Filtering time: ~8' | Filtering time: ~8' |

The two main disadvantages of filtering with XMController compared with filtering in Amira are the long filtering times (see table 2) and the fact that a large amount of the computer's resources are required during that time. Since XMController was only installed on the computer necessary for scanning, the scanning process was slowed down and caused system instability. The long filtering times make this method by far less flexible than the one in Amira.

**Table 3:** The Supermicro X8DA3 computer was used for 3D editing in Amira and Fiji/ ImageJ; the Dell Precision T5500 computer was used for scanning and reconstructing (with the XMController and the XMReconstructor)

| | Supermicro X8DA3 | Dell Precision T5500 |
|---|---|---|
| Operating system: | Windows 7 Professional SP1 (64 bit) | Windows XP SP3 (32 bit) |
| Processor: | 2x Intel Xeon X5680 | 2x Intel Xeon E5520 |
| RAM: | 96 GB (DDR3) | 4 GB (DDR3) |
| Graphics card: | NVIDIA Quadro FX 5800 | NVIDIA Quadro FX 5800 |
| Harddrive-type: | SSD | SSD |

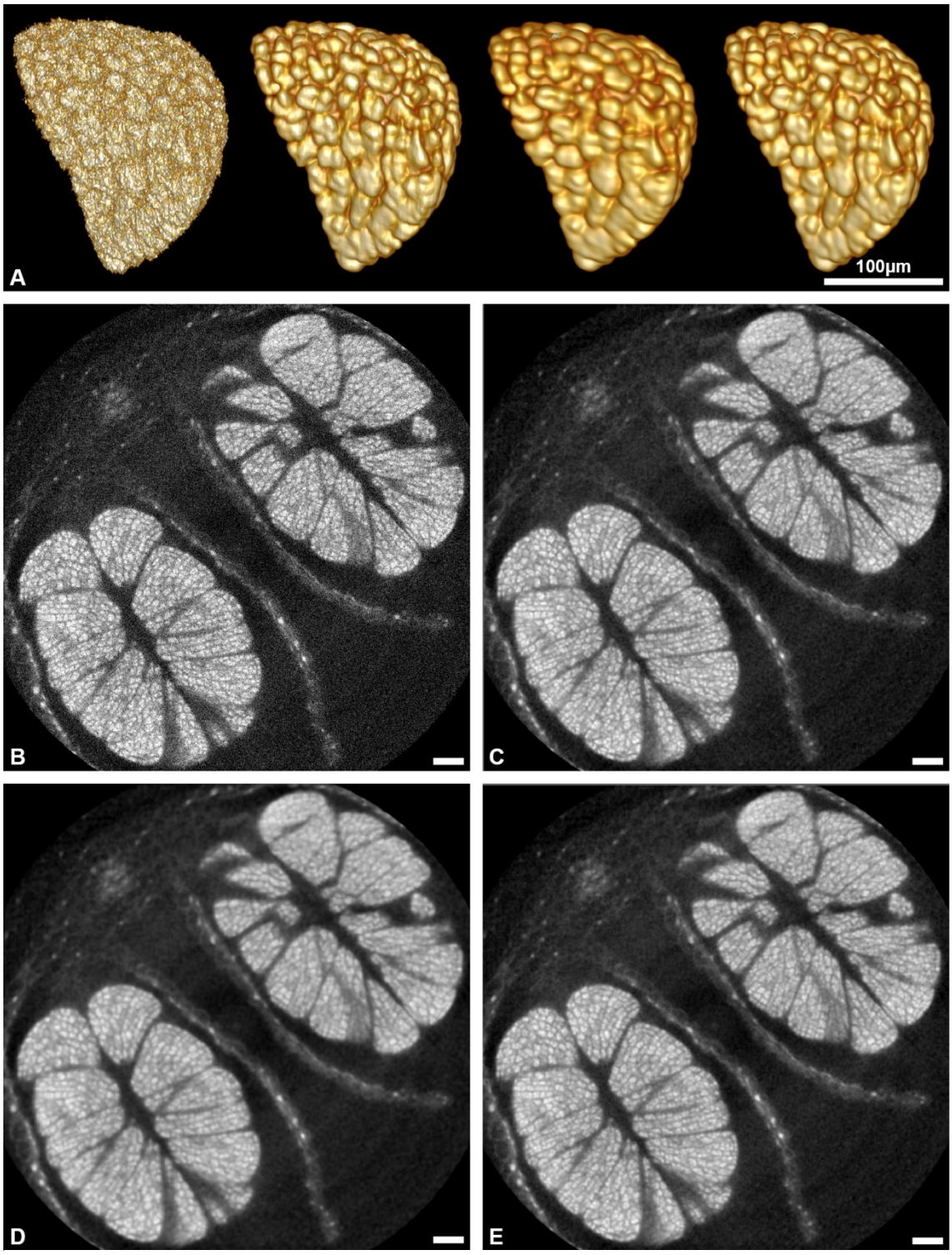



**Figure 6:** Different filtering methods illustrated on an *O. militaris* scan in high resolution (20x magnifications). The applied filters are according to table 2: (A) massula from left to right: unfiltered, filtered with XMReconstructor, with Fiji/ ImageJ and with Amira; (B-D) 2D sections of pollinia, (B) unfiltered, (C) filtered in XMReconstructor, (D) filtered in Fiji/ ImageJ, (E) filtered in Amira. Scale bars = 100µm

When counting pollen or ovules it is recommended to use the digital filters of Amira out of practicability although Fiji/ ImageJ filtering might give similarly good results. However, the same filtering conditions have to be present in the whole scan as well as in the counted domains. Otherwise filtering may influence the result.

Identical filtering of different parts of the sample does not influence the results of the counting process. The ovule number calculation of three gynoecia scans was conducted with as well as without filtering. The maximum difference between those results (filtered vs. unfiltered scans) was less than 0.4%.

In Amira, the two filters "3D Gaussian smoothing" and "3D noise reduction median" were used. Especially the "3D noise reduction median" filter was usually applied several times in order to obtain a better filtering quality (lower noise level). In Fiji/ ImageJ a combination of the filters "median 3D" or "mean 3D" and "Gaussian Blur 3D" was used.

## Difficulties of this method

### *Pollinium fragility and ovule development*

As mentioned above, pollinium fragility was a major problem. Not only was a large amount of data unusable, but it was also not always clear whether or not a pollinium was complete or several massulae were missing. This problem may be solved by collecting unopened buds right before opening. If parts of the pollinia would break away, they would remain inside the bud and could thus be counted. However, this may increase the risk of samples containing underdeveloped pollen grains or ovules. Possibly underdeveloped ovules were present in some of the buds of *E. palustris* that were analyzed. On those scans, usually only in the upper part of the gynoecium, ovules were highly contrasted. In the lower part of the gynoecium placental tissue was usually developed, but no (or less developed) ovules were found. In the transition area between fully developed ovules and underdeveloped ovules, less well contrasted ovules were present (see figure 17). It was also observed that ovules were present on all placentae but differently well contrasted and in different developmental stages (see figure 7). The risk of underdevelopment might also increase in pollen, However, Hsu et al. (2014) investigated different developmental stages in buds of several *Phalaenopsis*

species and discovered that the number of developed tetrads is almost the same in buds shortly before flowering and opened flowers. In the species we studied, although contrast of the ovules was sometimes poor, signs of pollen underdevelopment could never be detected; not even in early buds.

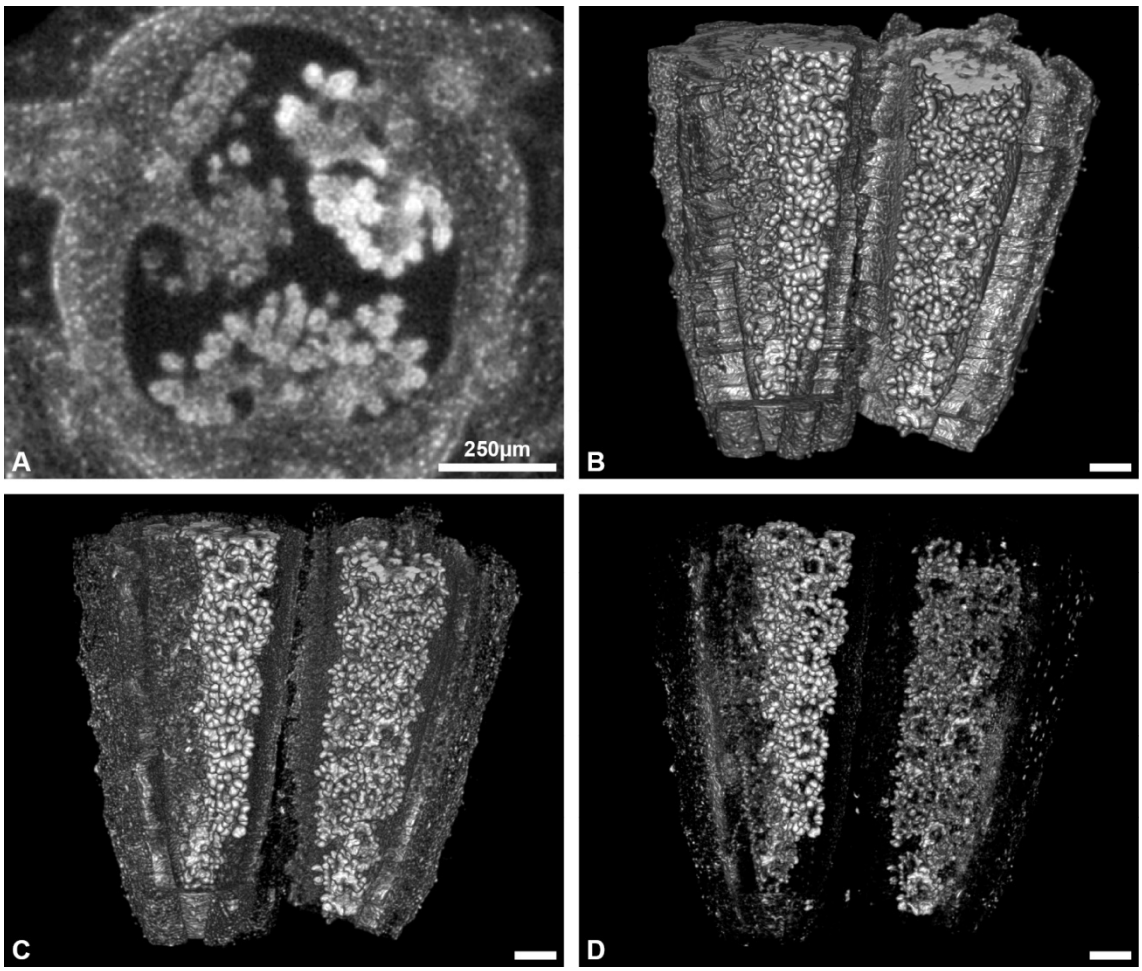



**Figure 7:** Gynoecium of *E. palustris*: (A) 2D section; (B-D) 3D images with different threshold levels, (B) low threshold level, (C) medium threshold level, (D) high threshold level. Scale bars = 250 µm

### *Pollen counting*

In order to be able to acquire the number of pollen grains in low resolution scans without performing additional high resolution scans in all samples, the determined average pollen grain volume of a reference sample (that was scanned in low as well as in high resolution) was transferred to the other samples of one species. An equal average pollen grain size within one population of orchids was therefore assumed, but not investigated any further. By collecting only late buds as mentioned above, the risk of different average pollen size

might be minimized since the collected samples would be in a more similar and comparable developmental state.

One of the main problems was the mentioned grayscale issue. Different pollinia as well as different parts within one pollinium were contrasted unequally. It was tried to solve this issue by using scaling methods (CT scaling and byte scaling). This still could not fix the problem. Especially the differences within one pollinium indicate that different parts were contrasted differently and thus also different pollinia might be contrasted differently. This implies that the determination of one threshold which suits all samples is difficult to achieve.

### *Ovule counting*

Although this method provides a robust way of counting ovules, movement of the sample during scanning can be a problem. It was observed on the majority of scans that the pictures on the top and on the bottom end of a scan domain were blurred due to reconstruction artefacts; the latter had a significant but small influence on the threshold of those pictures (see figure 8). Movement has to be avoided during scanning as much as possible. If bigger differences in threshold exist, however, it is possible to separate a domain from the rest of the scan and count ovules in this domain separately.

Furthermore, contrasting did not work very well for post anthetic flowers, and early and late fruits. The contrast in those samples was usually poor and it was difficult to select ovules without selecting large amount of placental material. Due to their development, ovules increased in size and occupied more space inside a gynoecium, sometimes reducing the ovule-free space within gynoecia to a minimum (see figure 26). Together with the weak contrast, it was thus difficult to separate the placentae and the corresponding ovules. In some species, as for instance in *D. viridis*, ovules are well developed at anthesis (Ziegenspeck 1936, p. 330). This can make the counting process difficult in certain species and is yet another argument for collecting buds right before opening, rather than already opened flowers; the analysis of developing fruits is not recommended.

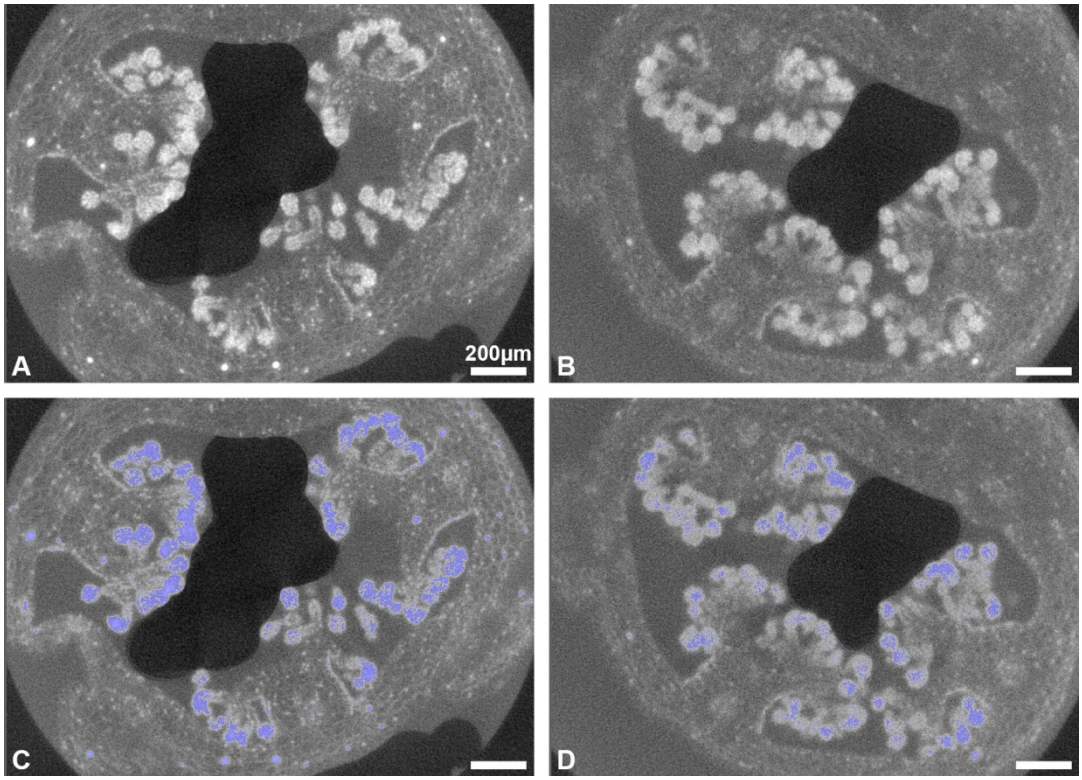



**Figure 8:** 2D sections of a gynoecium of *D. majalis*: (A) a midsection without threshold masking, (B) a bottom section without threshold masking, (C) a midsection with threshold masking, (D) a bottom section with threshold masking. Scale bars = 200µm

In *G. conopsea,* although the flowers did not seem further developed than flowers of other analyzed species, it was not possible to count the ovules due to poor staining. The ovules were not stained adequately whereas the ovary wall was stained better than in most other samples. The scanning quality appeared to the naked eye similar to or even better than in other samples. The ovary wall and its cells appeared to be very thick and solid. Those thicker and more massive ovary walls could be a reason for the poor contrasting, since the contrast agent may not have been able to pass through those structures appropriately. It is, however, also possible that the ovules simply were further developed and could not absorb the contrast agent as well as early ovules.

Another reason for the low contrasting of the ovules could be that the contrast agent was changed only two times in order to minimize the disturbances that might damage the pollinia. In most other samples the contrast agent was changed more often (up to eight times). However, also in *D. incarnata* the contrast agent was only changed twice. In this species, the contrasting was good and counting could be performed as usual. Additionally,

the samples lay in the contrast agent for more than five month. The fact that the ovule walls were stained well is yet another reason why the number of changes of FAA+PTA is not likely to be the reason for the low contrasting of the ovules.

It seems that FAA+PTA works in many, but not all samples as an efficient contrast agent. Performing incisions on the ovary wall could make the infiltration more efficient in such samples. This, however, increases the risk of ovule loss. Collecting earlier developmental stages in certain species or using other infiltration agents may be a solution to this problem.

### *Massulae counting*

Unfortunately, it was not possible to develop an automatic counting procedure. The time required for manual counting was dependent on the quality of a scan. In scans with lower quality the counting process was especially difficult and the time consumed by the counting process increased.

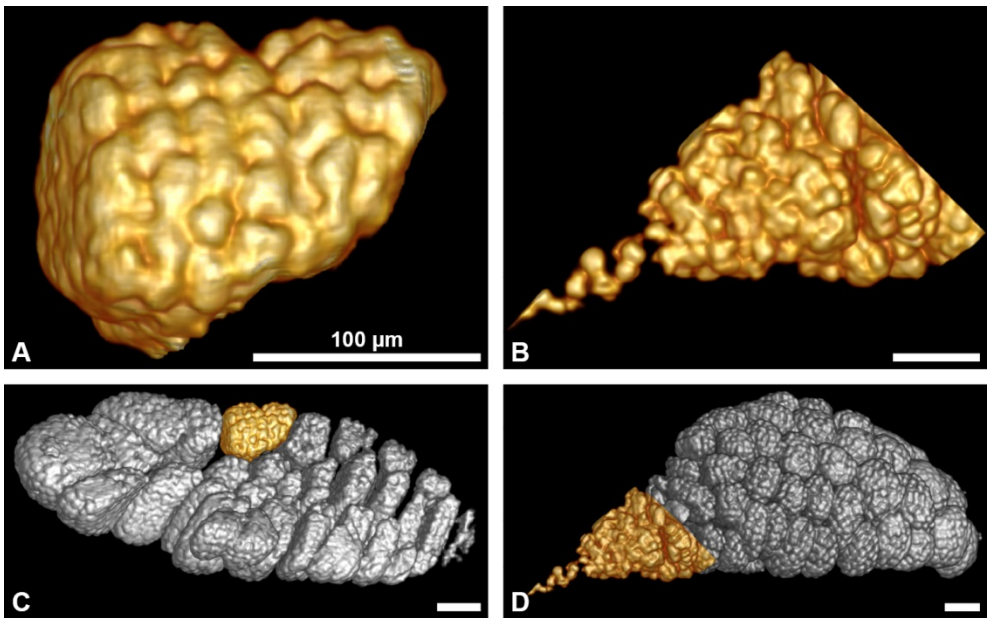



**Figure 9:** *A. pyramidalis*: (A) most likely two massulae, (B) basal part of a pollinium-half, (C-D) each showing one half of the same pollinium with the parts of (A-B) highlighted. Scale bars = 100 µm

Usually, single massulae could easily be distinguished. Under some circumstances, however, it was not always clear whether a structure was formed by only one or by two or more massulae (see figure 9 (A&C)).

A similar problem occurred at the base of pollinia. It was often difficult to judge whether a structure consisting of only a few pollen tetrads can be counted as one massulae or not. Additionally, the boarders between massulae seemed to vanish especially at the base of a pollinium (see figure 9 (B&D)).

#### *Large amount of data*

The last issue to mention is the large amount of data produced by scanning, reconstructing, and editing. A total amount of 6TB of data was produced during this study. Especially CT scaling increased the amount of data dramatically. Due to the fact that air has to be visible on all sides of all pictures it was not possible to restrict the domain of a scan that should be reconstructed. Handling, copying and storing the data can become difficult. Furthermore large amounts of RAM are recommended in order to be able to load the data in Amira (or in order to use the function "3D Iterative Thresholding" in Fiji/ ImageJ).

## Results of the analysis

As described above, flowers from the bottom, the middle and the top of an inflorescence were aimed for. P/Os were calculated and compared. Since P/Os reflect also the breeding system of plants (Cruden 1977), differences between rewarding and deceptive orchids within a genus may exist. If possible, the phenotypic gender ($G_p$) of a flower was determined and calculated.

The average ratio of ovules to pollen grains within a population that is necessary for determining the $G_p$ was calculated by combining all the data collected during this study concerning one species at a given location.

Additionally, the average difference between the levels bottom-middle-top was calculated and illustrates, if and how strong the average changes are from one level to the next.

#### *Epipactis palustris*

*E. palustris* was chosen not only because it was easily accessible in the botanical garden, but also because many species of *Epipactis* are autogamous (Bonatti, Sgarbi, and Del Prete 2006; Molnar and Sramko 2012; Squirrell et al. 2002; Claessens and Kleynen 2011, p.

102), whereas *E. palustris* and *E. helleborine* are allogamous. However, delayed autogamy[3] was also observed in the two latter species (Ziegenspeck 1936; Wiefelspütz 1970; Claessens and Kleynen 2011 pp. 96, 102, and references therein; Tałałaj and Brzosko 2008), which is a possible strategy if no visitors pollinate the flower after a certain amount of time (Lloyd 1992; Tałałaj and Brzosko 2008). A recent study that investigated the selfing potential in several *Epipactis* species observed major differences in fruit set between different *E. palustris* populations ranging from 0.5% to 82.5%, when only spontaneous autogamy was possible (Tałałaj and Brzosko 2008). This issue makes the investigation of the P/Os of *E. palustris* especially interesting. Furthermore, *E. palustris* offers a nectar reward to pollinators (Claessens and Kleynen 2011, p. 92; Brantjes 1981, p. 60). The main pollinators of this species are Diptera such as *Empis sp.*, species of Syrphidae, Hymenoptera (mainly honey bees) and also several Coleoptera (Jakubska-Busse and Kadej 2011, p. 54; Griebl 2013; Ziegenspeck 1936, p. 92). However, ants have also been observed as effective pollinators, inducing mainly self-, but also cross-pollination (Brantjes 1981, pp. 60, 65). It should be mentioned that in the present study, pollen bearing ants could be observed on *E. palustris* in the short time when the flowers were collected. Many plants were covered with aphids which most likely were the reason for larger amounts of ants visiting the plants.

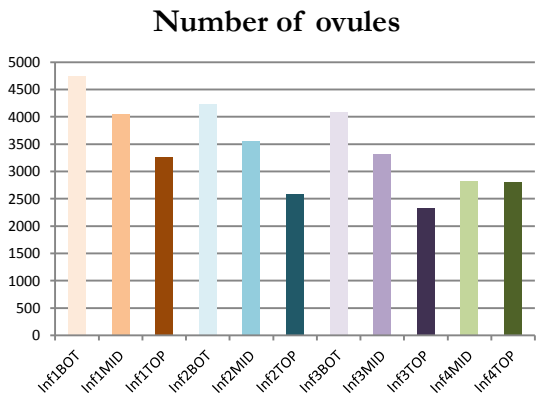
Only in one top flower was the P/O calculated since pollinia from open flowers were washed away and ovules in buds often showed signs of underdevelopment. The gynoecium contained around 6580 ovules and both pollinia a total number of around 173100 pollen grains. The P/O is therefore ca. 26.

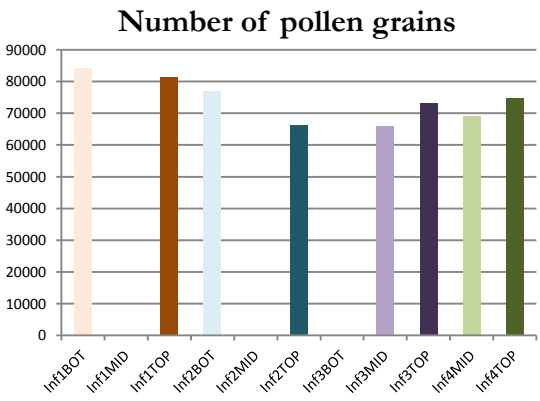
As mentioned further above, the problem of fragile pollinia was underestimated in *E. palustris* especially: the species has fragile and mealy pollinia. Thus, pollinia were missing in all collected samples with opened flowers. Only in buds, which were collected exclusively from the middle or the top of the inflorescences, was it possible to scan complete pollinia; however, several gynoecia in those samples seemed underdeveloped. This made a detailed analysis of *E. palustris* impossible.

[3] Autogamy takes place at the end of anthesis in case the pollen was not removed from the flower

### *Anacamptis pyramidalis*

Two *Anacamptis* species, *A. pyramidalis* and *A. morio,* were chosen because the genus *Anacamptis* includes deceptive species, such as the stated ones, and a rewarding species, *A. coriophora*, all of which can be found in Austria (Fischer, Oswald, and Adler 2008; Salzmann et al. 2007; Bell et al. 2009; Kowalkowska et al. 2012; Claessens and Kleynen 2011; Lind et al. 2007; Fay and Rankou 2010). Unfortunately, *A. coriophora* could not be collected during this study and thus could not be analyzed. In some publications *A. pyramidalis* is considered to be rewarding (Inda, Pimentel, and Chase 2012; Bateman and Rudall 2006). However, those studies either refer to van der Cingel (1995), which used Darwin (1886) as a main reference, or refer directly to Darwin. Newer publications describe *A. pyramidalis*, as abovementioned, as a non-rewarding orchid without any traces of nectar. Only insects with long proboscises are able to enter the long, nectarless spur of *A. pyramidalis* on their search for nectar. Thus, the main pollinators are butterflies and moths (Lepidoptera) (Bell et al. 2009; Claessens and Kleynen 2011, p. 206 et seqq. ; Darwin 1886). However, Griebl (2013) also mentions honey bees as observed pollinator.



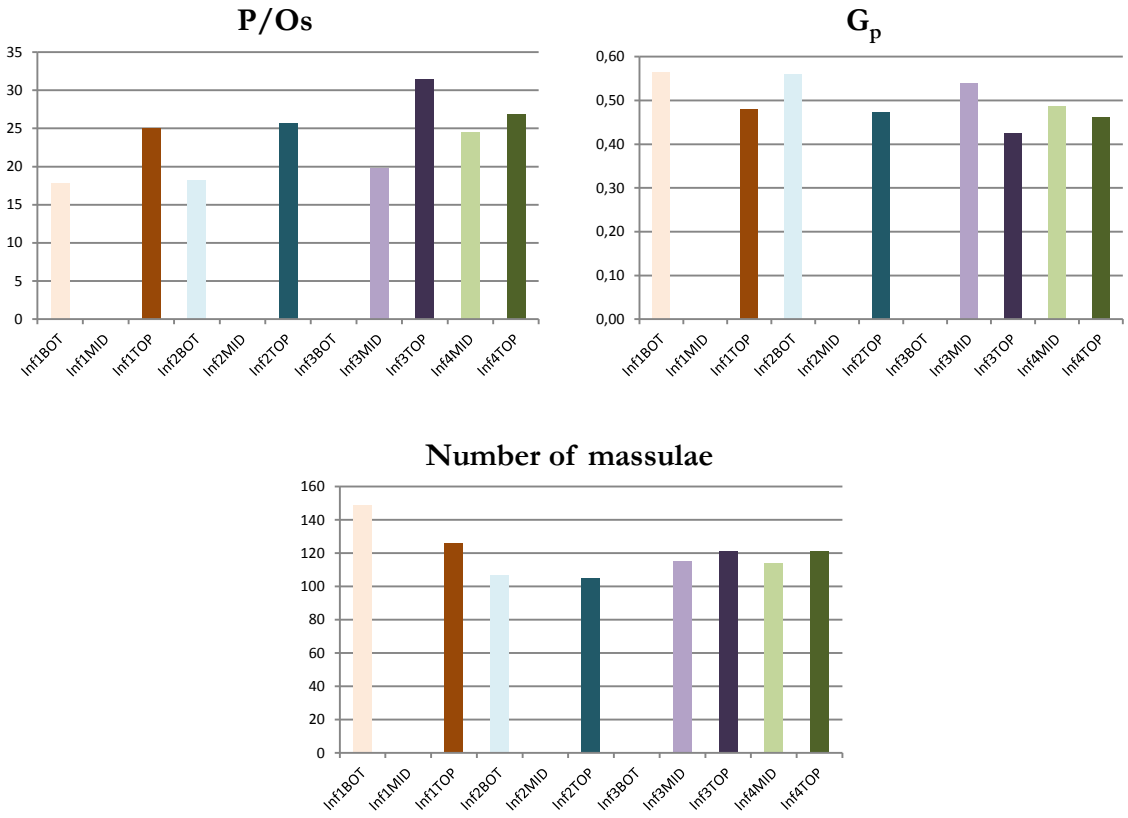



**Figure 10:** Overview of the gathered data of *A. pyramidalis*

The number of ovules declines from bottom to top in all analyzed inflorescences of *A. pyramidalis.* The average difference between the three levels bottom-middle-top was calculated and amounts to -16%. The number of counted ovules lies between 2330 and 4740 per ovary.

The number of pollen grains does not show such a clear pattern. An increase as well as a decrease could be detected. Also here the average difference was calculated and amounts to +3%. Hence, the number of ovules is the variable that influences the P/Os in this species whereas the number of pollen grains seems to vary without recognizable pattern.

The P/Os increase from bottom to top in all analyzed inflorescences of this species. In every inflorescence $G_p$ is lowest in the top flower. There seems to be a trend from more female flowers in the bottom to more male flowers in the top of the inflorescence due to the decline of ovule number and the +/- constant number of pollen grains.

In this species, also the total number of massulae was ascertained (ranging from 105 to 149 massulae per flower).

### *Anacamptis morio*

In order to enlarge the sample size of *Anacamptis*, *A. morio* was studied as well. The breeding system is characterized by a high level of outcrossing; selfing occurs rarely (Johnson, Peter, and Ågren 2004). Jersáková and Kindlmann (1998) could not find any evidence at all for the occurrence of autogamy and thus consider *A. morio* as a non-autogamous species. The species is, however, self-compatible. As mentioned above, *A. morio* is a deceptive orchid species producing no nectar. The main pollinators are bees (Hymenoptera, Apoidea) such as bumble bees, honey bees and solitary bees (Griebl 2013; Nilsson 1984) whereby Nilsson (1984) states that especially bumble bee queens play an important role as pollinators.

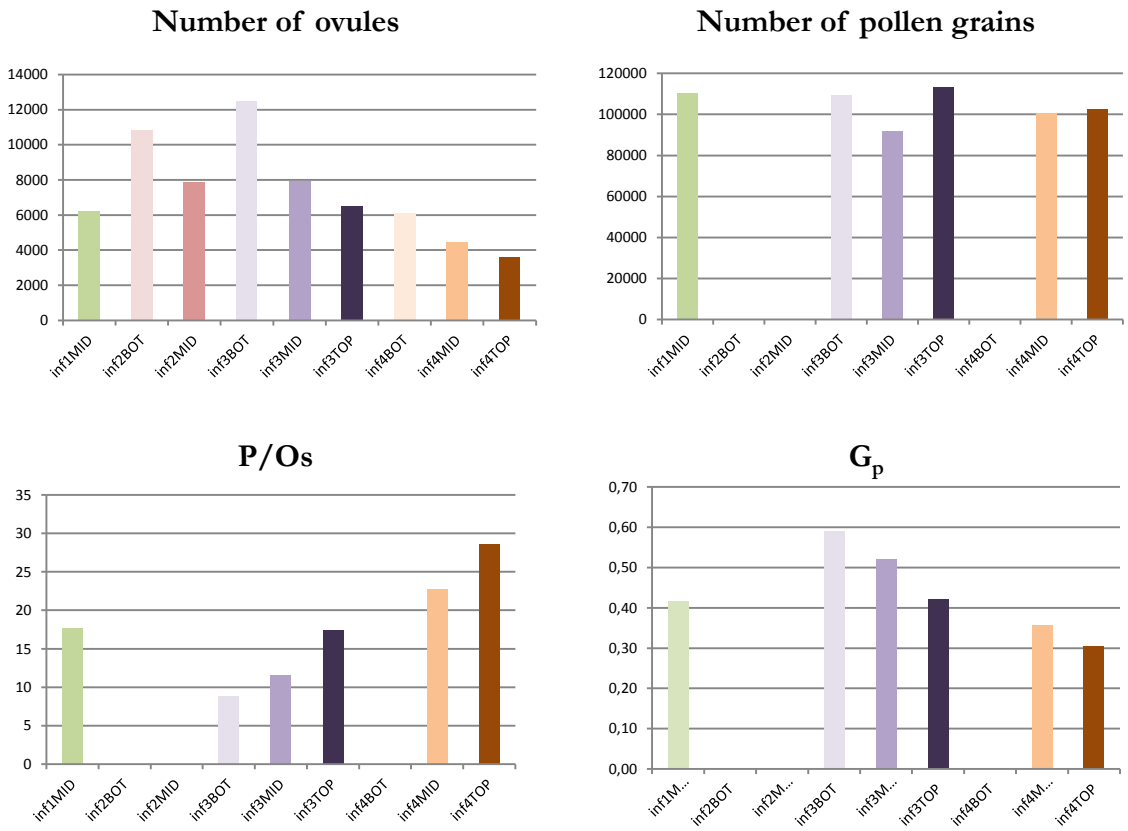



**Figure 11:** Overview of the gathered data of *A. morio*

The pattern present in *A. pyramidalis* was also observed in *A. morio*. The number of ovules declines from bottom to top. The average difference between two levels (going from bottom to top) is -26%. Additionally, there are large differences between inflorescences. In one inflorescence the bottom flower, hence the flower with the highest ovule count, has less ovules than the top flower of a different inflorescence. The ovule counts range from 3600 up to almost 12500 ovules; the lowest count is almost 3.5 times smaller than the highest count. In contrast to this, the number of pollen grains is more or less equal in all flowers, although there are fluctuations. The average difference between the levels bottom-middle-top is ca. +3%. Pollen counts reach from 92000 up to 110500 grains; hence the lowest count is 1.2 times smaller than the highest count.

Due to the large differences in ovule number and the more or less constant number of pollen grains, variation in P/Os is driven by ovule numbers. In all inflorescences the P/O increases from bottom to top.

The $G_p$ declines from bottom to top meaning that the bottom flowers are more female and the top flowers are more male. Only one flower showed a stronger tendency of being clearly more female than others ($G_p$=0.59) whereas four flowers show a $G_p$ of 0.42 or lower indicating a stronger tendency of being male. However, it has to be mentioned that the sample size with n=6 is small.

### *Orchis militaris*

The genus *Orchis* was chosen since it includes several deceptive species growing in Austria, but also one possibly rewarding species, *O. anthropophora* (Fischer, Oswald, and Adler 2008; Griebl 2013; Inda, Pimentel, and Chase 2012). However, it is not clear whether or not *O. anthropophora* is truly a rewarding species. Many authors claim *O. anthropophora* to be rewarding (Voeth 1999a; Bell et al. 2009; Inda, Pimentel, and Chase 2012; Jacquemyn, Brys, and Hutchings 2011; Baumann, Kretzschmar, and Blatt 2005); Claessens and Kleynen (2011) on the other hand report that from almost 1300 flowers, not a single possessed nectar; they thus assume *O. anthropophora* to be deceptive.

*O. militaris* is a nectarless, deceptive orchid (Claessens and Kleynen 2011; Bell et al. 2009; Fay 2013; Inda, Pimentel, and Chase 2012) that is mainly pollinated by bees (Voeth 1999a; Griebl 2013; Inda, Pimentel, and Chase 2012) and bumble bees (van der Cingel 1995, p. 100 et seq. ; Fay 2013).

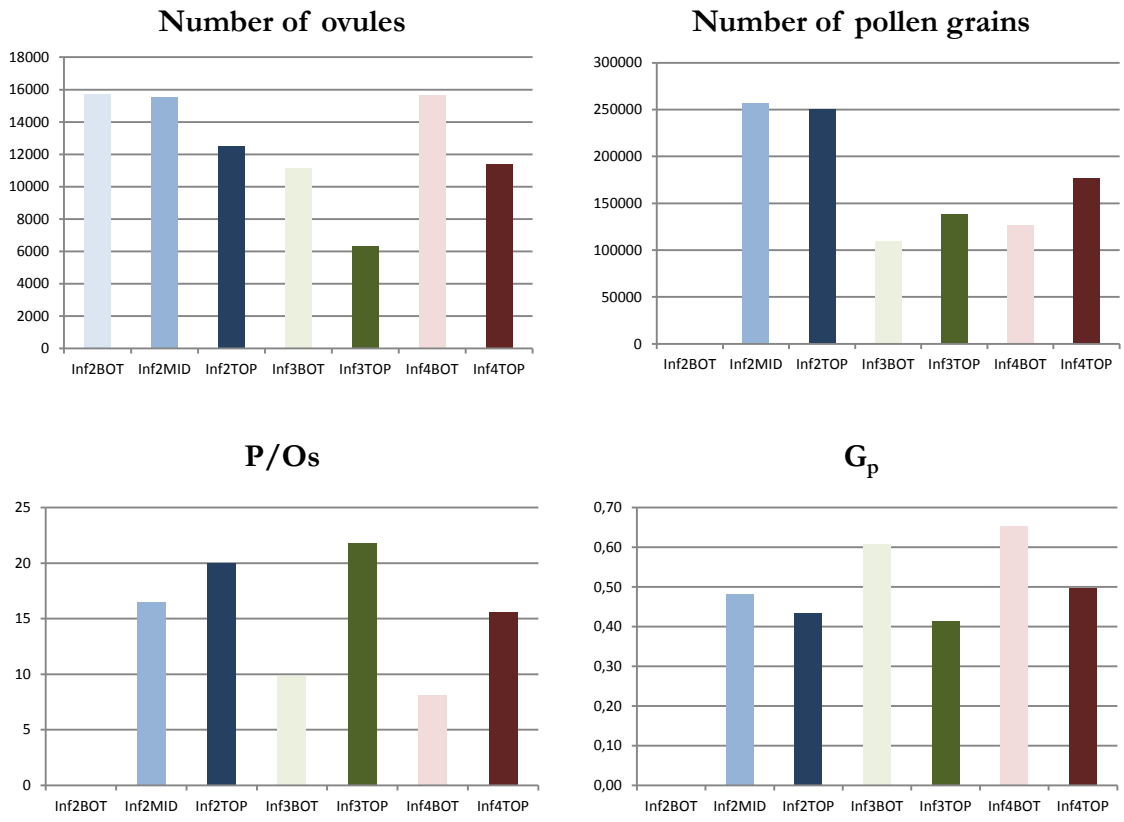



**Figure 12:** Overview of the gathered data of *O. militaris*

The number of ovules declines from bottom to top in all investigated samples. Between two levels going from bottom to top, the average difference is -17%. The highest ovule number counted is 15750, whereas the lowest count amounts to 6340 ovules. Hence, the highest and the lowest count differ by a factor of around 2.5.

The number of pollen grains increases in two samples from bottom to top, but also slightly declines in one. Thus, the average difference between two levels (bottom-middle and middle-top) equals +9%. The differences between flowers, especially between flowers from different inflorescences, are high. In no other species could such large differences in pollen grain number be detected. The highest count amounts to 256900 whereas the lowest count

amounts to 109500, thus they differ by a factor of 2.4. Compared to other analyzed orchid species in this study, this "lowest" value is still amongst the highest.

Although the total pollen grain number and the number of ovules differ by a similar factor, the P/Os show a similar picture as in the other species increasing from bottom to top whereas the $G_p$ declines. The reason behind this is that the differences in pollen grain numbers within one inflorescence are small compared to the differences between inflorescences. Also in this species the bottom flowers within an inflorescence tend to be more female than their corresponding top flowers.

### *Dactylorhiza incarnata*

Four *Dactylorhiza* species were chosen since the rewarding *D. viridis*, as well as the rewarding *Gymnadenia conopsea* (Bell et al. 2009; Claessens and Kleynen 2011, pp. 234, 290), which belongs to a sister group of the genus *Dactylorhiza* (Inda, Pimentel, and Chase 2012; Bateman and DiMichele 2002; Box et al. 2008; Schlegel et al. 1989; Bateman and Farrington 1989), can be found in Austria. Thus, in this group a good comparison of rewarding and deceptive species was hoped to be possible.

*D. incarnata* was chosen as one out of three collected nectarless, deceptive *Dactylorhiza* species (Inda, Pimentel, and Chase 2012; Bell et al. 2009; Claessens and Kleynen 2011; Lammi and Kuitunen 1995). Although the species is self-compatible, autogamy usually does not occur (Lammi and Kuitunen 1995). As pollinators bumble bees are mentioned and have also been observed as such (Griebl 2013; Vallius, Lammi, and Kuitunen 2007; Voeth 1999b, p. 123; Nilsson 1981).

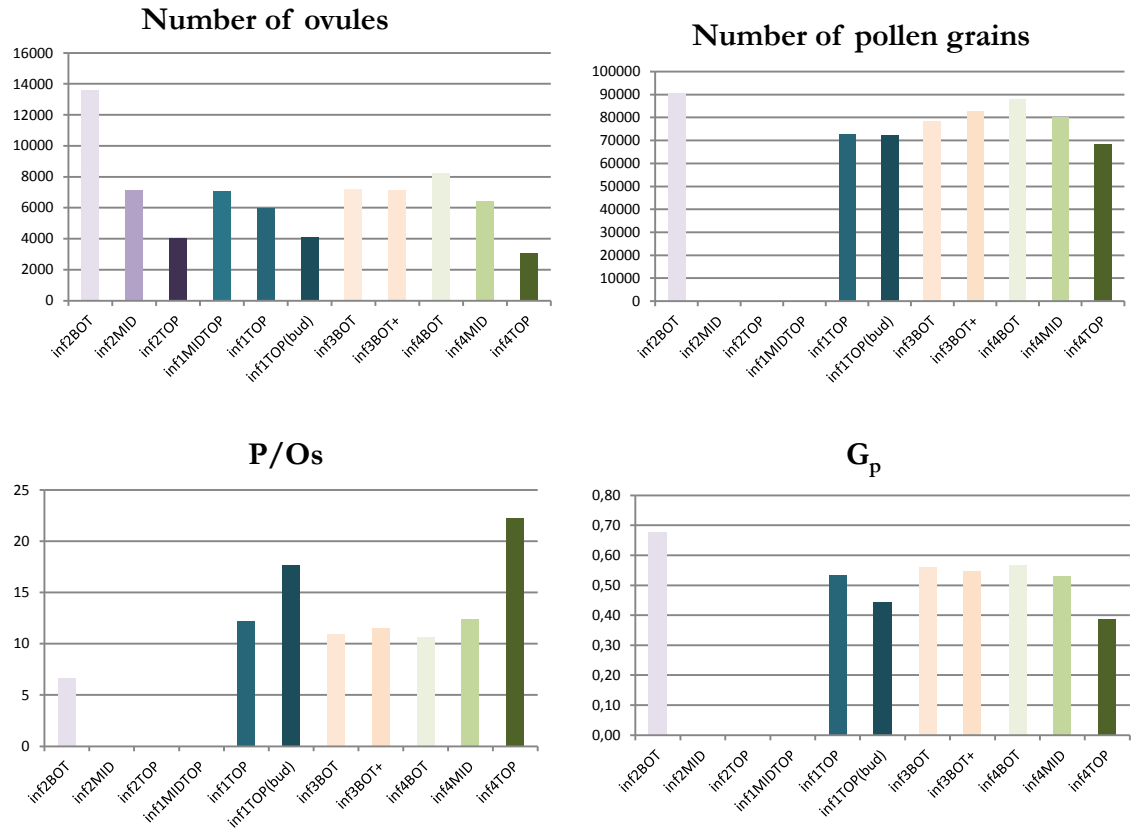



**Figure 13:** Overview of the gathered data of *D. incarnata*

The number of ovules declines from bottom to top. The average difference between two levels going from bottom to top amounts to -41%. In one investigated species the total difference in ovule number amounts to 9600 ovules. The analyzed flower in the top only produced 29% of the ovule amount that could be found in the bottom flower of the same inflorescence (in other words a difference by a factor of 3.4 was measured). The largest ovule count found in this species amounts to 13610 ovules whereas the lowest count that could be found amounts to 3060 resulting in a difference by a factor of 4.4. The number of ovules was additionally calculated for two adjacent flowers in the bottom providing the same number of ovules (a total difference of 1‰).

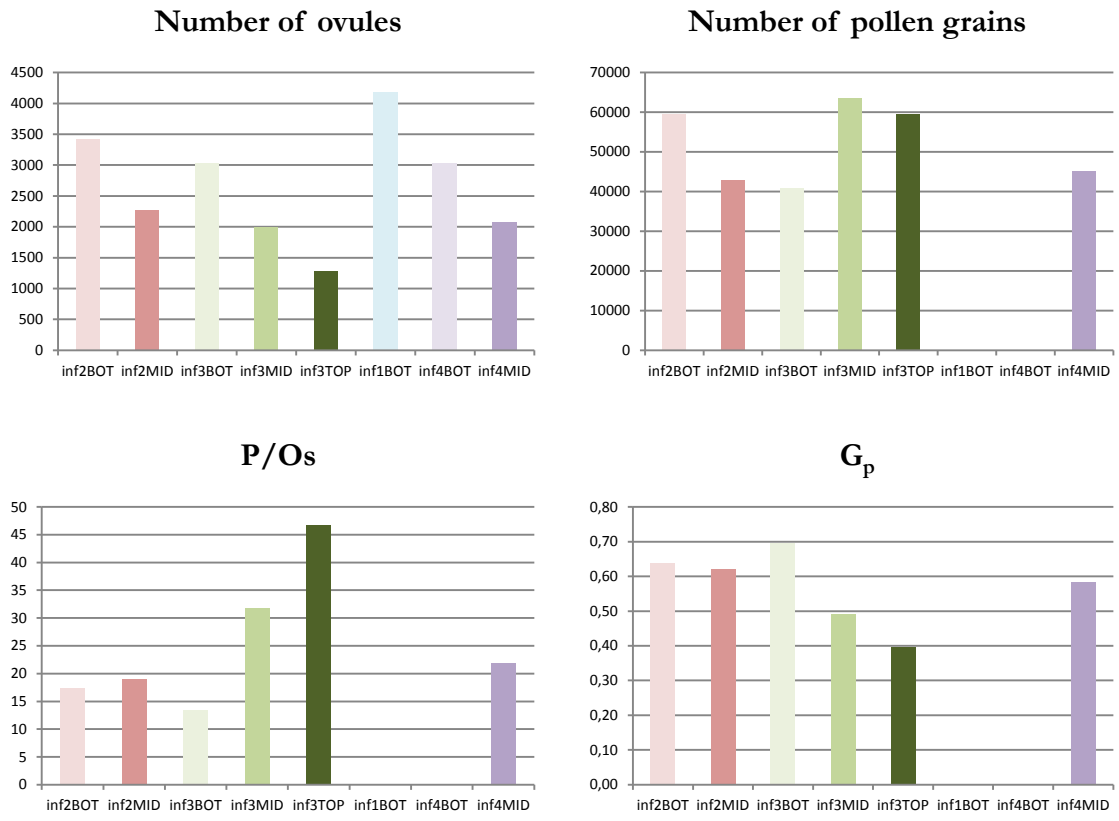
Only in one inflorescence could the number of pollen grains in distinct inflorescence positions (bottom, middle, top) be measured. Thus, the average difference between two levels going from bottom to top could only be calculated with the data set of one inflorescence and amounts to -12%. Due to damaged and missing pollinia it was not

possible to analyze more pollinia in different positions within one inflorescence. The counted number of pollen grains in this species varied between 72300 and 90600 grains, differing by a factor of 1.3.

Since extensive data of pollen grain numbers could not be obtained, the data set of P/Os is poor for this species. Due to the facts that the number of ovules declines dramatically from bottom to top and the number of pollen grains in different flowers are similar, P/O value patterns similar to those observed in other species are expected.

### *Dactylorhiza fuchsii*

*D. fuchsii* was chosen as one out of three collected deceptive *Dactylorhiza* species (Box et al. 2008), although some authors mention that the glucose rich stigmatic exudates act as a reward for certain pollinators (Dafni and Woodell 1986; Paulus 2005). The observed pollinators of this species are Hymenoptera, Diptera, and Coleoptera (Griebl 2013, p. 75; Paulus 2005; Dafni 1987; Neiland and Wilcock 1995; Dafni and Woodell 1986).



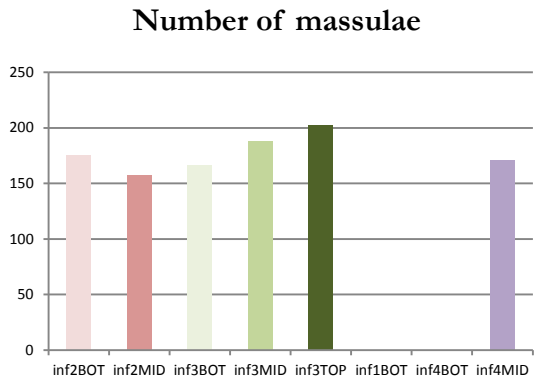



**Figure 14:** Overview of the gathered data of *D. fuchsii*

The number of ovules declines in all analyzed inflorescences from bottom to top with an average difference of -33% between two levels. The highest number counted amounts to 4170 ovules; the lowest number was 1270, resulting in a difference by a factor of 3.3.

Strong fluctuations of pollen grain numbers within one inflorescence could be detected. A strong decrease from bottom to middle, as well as a similar strong increase from bottom to middle was detected. However, although there are strong fluctuations, the average difference between two levels going from bottom to top amounts to only -2%. The mentioned fluctuations reach from 40800 pollen grains in the bottom flower up to 63600 pollen grains in the middle flower of one inflorescence differing by a factor of 1.6. The two values are also the absolute minimum and maximum number of pollen grains found in this species. If only this one inflorescence was analyzed, the average difference between two levels going from bottom to top would amount to +25%. A clear pattern of de- or increase could not be detected.

The P/O seems to increase from bottom to top. Due to the large difference in pollen grain number among one inflorescence, the bottom and the middle flower have similar P/Os in one inflorescence, although the number of ovules is smaller by a factor of 1.5 in the middle of the inflorescence. This shows, that also the random appearing fluctuations in pollen grain number can have an influence on the P/Os and thus also on the $G_p$. The $G_p$ shows a similar picture than in other analyzed species; especially the top flowers show a clearly stronger male function.

Additionally the massulae were counted in this species ranging from 157 up to 202.

### *Dactylorhiza majalis*

*D. majalis* was chosen as third deceptive species of the genus *Dactylorhiza* (Hansen and Olesen 1999; Claessens and Kleynen 2011). Bumble bees seem to be the main pollinators of this species (Griebl 2013; Hansen and Olesen 1999).

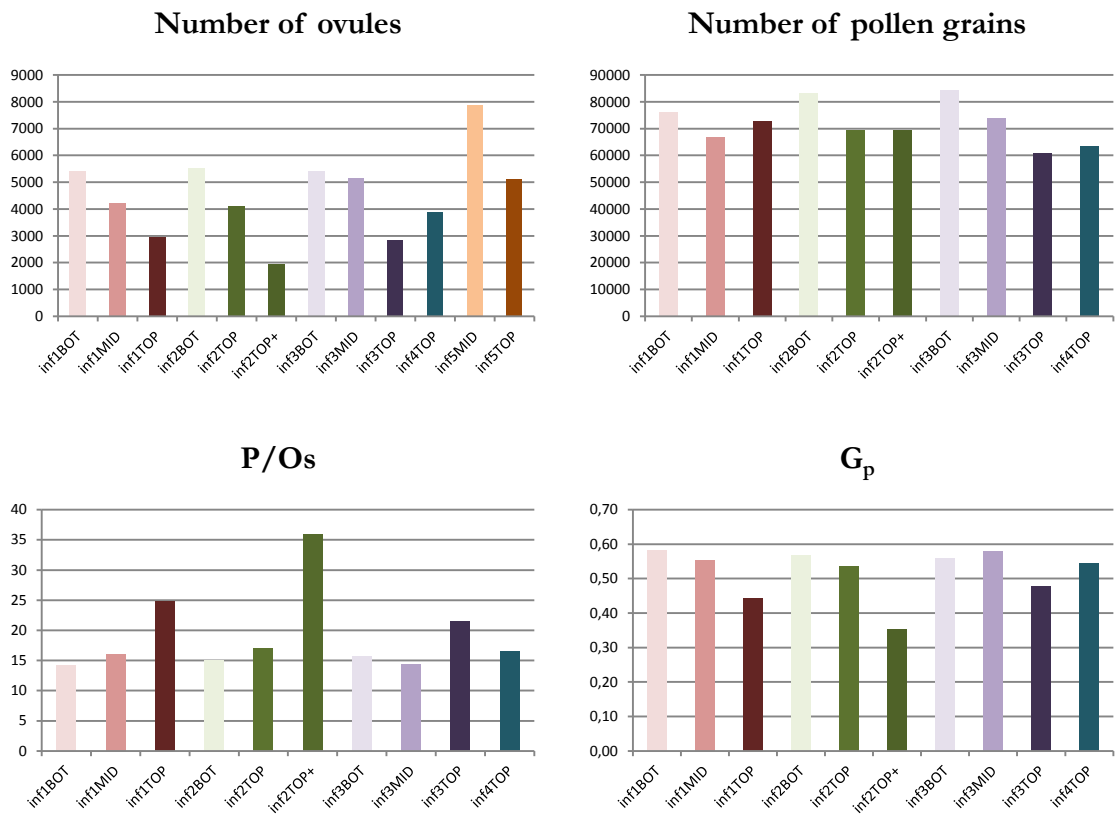



**Figure 15:** Overview of the gathered data of *D. majalis*

Unlike the other deceptive *Dactylorhiza* species analyzed, in many inflorescences undamaged pollinia could be found in lower and upper regions. The number of ovules decreases in all inflorescences from bottom to top. The average difference between two levels going from bottom to top amounts to -32%. Strong differences not only occur within, but also among different inflorescences. The number of ovules in top flowers varied between 1930 and 5110. The highest count with 7880 ovules could be observed in a flower from the middle section of an inflorescence. Unluckily, no flower from the bottom region of this inflorescence was scanned since the pollinia were damaged. The lowest count was

found in a top flower of a different inflorescence and amounts, as mentioned before, to 1930 ovules. Those two values differ by a factor of 4.1.

The number of pollen grains may decrease or increase from bottom to top. However, the largest count could be found in all investigated inflorescences in the bottom indicating that there might be a tendency of decrease. Also here the average difference was calculated and amounts to -8%. The highest number of pollen grains was 84300 whereas the lowest count amounts to 61000, differing by a factor of 1.4. The pollinia of the sample with the highest ovule number were destroyed and thus could not be analyzed.

The P/O seems to be more or less stable in bottom to middle flowers around a value of 15. In some flowers located at the top of the inflorescence higher values up to 36 could be detected. The $G_p$ shows a similar pattern. Three flowers had a $G_p$ under 0.5. All those flowers were from the upper region of an inflorescence, which indicates that at least in the topmost flowers the male functions becomes more important in respect to the female function.

### *Dactylorhiza viridis*

*D. viridis* (former *Coeloglossum viride*) was long considered to not be included in the genus *Dactylorhiza* but to form a sister group to it (Devos et al. 2006). However, molecular results based on combined DNA material suggest an inclusion of *D. viridis* in the genus *Dactylorhiza* (Inda, Pimentel, and Chase 2010, 2012; Bateman et al. 2003). *D. viridis* is the only rewarding species within the genus *Dactylorhiza* (Claessens and Kleynen 2011; Devos et al. 2006), offering traces of nectar (Bell et al. 2009; Ziegenspeck 1936) and was thus a species of high interest in the comparison of deceptive and rewarding orchids. Possible autogamy was discussed (Hagerup 1952; Reinhard 1991, p. 87) but could not be observed by Claessens and Kleynen (2011) nor by Hagerup (1952). Allogamous insect pollination appears to be the general reproductive strategy of the species. The main pollinators (also observed in Austria) are Coleoptera and Hymenoptera (family Ichneumonidae) (Griebl 2013; Claessens and Kleynen 2011, p. 242; Silén 1906). Noteworthy, Silén (1906) could observe pollinators mainly in the very early morning hours from 2 to 10 a.m. during his

observations near the village Kittilä, Finland. Afterwards the visitation stopped almost completely.

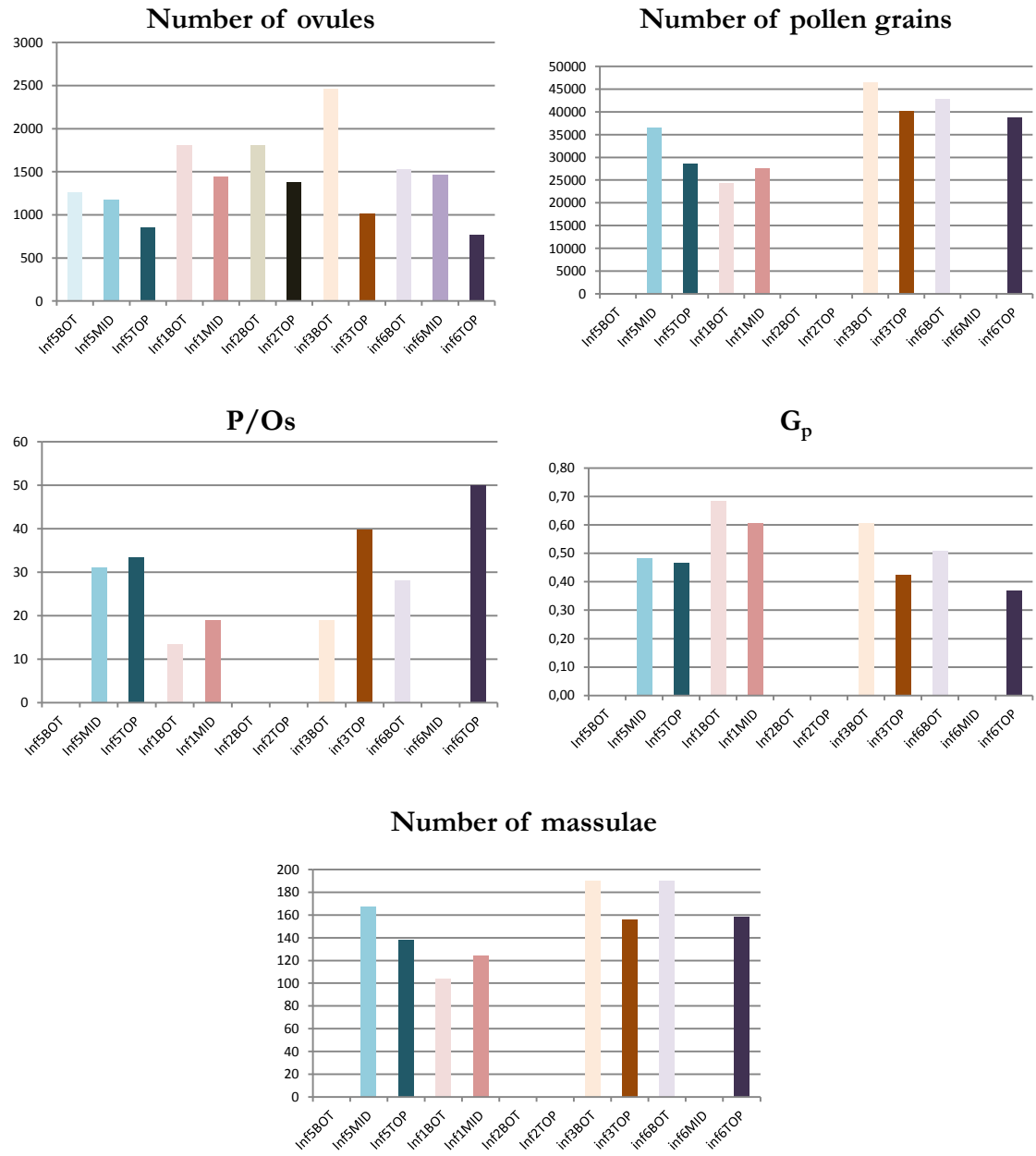



**Figure 16:** Overview of the gathered data of *D. viridis*

In *D. viridis* the number of ovules declines from bottom to top in all analyzed inflorescences. Although in two inflorescences the percentage of decline was only 4-7%. The average difference between two levels going from bottom to top amounts to -22%. The ovule numbers range from 770 up to 2460 which thus differ by a factor of 3.2.

The number of pollen grains declines 3 out of 4 times. However, in one sample an increase from bottom to top could be observed. The average difference between two levels going from bottom to top is slightly negative and amounts to -5%. The lowest value, 24200 pollen grains, and the highest value, 46400 pollen grains, differ by a factor of 1.9, indicating that there are large differences between inflorescences, but smaller differences within one.

The P/Os increase from bottom to top whereas the $G_p$ declines indicating that the bottom flowers tend to be more female than the top flowers. The P/Os calculated in this species range from 13 (in a bottom flower) up to 50 (in a top flower).

In this species also the number of massulae was counted; it ranged from 104 to 190.

### *Gymnadenia conopsea*

As abovementioned, the genus *Gymnadenia* is closely related to the mostly deceptive genus *Dactylorhiza*. *Gymnadenia* on the other hand comprises solely rewarding species (Claessens and Kleynen 2011, p. 289). *G. conopsea* is a rewarding orchid offering large amounts of nectar (Stpiczyńska and Matusiewicz 2001; Claessens and Kleynen 2011, p. 290; Bell et al. 2009; Meekers et al. 2012), which was a reason why *G. conopsea* was especially interesting in this study. Groll (1965) investigated the possible influence of autogamy in this species. In his study he could not find evidence for autogamy except the fact that the plant is self-compatible. When flowers were artificially self-pollinated, a reduction in seed set was observed by Groll (1965). Thus, allogamous pollination is assumed to be the main pollination mode (Groll 1965). This is also supported by of the flowers' high nectar production and the fact that the flowers are well adapted to pollination by Lepidoptera (Claessens and Kleynen 2011, p. 290); the main pollinators of *G. conopsea* (Huber et al. 2005; Claessens and Kleynen 2011, p. 290; Bateman and DiMichele 2002; Griebl 2013). However, Claessens and Kleynen (2011, p. 292) also observed bumble bees as pollinators of *G. conopsea*.

As mentioned above it was not possible to count the number of ovules in this species due to low contrasting. The scanning process was thus canceled after a few scanned samples. Since bismuth tartrate provided good results not only in contrasting more vacuolated tissues, but also in contrasting ovules and pollen (Staedler, Masson, and Schönenberger 2013) it was tried as contrast agent. The samples were infiltrated for 48 hours and then re-scanned. However, contrast decreased, not only for the ovules, but also for the entire samples. Additionally, the samples were damaged by the new contrast agent.

### *Placental anomalies*

In more than ten samples throughout almost all analyzed species, sterile areas within placentae could be found. These sterile "anomalies" are characterized by the absence not only of ovules, but also of developed placental tissue and could be found mostly in flowers from the top of an inflorescence (8 out of 10 times). However, such anomalies can also occur in flowers from the bottom of an inflorescence (2 out of 10 times). This indicates that these placental anomalies are not a result of underdevelopment. Additionally, ovules surrounding placental anomalies did not show any sign of underdevelopment. In *E. palustris* buds, well developed placental tissues are present from the bottom to top of the gynoecium, although some areas are devoid of ovules (see figure 17 (C)). Moreover, the transition from normal ovule-bearing regions of a placenta to regions devoid of ovules is continuous in *E. palustris* (see figure 17 (A&B)). In comparison to the situation found in buds of *E. palustris*, anomalies occur abruptly, without noticeable transition, and do not comprise well developed placental tissues (see figure 18 (C-G)).

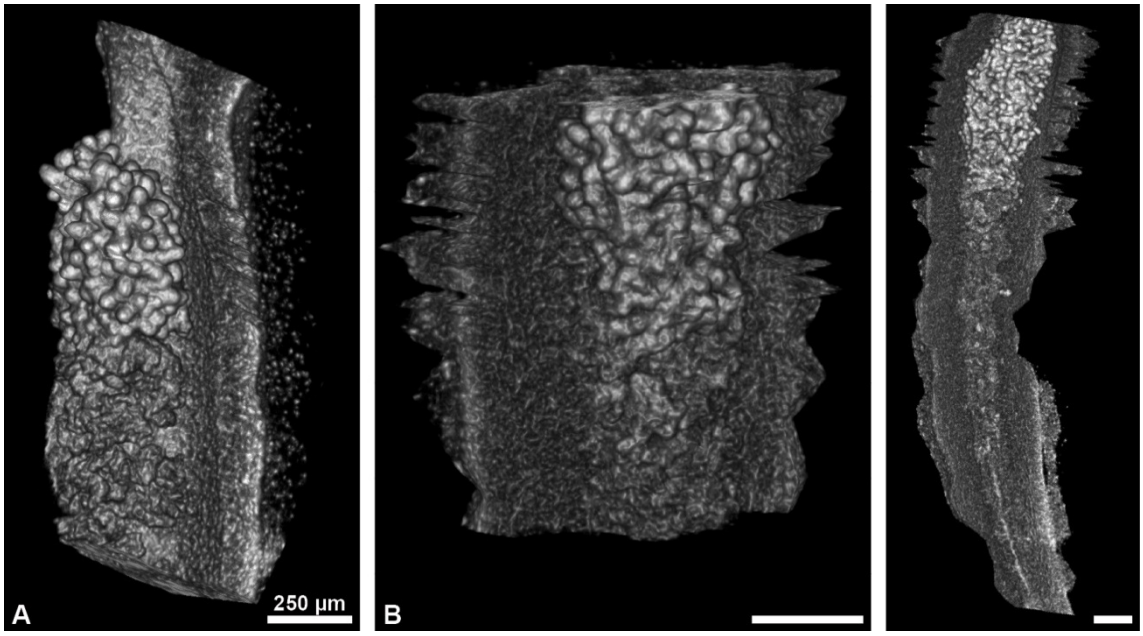



**Figure 17:** Placental scans of an underdeveloped *E. palustris*: (A) Top end of placenta: in the very top just the placental ridge without placental tissue is viable, followed by a domain of visible ovules which slowly disappear so that just placental tissue is visible at the very bottom, (B) transition area from ovules present to no ovules, (C) full view of an underdeveloped placenta. Scale bars = 250µm

The size of placental anomalies can vary strongly and several can occur within one placenta. The anomalies that could be found varied from very small ones that occupy around 2% of a placenta to larger ones that are present on 50% of a placenta or even more (see figure 18 (C-G)). In one sample, one placenta was totally absent and another placenta was extremely reduced (see figure 18 (B)). Compared to the largest placenta present in this gynoecium, the smaller placenta was reduced by 75%. This was the most extreme observed case. Smaller length differences between placentae within a gynoecium could, however, be detected in almost all scanned samples (see figure 18 (A)).

The number of placental anomalies was obtained just by brief observations of all gynoecia scans, and is most likely more numerous than stated.

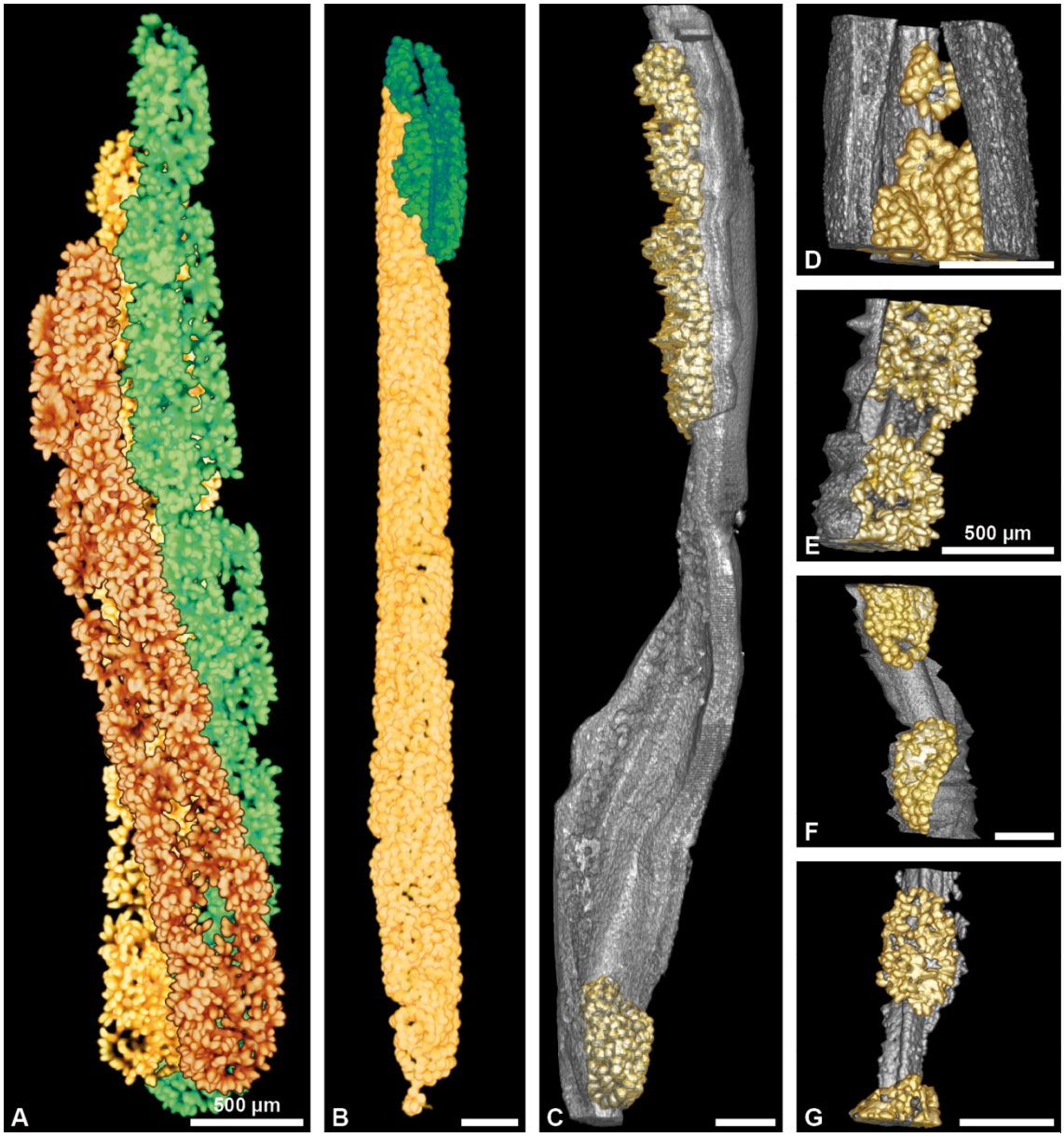



**Figure 18:** Different types of irregularities and placental anomalies in gynoecia of different species and in different inflorescence positions. (A) Top flower of *D. incarnata*: size differences between different placentae within one gynoecium (ratio of the placenta lengths = 1 : 0.88 : 0.78); (B) top flower of *D. majalis*: one placenta is totally missing, another one is reduced to less than 25% of the longest placenta; (C) *D. majalis*, one flower below (B): longest anomaly (which is surrounded by ovules on both ends) occupying more than 50% of the placenta; (D): smaller anomaly (occupying ~2% of the placenta) in a bottom flower of *A. pyramidalis*; (E) small to medium sized anomaly (occupying ~5.5% of the placenta) in the flower of (A); (F) medium sized anomaly (occupying ~8.5% of the placenta) in a bottom flower of *D. fuchsii*; (G) larger anomaly (occupying ~14% of the placenta) in a top flower of *D. incarnata.* Scale bars = 500µm

### *Summary and comparison of the results*

In all the inflorescences and in all species investigated, the number of ovules declined from bottom to top. Thus, the calculated difference from bottom to top was always negative (see table 6). The impact of the position on the number of ovules (see figure 19) was calculated in R and showed in five out of seven species a significant or even highly significant ($0.0039 < p < 0.039$) impact (see table 4). In *D. majalis* a tendency was detectable; in *O. militaris* no impact could be detected (see table 4). It should be mentioned however, that *O. militaris* was the species with the smallest samples size. In *D. majalis* the very large ovule numbers of one sample and its missing bottom sample could have influenced the result.

**Table 4:** Results of ANOVAs on flower position and number of ovules in different species

| | Degrees of freedom | p-value | $R^2$ |
|---|---|---|---|
| *A. pyramidalis* | $F_{2,8}$= 11.966 | 0.0047** | 0.74947 |
| *A. morio* | $F_{2,6}$=2.7152 | 0.0123* | 0.47509 |
| *O. militaris* | $F_{2,4}$= 2.0016 | 0.1658 | 0.5002 |
| *D. incarnata:* | $F_{2,8}$= 5.4401 | 0.0269* | 0.57627 |
| *D. fuchsii:* | $F_{2,5}$= 13.927 | 0.039* | 0.84781 |
| *D. majalis:* | $F_{2,9}$=4.5393 | 0.06969 | 0.50217 |
| *D. viridis:* | $F_{2,9}$= 5.6102 | 0.0039** | 0.55491 |

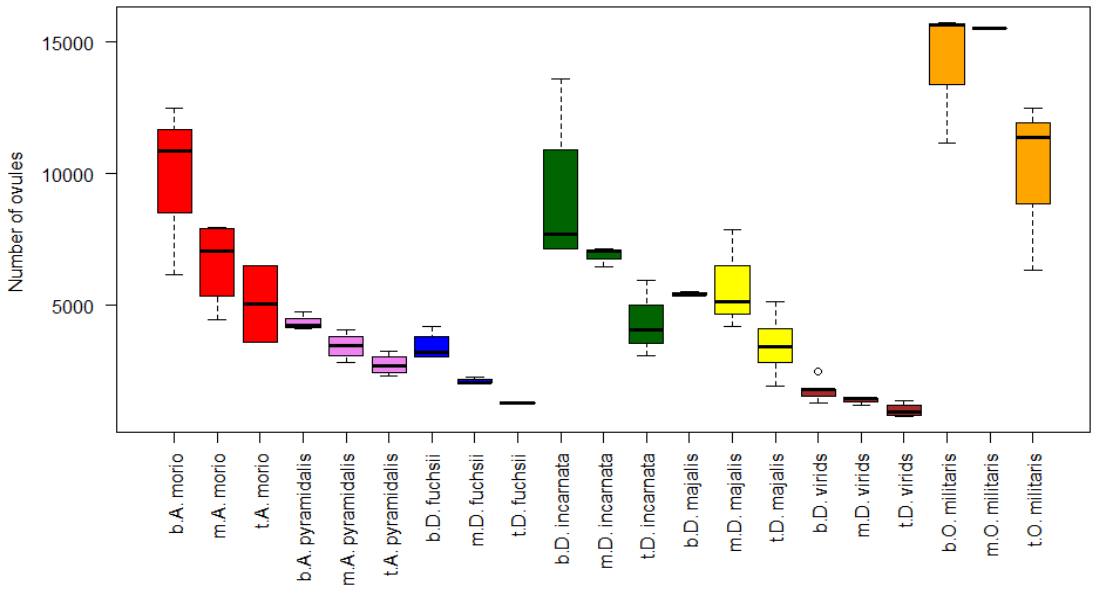



**Figure 19:** Number of ovules in the analyzed orchids. The letters b(ottom), m(iddle) and t(op) in front of species names indicate the flower positions.

The number of pollen grains does not show such a clear pattern (see figure 20). In all investigated species the number of pollen grains increased as well as decreased from bottom to top at least once. Thus, also the calculated difference is positive, as well as negative in some of the species investigated (see table 6). *D. incarnata* shows the highest difference in pollen grain number; however the results were obtained only on one inflorescence. *D. fuchsii* is furthermore a good example of how differently the pollen grain number can vary within different inflorescences, once declining and once increasing from bottom to middle in almost the same extent. A lager sample size would be necessary to detect the influence of the inflorescence position on the pollen grain number. A general trend could not be detected.

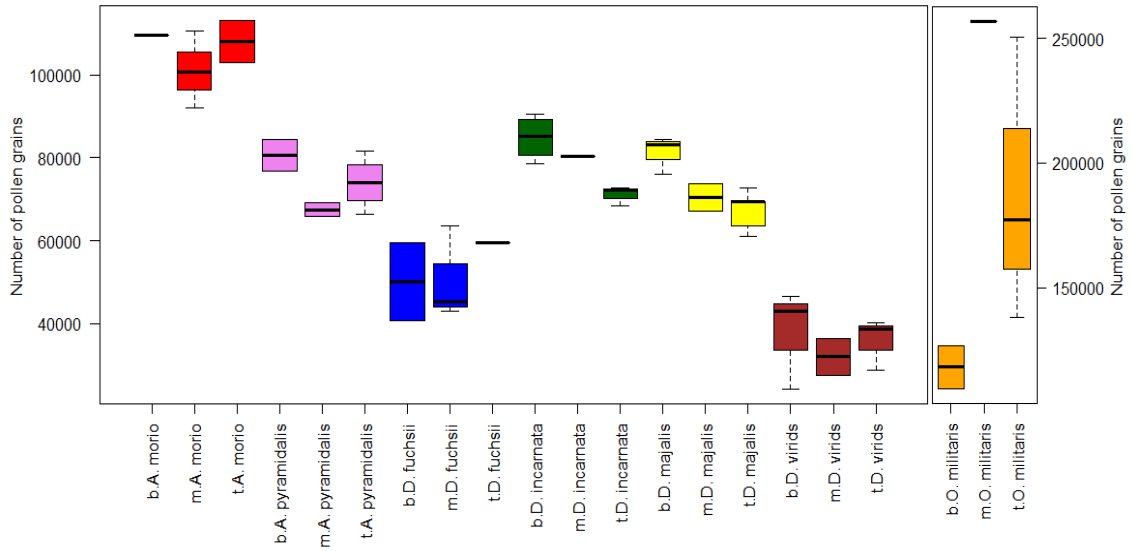



**Figure 20**: Number of pollen grains in the analyzed orchids. The letters b(ottom), m(iddle) and t(op) in front of species names indicate the flower positions. *O. militaris* is plot on a separate figure due to the larger inter individual differences (see *Orchis militaris* in the chapter „Results of the analysis")

Furthermore, the number of ovules appears to be the parameter influencing the P/Os the most (see table 6). Not only is the difference in ovule number in all investigated species larger (more influential) than it is in pollen grain number, but also is the maximum percentage difference between the lowest and the highest count in all investigated species lager in ovules than in pollen grains (illustrated by the factors in table 5).

**Table 5:** Variance in ovule and pollen grain numbers within the analysed species. Additional to the intervals the factors by which the minimum and the maximum value differ are stated

| | Ovule numbers | Factor | Pollen grain numbers | Factor |
|---|---|---|---|---|
| *A. pyramidalis* | 2330 – 4740 | 2.0 | 65800 – 84400 | 1.3 |
| *A. morio* | 3600 – 12500 | 3.5 | 92000 – 110500 | 1.2 |
| *O. militaris* | 6340 – 15750 | 2.5 | 109500 – 256900 | 2.4 |
| *D. incarnata* | 3060 – 13610 | 4.4 | 72300 – 90600 | 1.3 |
| *D. fuchsii* | 1270 – 4170 | 3.3 | 40800 – 63600 | 1.6 |
| *D. majalis* | 1930 – 7880 | 4.1 | 61000 – 84300 | 1.4 |
| *D. viridis* | 770 – 2460 | 3.2 | 24200 – 46400 | 1.9 |

**Table 6:** Summary of the differences between the levels bottom-middle-top in ovule and pollen grain numbers. Additionally to the percentage change, the factor necessary for reaching the next higher value is stated. This factor illustrates the actual impact of a given change (-50% ≙ +100%; the factor is 2 in both cases)

| | Difference between the levels bottom-middle-top | | | |
|---|---|---|---|---|
| | Ovule numbers | | Pollen grain numbers | |
| | % | Factor | % | Factor |
| *A. pyramidalis* | -16% | 1.19 | +3% | 1.03 |
| *A. morio* | -26% | 1.35 | +3% | 1.03 |
| *O. militaris* | -17% | 1.20 | +9% | 1.09 |
| *D. incarnata* | -41% | 1.69 | -12% | 1.14 |
| *D. fuchsii* | -33% | 1.49 | -2% | 1.02 |
| *D. majalis* | -32% | 1.47 | -8% | 1.09 |
| *D. viridis* | -22% | 1.28 | -5% | 1.05 |

The P/Os did not show any obvious indication of being any different in the rewarding species *D. viridis* as compared to deceptive species. The results of *D. viridis* were compared with the results of the deceptive *D. fuchsii* in R since the P/Os of those two species were the most similar (see figure 21). No differences between those two species could be detected ($F_{1,12}$= 0.38363, p= 0.5508, $R^2$= 0.03098). Additionally, *D. viridis* has, most likely due to its small overall size of 5-25cm (Griebl 2013), the lowest total number of pollen as well as ovules. Thus, the size of *D. viridis* might have also influenced the P/Os. The analysis of more rewarding species is necessary in order to be able to determine the hypothesized influence of reward on the P/Os in orchids.

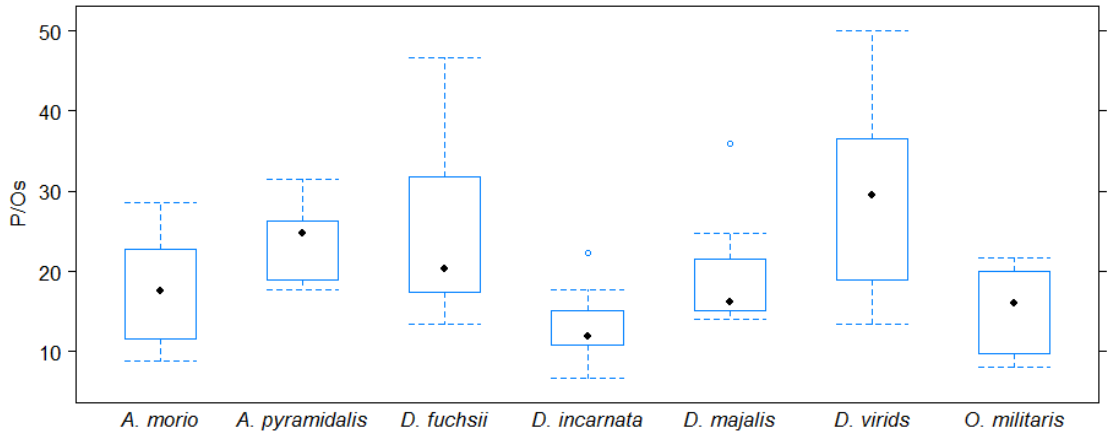



**Figure 21:** P/O-boxplots of the analyzed species

The number of massulae was determined in species of two different genera (*Anacamptis* and *Dactylorhiza*). *D. viridis* was chosen since it was the only rewarding species available. It was tried to determine whether possible trends might be detected between rewarding and deceptive species that might be interesting for further investigation. In this brief investigation no hints for possible major differences could be detected. The number of massulae per flower ranged in *D. viridis* from 104 to 190 and thus lay precisely in the middle of the two deceptive species *A. pyramidalis* (105 – 149 massulae per flower) and *D. fuchsii* (157 – 202 massulae per flower). However, a positive correlation between number of massulae per flower and the total number of pollen grains could be detected (see figure 22). In two species this correlation was significant (*A. pyramidalis*: $F_{1,6}$=7.77, p=0.03168, $R^2$=0.4916; *D. viridis*: $F_{1,6}$=56.57, p=0.0.000286, $R^2$=0.8881), in *D. fuchsii* only a tendency could be detected (*D. fuchsii*: $F_{1,4}$=6.942, p=0.0579, $R^2$=0.543).

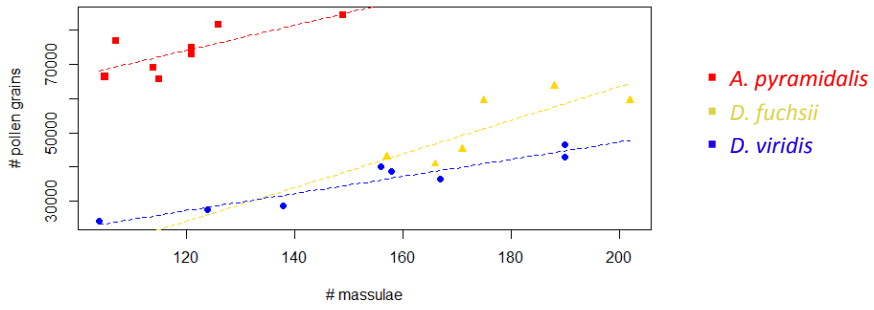



**Figure 22:** Regression lines showing the relation between the number of massulae and the number of pollen grains

## Discussion

The orchid family is one of the largest (if not the largest) plant family on Earth with more than 25.000 species (WCSP 2011; Sheehan and Sheehan 1994; Dietrich 2005; Chase 2005). The number of estimated species increased dramatically over the last decades (Swamy 1943; Hunt 1969) and still does (Dressler 2005). All in all, Orchidaceae represent around 7% of the extant angiosperm species diversity (Paton et al. 2008; van der Pijl and Dodson 1966, p. 1).

However, according to Erbar and Langlotz (2005), up to 2005, only 26 species had been studied for P/Os. It has to be mentioned, however, that (1) some studies were not considered in their review such as Lehnebach and Riveros (2003) or Lehnebach and Robertson (2004), and (2) further studies on the P/Os of orchids have been published since (Lehnebach, Robertson, and Hedderley 2005). Although the number of known P/Os in Orchidaceae is slightly higher than mentioned by Erbar and Langlotz (2005), P/Os are only known for 1‰-2‰ of orchid species. This illustrates how little is known about male and female reproductive investment strategies in Orchidaceae. One reason for this lack of knowledge is the fact that the ovules in orchids are not (fully) developed until the flower is pollinated (Zhang and O'Neill 1993; Arditti 1992, p. 500; Wirth and Withner 1959; O'Neill 1997 and references therein). This often makes the counting process of ovules difficult or even impossible before seed stage (Gregg 1989; Lehnebach and Riveros 2003; Proctor and Harder 1994). At the same time, orchid pollen grains are relatively small and usually aggregated in pollinia (and often additionally in massulae-subunits), making it very difficult to confidently establish pollen grain numbers (Proctor and Harder 1994; Dafni, Kevan, and Husband 2005, p. 87). Therefore, efficient and fast ways of counting ovules and pollen grains in orchids are greatly needed.

### Method

In the method presented here, the infiltration process is time consuming, albeit little work is required, except from changing the medium once every 2-3 days. Moreover, during the infiltration of one species, other species can be scanned and analyzed. In order to accelerate

the process of analysis, automatic pollen counting with Fiji/ ImageJ can be conducted and manual ovule counting in Amira can be performed simultaneously. Besides, the method we describe provides a robust way of counting even very large amounts of pollen grains or ovules. Although automatic counting of pollen grains in Fiji/ ImageJ requires high resolution and high quality scans, manual counting of ovules could be performed on low resolution scans (even of poor quality). When the scanning quality is poor, filtering in Amira is recommended which can ease the manual counting process dramatically (see figure 23).

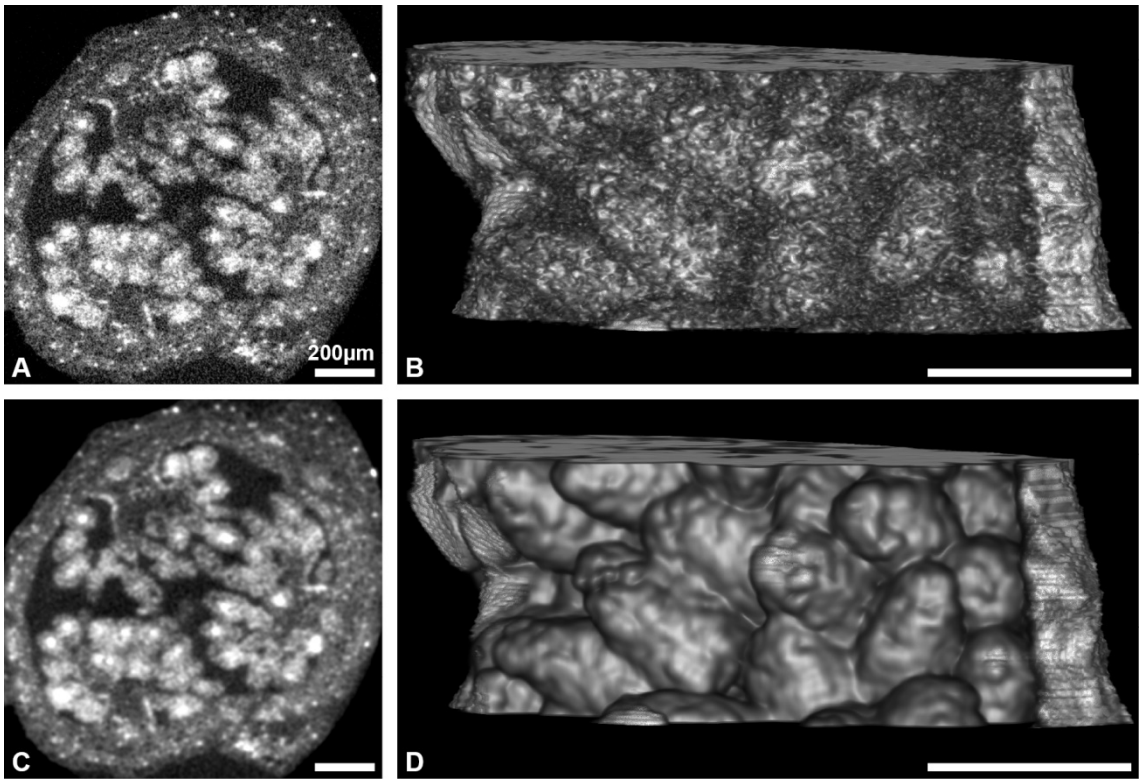



**Figure 23:** Scan of a gynoecium of a bottom flower of *D. viridis*. (A) unfiltered 2D section, (B) 3D image of an cropped and unfiltered domain, (C) filtered 2D section, (D) 3D image of a cropped and filtered domain. Scale bars = 200µm

### *Counting pollen grains*

As mentioned above, the P/Os of only 1‰-2‰ of the world's orchids are known. This is especially surprising, considering that pollen grain and ovule numbers in orchids were already determined in the 19th century (Amici 1847; Darwin 1886, p. 277 et seq. ; Darwin and Gray 1868, p. 453 et seq.). A ratio of pollen grains to ovules (using seed count as a proxy) in *D. maculata* is already mentioned by Darwin (1886, p. 278). Although several

new methods have been tried to ease and improve the counting process of pollen grains in orchids (Cruden 1977; Mehrhoff 1983; Lehnebach and Riveros 2003), many authors still employ methods similar to the ones that were used in the mid of the 19th century. Several massulae are crushed and an average number of pollen grains per massulae as well as an average number of massulae per pollinium are determined. By extrapolation the number of pollen grains in the whole pollinia can be calculated (Nazarov and Gerlach 1997; Neiland and Wilcock 1995). Although the number of massulae and the number of pollen grains are correlated (this study, see figure 22), the massulae size, and thus the number of pollen grain in a massulae, can vary considerably within one pollinium (this study, see figure 24 (A&B) and see also Nazarov and Gerlach (1997)). Additionally, although the pollinia were treated with great caution during this study, single pollen grains not connected in a tetrad state could be found. Crushing the massulae might increase this number dramatically making the counting process less accurate, if counting assumes that the units counted are solely tetrads. When counting is performed with Amira or Fiji/ ImageJ, single pollen grains can be counted instead of tetrads (see figure 24 (C&D)).

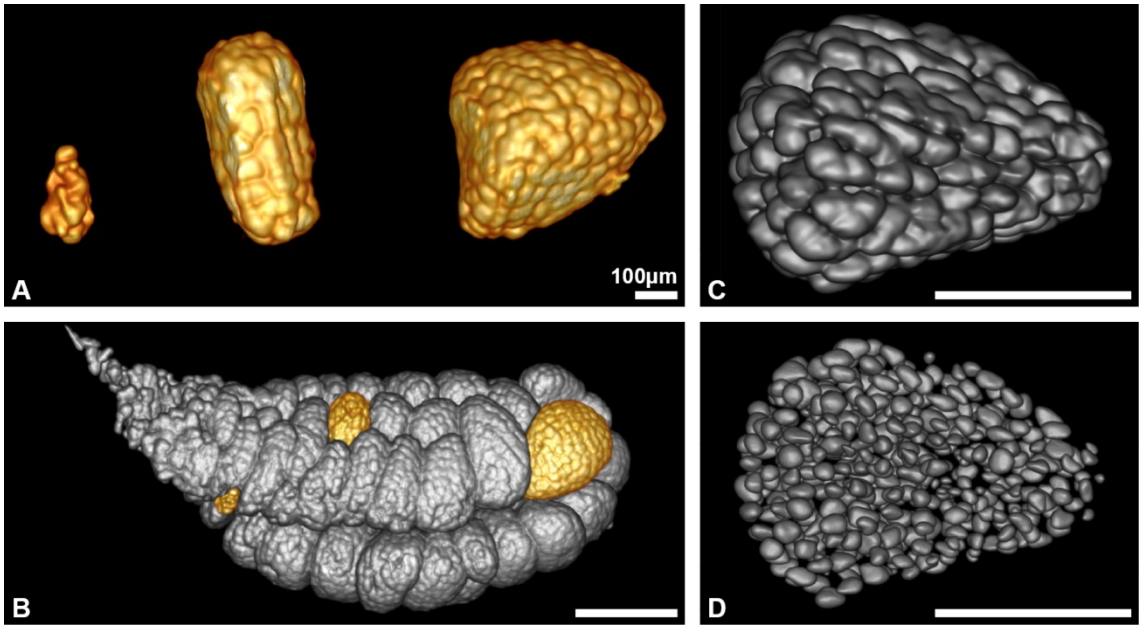



**Figure 24:** (A) Different massulae from the same pollinium of *A. pyramidalis,* (B) pollinium of *A. pyramidalis* with the massulae of (A) highlighted, (C) filtered high resolution massula of *D. viridis*, (D) the massula of *D. viridis* shown in (C) after iterative-thresholding-separation. Scale bars = 100µm

### *Counting ovules – former methods*

In all species studied the number of pollen grains exceeded the number of ovules by far (see chapter "Results of the analysis"). However, counting ovules is also problematic. Firstly, the number of ovules per ovary can be extremely high in some orchid species. Scott calculated the number of seeds in the tropical orchid *Cycnoches chlorochilon* to be more than 3.7 million per capsule (Rolfe 1909) and a compilation of Arditti and Ghani (2000) illustrates that such numbers were obtained more than once in orchid species. Secondly, ovules in orchids are often very little developed before pollination takes place. This makes the counting process especially difficult and often seeds instead of ovules are counted (Proctor and Harder 1994; Meléndez-Ackerman and Ackerman 2001; Sipes and Tepedino 1995; Darwin and Gray 1868, p. 453; Darwin 1886, p. 277; Rolfe 1909).

Over the last centuries, ovule and seed counting has been performed via several methods. As early as the 19th century Scott (as mentioned in Darwin and Gray 1868, p. 453 et. seq. ; Darwin 1886, p. 277; Rolfe 1909) used at least two different counting methods. One of those methods (used by Scott and Darwin, see Darwin and Gray 1868, p. 453 et. seq. ; Darwin 1886, p. 277) is to line up all the seeds of a capsule and to count the number of seeds on a certain length. By extrapolation the total number of seeds can be determined. However, Nazarov (1998) criticizes this method for its high risk of error. Another method described by Scott (Rolfe 1909) calculates the number of seeds by using the seeds weight. Subsamples of seeds are counted and weighed. However, high precision weighing is necessary since the weight of one seed is usually in the area of the microgram (Salisbury 1942, p. 5; Burgeff 1936, p. 31; Stoutamire 1964; Rolfe 1909; Koch and Schulz 1975). Hence, several hundreds of seeds are necessary in order to reach the mg range. Additionally, the seed weight has to be measured for each capsule, since it can vary considerably between different capsules. Pollination type (self-pollination vs. cross-pollination) has a large influence on the seeds weight due to different seed sizes and the different number of aborted seeds (Nazarov 1989). Furthermore, also the degree of (de-)hydration cannot be disregarded (Rasmussen 1995, p. 8). Additionally, due to the small size and light weight of the seeds, air streams and static electricity can be problematic when handling dry seeds

(Nazarov 1998; Proctor and Harder 1994). This illustrates that the loss of seeds is a risk. Since even mature seeds have small sizes and low weights, counting ovules with these traditional techniques seems even more difficult.

Another technique is counting seeds (or ovules) under the microscope (Proctor and Harder 1994; Sipes and Tepedino 1995; Willems and Melser 1998; Lehnebach, Robertson, and Hedderley 2005; Kopylov-Gus'kov et al. 2006). Different ways of sample preparation are mentioned by different authors. Often a liquid is mentioned in or on which the seeds (or ovules) are dispersed (glycerin, ethanol-glycerin or water). Sometimes it is noted that this liquid was evaporated before counting in order to reduce movement. Moreover, subsampling is mentioned in all of these studies except in Kopylov-Gus'kov et al. (2006). In the latter study and also in Lehnebach at al. (2003; 2004; 2005) ovules were counted directly instead of seeds.

Nazarov (1989) counted the number of ovules as well as the number of seeds on a photograph and used a modified calculator to fasten and ease the counting process by marking the counted seeds or ovules. For counting seeds he used water on which surface the seeds (especially those of European orchids) remain (Burgeff 1936, p. 41 et seqq.). Nazarov assumes that seeds are equally distributed on the water surface.

A different way of counting ovules is also described by Nazarov (1989). Pressure preparations of single placentae were made, photographed and the ovules counted. By counting one placenta per flower and extrapolation a more time saving way of proceeding is proposed. By extrapolation, a total error of no more than 10% is assumed due to placental differences; a result similar to observations of Kopylov-Gus'kov et al. (2006). However, in the latter study also large differences between placentae of *Epipogium* could be observed. Although counting ovules separately in different placentae was not performed in the present study, the placental anomalies observed in this study indicate that relying on only one placenta can lead to dramatic errors (see figure 18). Moreover it might be very difficult to picture all ovules without hidden ones on one photograph due to the fact that even within one placenta the ovules tend to strongly overlap (see figure 25).

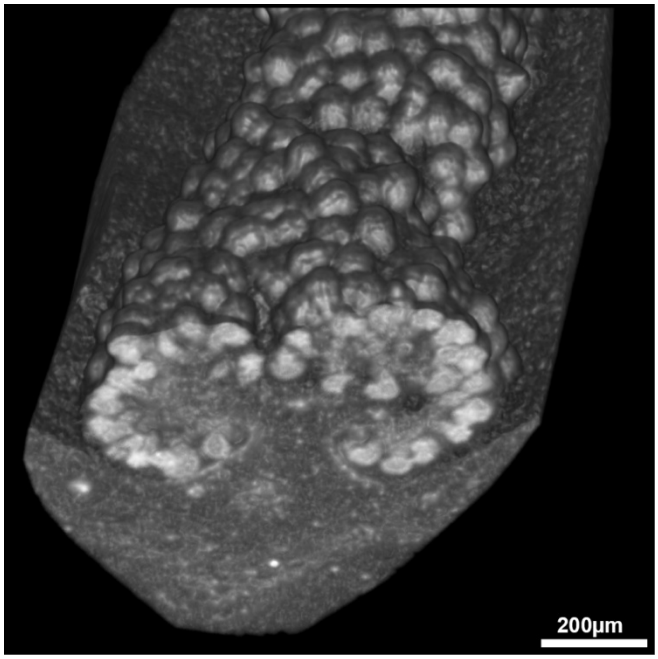



**Figure 25:** *D. incarnata*: Placenta with numerous, partly overlapping ovules. Scale bar = 200µm

Mehrhoff (1983) used a laser particle counter to obtain seed and ovule numbers. Subsampling was also necessary, but the counting process was more straightforward and possibly more precise. However, despite these apparent advantages, this method was -to our knowledge- not used in later studies.

### *Micro-CT scanning and computer-aided counting*

In all the abovementioned methods, the risk of seed (or ovule) loss is present. Furthermore, it might be difficult to distribute seeds (or ovules) equally on a petri dish in/on a liquid. With the method presented in this paper those two issues are irrelevant, because ovaries can be scanned without opening the capsules; there is therefore no chance of ovule loss if the flowers are harvested properly. Furthermore, since the average ovule size is determined and the whole volume of all ovules can be selected, the ovules' distribution is not of great importance. Even large placental anomalies are of no concern and do not influence the result. Moreover, on the scanned material, further analyses can be performed, for example: (1) the analysis of different ovule numbers on different placentae (an issue mentioned by Kopylov-Gus'kov et al. (2006)), (2) the distribution of ovule size, or (3) general morphometrics on other parts of the flower (*e.g.,* stigmatic cavity). In addition, calculations

can easily be repeated, and precision can be improved by increasing the size of the subsample. The first point (the analysis of different ovule numbers on different placentae) requires a separation of different placentae. Whether this is easy to carry out depends very much on the inner structure of a gynoecium (see figure 26).

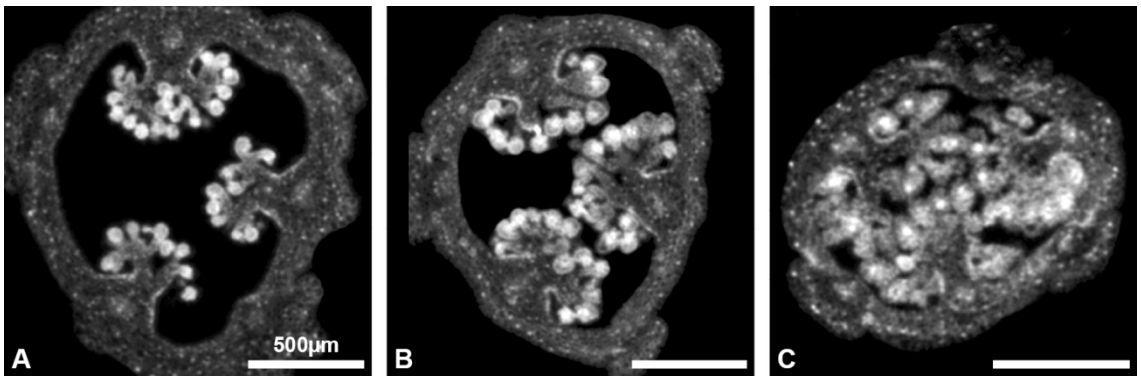



**Figure 26:** 2D sections of different *D. viridis* placentae, all from bottom flowers. Scale bars = 500µm

The fact that additional analysis can be performed after ovule and pollen counting is of special relevance in orchids for which material may be scarce due to the endangered status of many species and their protection from collection.

Ovules of orchids are very little developed at anthesis. With the method we present, gynoecia can be analyzed at anthesis, thereby providing a robust way of counting ovules instead of seeds. To improve the counting process it would be of advantage if counted ovules, pollen grains or massulae could easily be marked in Amira. This could improve the counting process not only by accelerating it, but also by reducing the probability of human error. This is unfortunately not yet possible.

### *Scaling methods*

Byte scaling worked very well on gynoecia and made stitching easily possible. Additionally, it allowed further cropping before reconstruction which decreased the file size enormously. However, changing parameters such as the source distance, exposure times etc. are not possible, if comparable grayscale values are wanted. CT scaling is independent of many of these variables and thus proves advantageous in certain situations. One main disadvantage is, however, that air has to be visible in the field of view, on both sides, during all the scanning process. Thus, it is not possible to use optimal source and detector distances. Furthermore, during reconstruction, the scanning volume cannot be cropped, and the whole scanning volume has to be reconstructed. This not only increases reconstruction time, but severely increases data size.

### *Remaining problems and possible solutions*

Some problems still require to be addressed. The problem of pollinium fragility can be solved by collecting unopened buds; using this type of material allowed us to obtain scans of undamaged pollinia even for *E. palustris* with its soft and mealy pollinia (see figure 27). In comparison, open flowers that were collected and stored directly in FAA+PTA contained no parts of pollinia anymore when scanning was performed.


**Figure 27:** Soft pollinia of *E. palustris*. Scale bar = 500µm

If open flowers need to be collected, further protection of the pollinia is advisable. Investigating the minimum amounts of FFA+PTA changes necessary for reasonable staining quality would be helpful, although this may vary between species and developmental stages. The effect of decreasing contrast after a longer time of infiltration with FAA+PTA observed by Staedler (2013) should also be considered. If changing FAA+PTA is not necessary for staining

pollen grains at all, removing the pollinia before harvesting a flower and storing them in separate vessels might be an option worth considering.

Another problem to be addressed is the retarded development of orchid ovules before the beginning of the pollination process (not necessarily the fertilization process). During this study, however, only in buds of *E. palustris* were the ovules partly so underdeveloped that counting could not be performed. In all the other investigated samples (even in buds of other species) underdevelopment never hindered counting. In some samples (*D. viridis* and *G. conopsea*), the opposite effect was observed: developmentally advanced ovules showed poor staining quality. The right time for harvesting flowers seems thus to vary among species.

Although some issues remain to be addressed (see chapter "Difficulties of this method"), a computer-aided counting process is a promising method: future advances in software technology will make it possible not only to speed up, but also to increase the precision of the counting process by counting not only parts of the pollinia, but all the actual pollen grains that constitute them. Additionally, advances in object recognition software may make it possible to automatically establish ovule and massulae numbers.

### Observed differences

A decline in ovule numbers from bottom to top has already been observed (Nazarov and Gerlach 1997; Salisbury 1942, p. 96; Kopylov-Gus'kov et al. 2006) and could be confirmed in this study in all analyzed species. One reason might be size differences between bottom and top flowers (Bateman and Rudall 2006; Salisbury 1942, p. 96). This, however, cannot explain why the pollen grain numbers do not show such a clear decline and why male function is relatively more pronounced in the top of the inflorescence in all analyzed species. Such a trend was already suggested in previous studies (Vallius 2000; Berry and Calvo 1991). Hymenoptera usually start probing the bottom of the inflorescence and then move upwards (Pyke 1978; Claessens and Kleynen 2011, p. 35; Light and MacConaill 1998; Catling 1983) leading tendentially to higher pollination frequencies and fruit set in lower flowers (Tremblay 2006; Jersáková and Kindlmann 1998; Dafni and

Woodell 1986). However, in the mostly coleopteran-pollinated species *D. maculata* no effect of floral position on the frequency of fruit set was detected (Vallius 2000). The seed set on the other hand was smaller in upper flowers, most likely due to resource preference for lower flowers. Vallius (2000; following Ehrlen 1991) suggests that the uppermost flowers may function as an ovary reserve if herbivory occurs in lower flowers. It has already been observed that removal of bottom flowers induces a growth in flower size of the flowers immediately above the excisions (Bateman et al. 2003). This may compensate the loss of floral display size.

Although Vallius (2000) discovered a tendency for the weight of the pollinia to be lighter in the top of the inflorescences of *D. maculata*, a tendency of decreasing pollinium volume at the top of an orchid's inflorescence could not be detected in the present study. Within an inflorescence no pattern of decline or increase in pollen grain numbers could be detected. Since pollen removal is more likely than pollen deposition (Proctor and Harder 1995), the flowers in the top could mainly function as pollen donors. This would explain why the number of ovules decreases in order to save resources, but the number of pollen grains remains +/- constant. A similar effect was observed by Willson and Price (1977) in species of Apocynaceae; a family in which pollen grains are also packed in pollinia. The presence of anomalies may additionally decrease the number of ovules in top flowers.

Although it was also observed that addition of nectar into the spurs of rewardless orchids can increase the removal rate of pollinia (Johnson and Nilsson 1999; Johnson, Peter, and Ågren 2004), Smithson and Gigord (2001) detected negative effects for plant fitness. In their study, twice as many pollinia were removed in nectarless plants compared to plants in which nectar was added on a daily basis. Although the visitation rate was higher in the artificially rewarding plants, the likelihood of pollinia-removal during a flower's visit was approximately ten times less. Due to an intensified searching behavior of bumble bees on rewardless individuals, pollinia attachment was significantly increased. Thus, the function of upper flowers as pollen donors might be even stronger in deceptive species.

Orchids in which flowers open from the top of the inflorescence downwards are uncommon, but have been documented (*e.g.*, *Myrosmodes cochleare*, *Orchis simia*). In *Myrosmodes cochleare*, the ability of setting fruits, however, increases from 0% in the top to 100% in the bottom of the inflorescence, whereas the viability of pollinia remained constant (Berry and Calvo 1991). In the European genus *Orchis* a basipetal flowering species, *O. simia*, exist (Griebl 2013). Nazarov and Gerlach (1997) mention that this was the only species investigated that did not follow the rule of decreasing ovule primordia from bottom to top. However, they do not specify if an inverse pattern was detected. It would be thus of special interest to determine if the order in which the flowers open on an inflorescence has an impact on the differences in P/Os within an inflorescence.

## Conclusions

1. The method is new and it works

We herewith describe a new method to count pollen and ovules in flowers based on X-Ray Computed Tomography (CT); this new method possesses a set of advantages and possibilities not provided by previously available methods. Very large pollen numbers can be counted, on a whole evidence basis (total volume), independent of aggregation state: pollen can be counted in single grain, tetrads, massulae, pollinia, or in a mixture of aggregation states, without the need to destroy aggregation. Very large numbers of very small ovules can be directly counted without the need of preparation of the ovary (risk of ovule loss), nor having to rely on seed numbers as an approximation of ovule numbers. Furthermore, unlike usual extrapolation methods, our CT-based method is sensitive to irregularities in ovule distribution inside the ovary. Moreover, the floral material is not destroyed during the scanning process, which enables the gathering of other datasets, *e.g.*, morphometric datasets from the same flowers.

2. We found a new pattern of variation of pollen ovule ratios in orchids and it means something

The method we present opens new alleys in the study of reproductive investment in plants, *e.g.*, to test whether the differences in pollinator behavior in rewarding and deceptive

species of Orchidinae (Orchidoideae, Orchidaceae) influence the intra-inflorescence variation of P/Os. Pollen grain numbers showed intra- and inter-individual variation in all species, but variation did not show any trend along the inflorescences. Massulae numbers were however well correlated with pollen grain numbers. Ovule distribution anomalies within gynoecia were present in all species studied and thereby invalidate extrapolation methods for calculating ovule numbers. Ovule numbers showed a clear trend of decrease towards the distal end of the inflorescence; thereby resulting in increasing P/Os towards the distal end of the inflorescence. Position on the inflorescence is therefore an important determinant of P/Os in Orchidinae. Apical flowers are relatively more male, possibly acting mostly as pollen donors or ovule reserve (in case of predation of the lower, more female flowers). Thus, a single P/O value without position in the inflorescence may be misleading; stating a P/O range instead of a single value would be more accurate. If a single value is required, equal amounts of bottom, middle and top flowers of each plant should be used for calculating. A difference in P/Os between deceptive and rewarding species of Orchidinae could not be detected.

## Future work

1. Improve the method

The method we describe allowed us to gather data previously only poorly accessible, or even inaccessible to science (pollen and ovule counts in orchids). However, more work is required in order to improve the reliability of the method and streamline data acquisition. The possibility to mark counted objects in 3D visualization software would reduce the possibility of mistakes and streamline counting. The assumption of equal pollen grain volume within a population would require rigorous testing in order to support our results. Methods should be developed to contrast the ovules in a more homogeneous way in order to increase speed of processing and accuracy of counts. Sample collection and preservation need to be improved in order to prevent the dislocation of pollinia, *e.g.*, either by targeting very late buds for collection, or by collecting pollinia separately.

2. Improve the results

In order to determine if the presence of a reward influences the distribution of reproductive investment in orchids, more species need to be sampled. Sampling should focus on rewarding species of the mostly deceptive genera that we studied (*e.g.*, *A. coriophora*). Evolution of differential reproductive investment may be slow; therefore species from a rewarding genus (e.g., *Platanthera*) should also be included in the dataset. Furthermore, sexually deceptive (*Ophrys spp.*, *Serapias lingua*) should also be included in order to assess if different types of deception (food or mating partner) could lead to different patterns of reproductive investment. Additionally, an autogamous orchid species should be included in the analysis.

Analysis of pollen and ovule counts of all flowers of an inflorescence would allow to better understand possible patterns of variation of pollen numbers across an inflorescence. It may additionally show the frequency of larger placental anomalies and their impact on ovule numbers. The assumption of equal average pollen grain size in a population still requires to be tested.

The new method we describe allows further analysis of the scanned data. Size differences of massulae within one pollinium could be measured as well as the pollen grain number of different massulae. Morphometric studies of flowers can also be performed. Detection of differences in ovule number on distinct placentae could also be investigated as well as possible differences in the ovule's volume.

This method opens new alleys of research by allowing to gather several datasets from the same sample; the latter should prove useful especially for studies of endangered species. Future improvement in soft- and hardware will make it possible to streamline the counting process even further.

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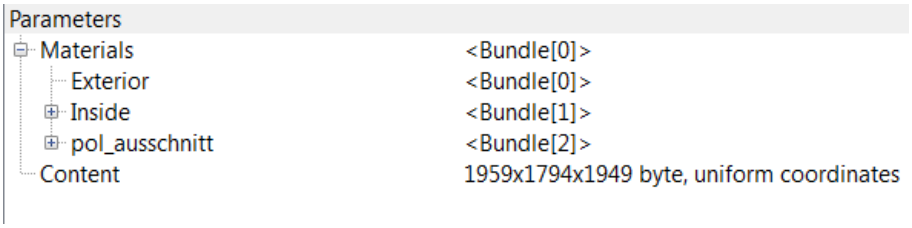
# Appendix

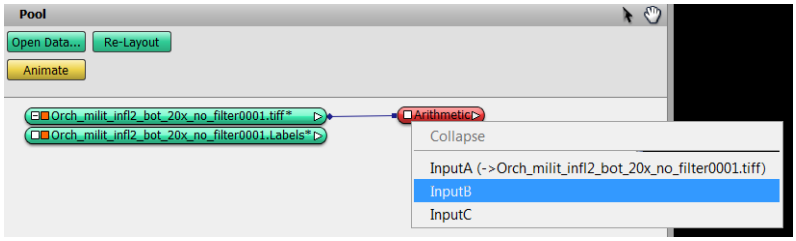
## Pollenzählen mit ImageJ

WICHTIG: Es ist zu empfehlen, zuerst mit einem sehr sehr kleinen Ausschnitt einen Test-run zu machen, um zu sehen, welche Filtereinstellungen ausgewählt werden müssen, um ein gutes Ergebnis zu erlangen! → gut **DOKUMENTIEREN**, welche Filter in welcher Reihenfolge verwendet wurden.

1. Ausschnitt in Amira möglichst knapp beschneiden (je kleiner die Datei, desto schneller geht alles in den späteren Schritten)
    a. Grobes Beschneiden mit dem Crop-Editor  im Segmentation-Editor 
    b. Exkurs: richtiges Beschneiden und Speichern eines speziellen Materials mit Amira
        - Im Segmentation-Editor  jenen Teil auswählen, den man untersuchen will und einem neuen Material hinzufügen
        - Im Object Pool  das Label auswählen und den Parameter Editor  auswählen.
          Dort sieht man, welches Material welcher Bundle-Nummer zugeordnet ist. (<Bundle[Nummer]>)

        - Die gewünschte Bundle-Nummer notieren und Parametereditor schließen
        - Nun rM (rechte Maustaste) auf die Bildstapeldatei in Amira (also die zur Labledatei zugehörige Hauptdatei) - Compute – Arithmetic
        - Nun muss die Arithemtic-Datei mit dem Lable verbunden werden (dazu auf das weiße Kästchen neben „Arithmetic“ klicken und als „InputB“ das jeweilige Lable wählen)



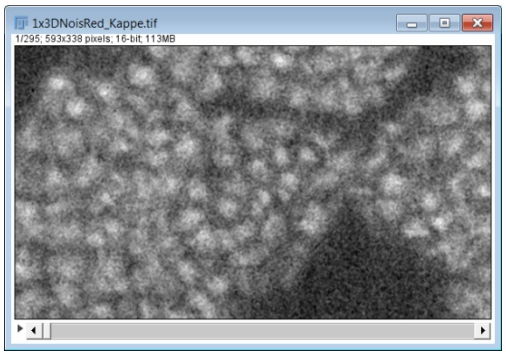

- Im Feld Expression folgendes Eintragen: (b== <#>) *a

  b … InputB; a … InputA; <#>… Bundlenummer von oben

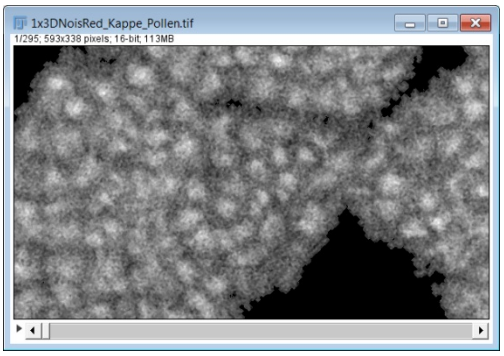
  Beispiel: (b==2)*a

- Nun ist das ausgeschnittene Lable eine eigene Datei

Vorher




Nachher




**TIPP**: Es empfiehlt sich, beim Pollenzählen schon in Amira den Pollen mit einem groben (oder auch feineren) TH auszuwählen und so die +/- grobe Pollenauswahl mit dem oben genannten Verfahren zu exportieren. Dadurch wird weitgehend verhindert, dass störendes Material vorhanden ist, das beim Zählvorgang zu falschen Ergebnissen führen kann.

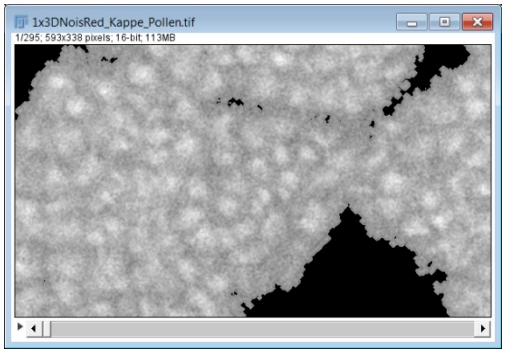
2. (Filtern in Amira  (NoiseReduction (3D)+ Gauss(3D))) … es ist oft nicht möglich, ein Material ohne größere Mengen von Noise (=unerwünschtes Störmaterial) auszuwählen, wenn nicht gefiltert wurde. Manchmal kommt man also nicht umhin, schon bei Amira zu filtern. Ich persönlich versuche jedoch, den Filterprozess möglichst in ImageJ durchzuführen. Wichtig: **GUT DOKUMENTIEREN in welcher Reihenfolge welche Filter angewandt wurden!**)
3. Amiradatei als Tiff-Datei speichern
4. ImageJ (Fiji-Version) öffnen
5. File – Import –
   - Tiff-files können direkt in ImageJ mit drag and drop geladen werden.

oder für Bildstapel (z.B. DICOM-Bildstapel):

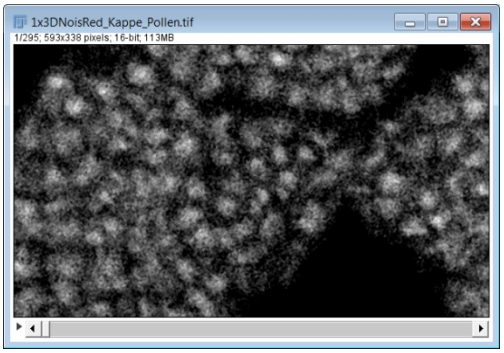
- Image sequence (für Bildstapel) – erste Datei des DICOM-Stapels auswählen – OK

6. Nun muss der Kontrast richtig eingestellt werden unter: image – adjust – Brightness/Contrast (Durch das richtige Einstellen des Kontrastes kann der vorhandene Noise noch weiter reduziert und Fehler im Separationsvorgang (mittels Iterative Thresholding: siehe später) vermieden werden.)

Vorher

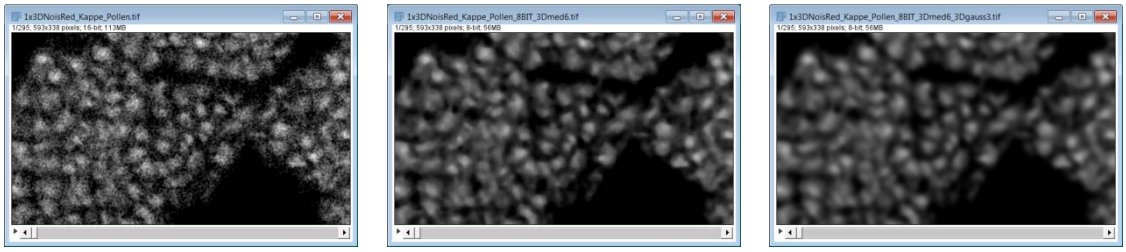



Nachher




7. Erst jetzt sollte die Datei von 16BIT auf 8BIT umgestellt werden unter: Image – Type – 8BIT → Datei wird kleiner → schnelleres Bearbeiten möglich. Zudem wird seltsamerweise erst durch das Umstellen auf 8BIT der vorhin gewählte Kontrastbereich tatsächlich ausgewählt und die Graustufenbereiche außerhalb verworfen
   ABER: Einstellen auf 8-BIT VOR dem weiteren Filtern. Filtert man vor dem Umstellen auf 8BIT, so werden beim Filtern auch jene Bereiche mitberücksichtigt, die man durch das auswählen eines guten Kontrastes eigentlich ausschließen wollte.
8. Filtern mit ImageJ: Process – filters (Es ist nicht ratsam, mehrere gleiche Filter übereinander zu legen; also z.B. Gauss mit Kernel2 und dann Gauss mit Kernel3, sondern tendenziell eher besser, EINMAL zu filtern, aber einen höheren Kernel zu wählen)
   WICHTIG: Es muss wirklich GUT gefiltert werden (3DMedian oder 3DMean + 3DGaussianBlur!). Das Bild kann und soll dadurch durchaus verschwommen werden!
   Beispielbild nach dem Filtern:

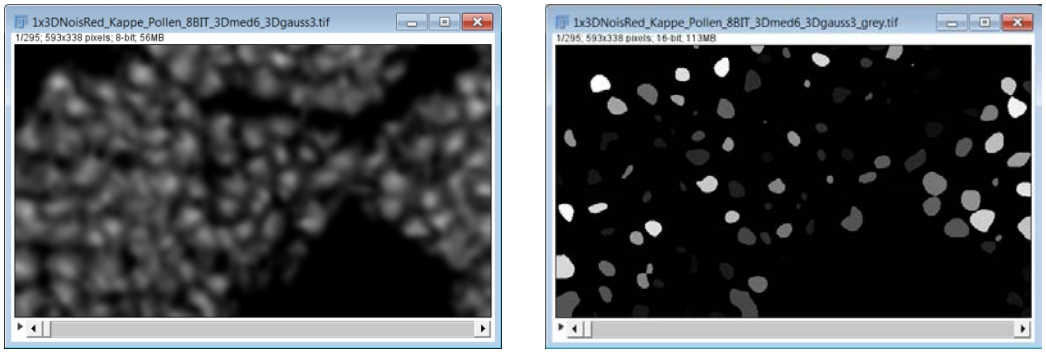
Vorher | Nach 3Dmedian | Nach 3Dmedian6 & 3Dgauss3



9. Speichern als neue Datei !! (als Tiff)
10. Plugins – 3D – 3D iterative Thresholding:

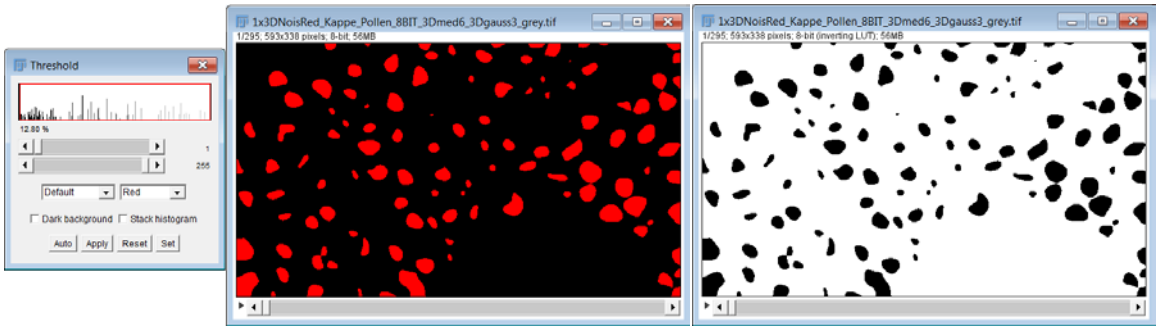
    Einstellungen können meist auf den Default-Einstellungen belassen werden (zumindest hatte ich noch nie ein Problem damit); Thresholdingmethode: STEP verwenden. Dieser Vorgang kann mitunter viel Zeit und auch viel RAM-Speicher benötigen. Man sollte daher den verwendeten RAM-Speicher im Auge behalten um das Limit nicht zu überschreiten und dadurch Systeminstabilität zu verursachen.
11. Danach sollte eine Datei ausgegeben werden, bei der nur noch einzelne (in unterschiedlichen Graustufen eingefärbte) Klumpen vorhanden sind.

Vorher | Nachher



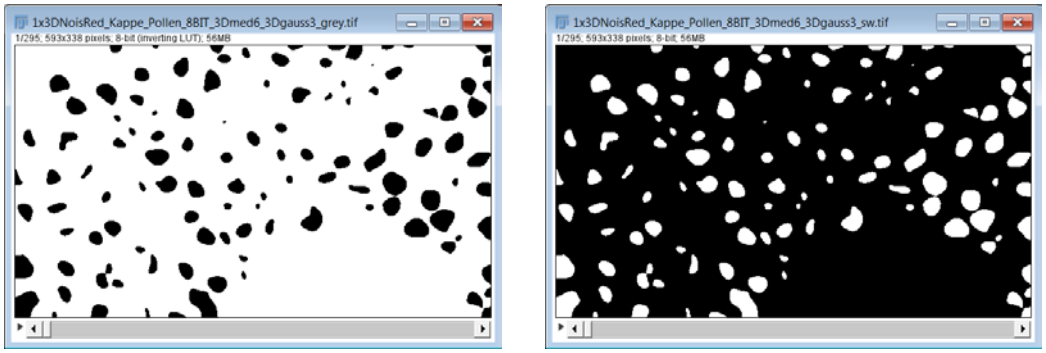
12. Es könnte sein, dass es hier wichtig ist, dass die Datei wieder auf 8-BIT umgestellt wird, da sonst bei der TH-Einstellung (nächster Schritt) Teile nicht ausgewählt werden können (zumindest hatte ich ein Problem, dass ich so zu lösen glaubte)
13. Nun muss der Threshold (=TH) angepasst werden. Dazu: image – adjust – Threshold (richtigen TH auswählen, sodass nur mehr Pollen ausgewählt ist → Vorsicht: Nicht zu viel und nicht zu wenig!; wurde jedoch in den Schritten zuvor gut gearbeitet (gutes Beschneiden in Amira mit dem „Compute"-Befehl (siehe Punkt 1.b) & Kontrastanpassung in ImageJ (siehe Punkte 6&7)) muss der TH nur sehr geringfügig angepasst werden

Vorher Nachher

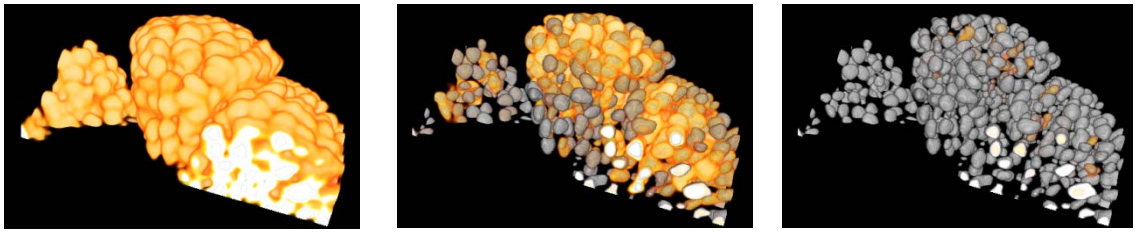



14. Einfärbung der Graustufen ändern: Image – Lookup Tables (Ich habe üblicherweise den Loop Table „grays“ verwendet)

Vorher Nachher




15. (Wir noch immer (aus welchem Grund auch immer) der Hintergrund hell und die Pollen dunkel dargestellt, müssen die Helligkeiten invertiert werden.
    ➔ Edit – invert) … dieser Schritt ist meiner jetzigen Erfahrung nach nicht nötig. Es wird zwar die Anzeige invertiert, aber es genügt, einfach den lookup table auf „grays“ zurück zu setzen und alles ist wieder wie gewohnt
16. Speichern als neue Datei (Tiff format)
17. Nun muss in Amira abgeglichen werden, ob der Separationsvorgang erfolgreich war. Dazu müssen die Tiff-Datei vor und jene nach dem Iterative-Thresholding-Prozess in ein Amira-Fenster geladen werden. Am besten gibt man dort jeder der beiden Datei einen unterschiedlichen Volren-typ (z.B. „grey“ und „volrenRed“). Durch geschicktes verschieben des TH kann nun geschaut werden, ob einzelne Pollenkörner vergessen wurden (oder ev. auch, ob sie gespalten worden sind und somit doppelt gezählt werden würden)



18. Danach wieder zurück zu ImageJ: Plugins – 3D object counter. Durch zweimaliges Zählen (einmal mit der Option „exclude edges“ und einmal ohne) kann die Anzahl ermittelt werden.

## Table of scanning conditions

~~crossed out~~: sample was rescanned
**bold**: high resolution scan

camera binning: 1
fixation FAA
contrast agent phosphotungstic acid (PTA)

| species | inf | position | type | acceleration voltage [kV] | source power [W] | exposure time [s] | pictures per sample | optical magnification | source distance [mm] | detector distance [mm] |
|---|---|---|---|---|---|---|---|---|---|---|
| A. morio | 1 | mid | gyn | 47 | 1,41 | 12 | 728 | 4 | 25 | 11,5 |
| A. morio | 1 | mid | gyn | 47 | 1,41 | 12 | 728 | 4 | 25 | 11,5 |
| A. morio | 1 | mid | pol | 47 | 1,41 | 12 | 728 | 4 | 22 | 30 |
| A. morio | 2 | bot | gyn | 47 | 1,41 | 12 | 728 | 4 | 30 | 10,5 |
| A. morio | 2 | bot | gyn | 47 | 1,41 | 12 | 728 | 4 | 30 | 10,5 |
| A. morio | 2 | mid | gyn | 47 | 1,41 | 12 | 728 | 4 | 25 | 12 |
| A. morio | 2 | mid | gyn | 47 | 1,41 | 12 | 728 | 4 | 25 | 12 |
| A. morio | 2 | mid | pol | 47 | 1,41 | 12 | 728 | 4 | 24 | 15 |
| A. morio | 3 | bot | gyn | 47 | 1,551 | 12 | 728 | 4 | 30 | 10 |
| A. morio | 3 | bot | gyn | 47 | 1,551 | 12 | 728 | 4 | 30 | 10 |
| A. morio | 3 | bot | pol | 47 | 1,551 | 12 | 728 | 4 | 22,5 | 30 |
| A. morio | 3 | mid | gyn | 47 | 1,551 | 12 | 728 | 4 | 31 | 12 |
| A. morio | 3 | mid | gyn | 47 | 1,551 | 12 | 728 | 4 | 31 | 12 |

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|---|---|---|---|---|---|---|---|---|---|---|
| A. morio | 3 | mid | pol | 47 | 1,551 | 12 | 728 | 4 | 22 | 30 |
| A. morio | 3 | top | gyn | 47 | 1,551 | 12 | 728 | 4 | 25 | 12,5 |
| A. morio | 3 | top | gyn | 47 | 1,551 | 12 | 728 | 4 | 25 | 12,5 |
| A. morio | 3 | top | pol | 47 | 1,551 | 12 | 728 | 4 | 22,5 | 30 |
| A. morio | 4 | bot | gyn | 47 | 1,504 | 12 | 728 | 4 | 30 | 10,5 |
| A. morio | 4 | bot | gyn | 47 | 1,504 | 12 | 728 | 4 | 30 | 10,5 |
| A. morio | 4 | bot | pol | 47 | 1,504 | 12 | 728 | 4 | 22 | 30 |
| A. morio | 4 | mid | gyn | 47 | 1,504 | 12 | 728 | 4 | 25 | 10,5 |
| A. morio | 4 | mid | gyn | 47 | 1,504 | 12 | 728 | 4 | 25 | 10,5 |
| A. morio | 4 | mid | pol | 47 | 1,504 | 12 | 728 | 4 | 21,5 | 30 |
| A. morio | 4 | top | gyn | 47 | 1,504 | 12 | 728 | 4 | 23,5 | 11 |
| A. morio | 4 | top | gyn | 47 | 1,504 | 12 | 728 | 4 | 23,5 | 11 |
| ~~A. morio~~ | ~~4~~ | ~~top~~ | ~~pol~~ | ~~47~~ | ~~1,504~~ | ~~12~~ | ~~728~~ | ~~4~~ | ~~22~~ | ~~30~~ |
| A. morio | 4 | top | pol | 47 | 1,41 | 12 | 728 | 4 | 26 | 14 |
| **A. morio** | **4** | **top** | **pol** | **60** | **1,98** | **55** | **728** | **10** | **26** | **12** |
| **A. morio** | **4** | **top** | **gyn** | **50** | **1,55** | **65** | **728** | **10** | **26** | **12** |
| A. pyramidalis | 1 | bot | gyn | 35 | 0,77 | 20 | 728 | 4 | 25 | 8 |
| A. pyramidalis | 1 | bot | gyn | 35 | 0,77 | 20 | 728 | 4 | 25 | 8 |
| A. pyramidalis | 1 | bot | pol | 45 | 1,17 | 12 | 728 | 4 | 25 | 8 |
| A. pyramidalis | 1 | mid | gyn | 35 | 0,77 | 20 | 728 | 4 | 35 | 7 |
| A. pyramidalis | 1 | mid | pol | 45 | 1,17 | 12 | 728 | 4 | 35 | 7 |
| A. pyramidalis | 1 | top | gyn | 35 | 0,77 | 20 | 728 | 4 | 35 | 7 |
| A. pyramidalis | 1 | top | pol | 45 | 1,17 | 12 | 728 | 4 | 35 | 7 |
| **A. pyramidalis** | **1** | **bot** | **pol** | **52** | **1,664** | **50** | **728** | **10** | **19** | **8** |
| **A. pyramidalis** | **1** | **bot** | **gyn** | **35** | **0,98** | **80** | **728** | **10** | **19** | **8** |

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|---|---|---|---|---|---|---|---|---|---|---|
| A. pyramidalis | 2 | bot | gyn | 35 | 0,77 | 20 | 728 | 4 | 25 | 8 |
| A. pyramidalis | 2 | bot | gyn | 35 | 0,77 | 20 | 728 | 4 | 25 | 8 |
| A. pyramidalis | 2 | bot | pol | 45 | 1,17 | 12 | 728 | 4 | 35 | 8 |
| A. pyramidalis | 2 | mid | gyn | 35 | 0,77 | 20 | 728 | 4 | 35 | 7,5 |
| A. pyramidalis | 2 | mid | pol | 45 | 1,17 | 12 | 728 | 4 | 30 | 7,5 |
| A. pyramidalis | 2 | top | gyn | 35 | 0,77 | 20 | 728 | 4 | 35 | 7 |
| A. pyramidalis | 2 | top | pol | 45 | 1,17 | 12 | 728 | 4 | 30 | 7 |
| A. pyramidalis | 3 | bot | gyn | 35 | 0,77 | 20 | 728 | 4 | 20 | 8,5 |
| A. pyramidalis | 3 | bot | gyn | 35 | 0,77 | 20 | 728 | 4 | 20 | 8,5 |
| A. pyramidalis | 3 | bot | pol | 45 | 1,17 | 12 | 728 | 4 | 25 | 8,5 |
| A. pyramidalis | 3 | mid | gyn | 35 | 0,77 | 20 | 728 | 4 | 20 | 7 |
| A. pyramidalis | 3 | mid | pol | 45 | 1,17 | 12 | 728 | 4 | 20 | 7 |
| A. pyramidalis | 3 | top | gyn | 35 | 0,77 | 20 | 728 | 4 | 20 | 7 |
| A. pyramidalis | 3 | top | pol | 45 | 1,17 | 12 | 728 | 4 | 20 | 7 |
| A. pyramidalis | 4 | mid | gyn | 35 | 0,77 | 15 | 728 | 4 | 22 | 7,25 |
| A. pyramidalis | 4 | mid | pol | 45 | 1,17 | 7 | 728 | 4 | 18 | 7,25 |
| A. pyramidalis | 4 | top | gyn | 35 | 0,77 | 12 | 728 | 4 | 18 | 7 |
| A. pyramidalis | 4 | top | pol | 45 | 1,17 | 7 | 728 | 4 | 18 | 7 |
| D. viridis | 1 | bot | gyn | 35 | 0,63 | 12 | 728 | 4 | 18,5 | 8 |
| D. viridis | 1 | bot | pol | 40 | 0,8 | 12 | 728 | 4 | 21 | 8 |
| D. viridis | 1 | mid | gyn | 35 | 0,63 | 12 | 728 | 4 | 18,5 | 8 |
| D. viridis | 1 | mid | pol | 40 | 0,8 | 12 | 728 | 4 | 21 | 8 |
| D. viridis | 2 | bot | gyn | 35 | 0,63 | 12 | 728 | 4 | 18 | 7 |
| D. viridis | 2 | bot | pol | 40 | 0,8 | 12 | 728 | 4 | 21 | 8 |
| D. viridis | 2 | top | gyn | 35 | 0,63 | 12 | 728 | 4 | 19 | 8 |

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|---|---|---|---|---|---|---|---|---|---|---|
| D. viridis | 2 | top | pol | 40 | 0,8 | 12 | 728 | 4 | 21 | 8 |
| D. viridis | 3 | bot | gyn | 35 | 0,63 | 12 | 728 | 4 | 18 | 7 |
| D. viridis | 3 | bot | pol | 40 | 0,8 | 12 | 728 | 4 | 21 | 8 |
| D. viridis | 3 | top | gyn | 35 | 0,63 | 12 | 728 | 4 | 18 | 7 |
| D. viridis | 3 | top | pol | 40 | 0,8 | 12 | 728 | 4 | 21 | 8 |
| **D. viridis** | **3** | **bot** | **pol** | **85** | **2,55** | **90** | **728** | **20** | **18** | **7** |
| D. viridis | 5 | bot | gyn | 35 | 0,65 | 12 | 728 | 4 | 21 | 8 |
| D. viridis | 5 | bot | pol | 40 | 0,8 | 12 | 728 | 4 | 21 | 8 |
| D. viridis | 5 | mid | gyn | 35 | 0,65 | 12 | 728 | 4 | 21 | 8 |
| D. viridis | 5 | mid | pol | 40 | 0,8 | 12 | 728 | 4 | 21 | 8 |
| D. viridis | 5 | top | gyn | 35 | 0,65 | 12 | 728 | 4 | 21 | 8 |
| D. viridis | 5 | top | pol | 40 | 0,8 | 12 | 728 | 4 | 21 | 8 |
| D. viridis | 6 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 8 |
| D. viridis | 6 | bot | pol | 40 | 0,8 | 12 | 728 | 4 | 25 | 8 |
| D. viridis | 6 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7 |
| D. viridis | 6 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7 |
| D. viridis | 6 | top | pol | 40 | 0,8 | 12 | 728 | 4 | 21 | 8 |
| D. incarnata | 1 | midtop | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 7,75 |
| D. incarnata | 1 | midtop | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 7,75 |
| D. incarnata | 1 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 20 | 7,25 |
| D. incarnata | 1 | top | pol | 50 | 1 | 18 | 728 | 4 | 35 | 7,25 |
| D. incarnata | 1 | top(bud) | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 7 |
| D. incarnata | 1 | top(bud) | pol | 50 | 1 | 18 | 728 | 4 | 35 | 7,25 |
| D. incarnata | 2 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,75 | 8 |
| D. incarnata | 2 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,75 | 8 |

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|---|---|---|---|---|---|---|---|---|---|---|
| D. incarnata | 2 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,75 | 8 |
| D. incarnata | 2 | bot | pol | 50 | 1 | 18 | 728 | 4 | 35 | 7,25 |
| D. incarnata | 2 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 19 | 8,25 |
| D. incarnata | 2 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 19 | 8,25 |
| D. incarnata | 2 | mid | pol | 50 | 1 | 18 | 728 | 4 | 35 | 7,25 |
| D. incarnata | 2 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 22 | 7 |
| D. incarnata | 2 | top | pol | 50 | 1 | 18 | 728 | 4 | 35 | 7,25 |
| D. incarnata | 3 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 8 |
| D. incarnata | 3 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 8 |
| D. incarnata | 3 | bot | pol | 50 | 1 | 18 | 728 | 4 | 35 | 7,25 |
| D. incarnata | 3 | bot+ | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 8 |
| D. incarnata | 3 | bot+ | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 8 |
| D. incarnata | 3 | bot+ | pol | 50 | 1 | 18 | 728 | 4 | 35 | 7,25 |
| D. incarnata | 4 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,25 | 7,25 |
| D. incarnata | 4 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,25 | 7,25 |
| D. incarnata | 4 | bot | pol | 50 | 1 | 18 | 728 | 4 | 35 | 7,25 |
| D. incarnata | 4 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,25 | 7,5 |
| D. incarnata | 4 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,25 | 7,5 |
| D. incarnata | 4 | mid | pol | 50 | 1 | 18 | 728 | 4 | 35 | 7,25 |
| D. incarnata | 4 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7 |
| D. incarnata | 4 | top | pol | 50 | 1 | 18 | 728 | 4 | 35 | 7,25 |
| **D. incarnata** | **1** | **top** | **pol** | **80** | **2** | **87** | **728** | **20** | **18** | **7,5** |
| D. fuchsii | 1 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,25 | 7,5 |
| D. fuchsii | 1 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,25 | 7,5 |
| D. fuchsii | 1 | bot | pol | 40 | 0,8 | 18 | 728 | 4 | 26 | 7,25 |

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|---|---|---|---|---|---|---|---|---|---|---|
| D. fuchsii | 4 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 8 |
| D. fuchsii | 4 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 8 |
| D. fuchsii | 4 | bot | pol | 40 | 0,8 | 18 | 728 | 4 | 22 | 8 |
| D. fuchsii | 4 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7,25 |
| D. fuchsii | 4 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7,25 |
| D. fuchsii | 4 | mid | pol | 40 | 0,8 | 18 | 728 | 4 | 22 | 8 |
| D. fuchsii | 2 | bot | pol | 40 | 0,8 | 18 | 728 | 4 | 22 | 8 |
| D. fuchsii | 2 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 7 |
| D. fuchsii | 2 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 7 |
| D. fuchsii | 2 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 7 |
| D. fuchsii | 2 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 7 |
| D. fuchsii | 2 | mid | pol | 40 | 0,8 | 18 | 728 | 4 | 22 | 7 |
| **D. fuchsii** | **2** | **bot** | **pol** | **75** | **1,95** | **90** | **728** | **20** | **18,25** | **7,75** |
| D. fuchsii | 3 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 8 |
| D. fuchsii | 3 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 8 |
| D. fuchsii | 3 | bot | pol | 40 | 0,8 | 18 | 728 | 4 | 22 | 8 |
| D. fuchsii | 3 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7 |
| D. fuchsii | 3 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7 |
| D. fuchsii | 3 | mid | pol | 40 | 0,8 | 18 | 728 | 4 | 22 | 8 |
| D. fuchsii | 3 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7 |
| D. fuchsii | 3 | top | pol | 40 | 0,8 | 18 | 728 | 4 | 22 | 8 |
| D. majalis | 1 | bot | gyn | 35 | 0,595 | 20 | 728 | 4 | 25 | 7,75 |
| D. majalis | 1 | bot | gyn | 35 | 0,595 | 20 | 728 | 4 | 25 | 7,75 |
| D. majalis | 1 | bot | pol | 48 | 0,85 | 15 | 728 | 4 | 35 | 7,25 |
| D. majalis | 1 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 8 |

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|---|---|---|---|---|---|---|---|---|---|---|
| D. majalis | 1 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 8 |
| D. majalis | 1 | mid | pol | 48 | 0,85 | 15 | 728 | 4 | 25 | 8 |
| D. majalis | 1 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7,25 |
| D. majalis | 1 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7,25 |
| D. majalis | 1 | top | pol | 48 | 0,85 | 15 | 728 | 4 | 20 | 6,5 |
| D. majalis | 2 | bot | gyn | 35 | 0,595 | 20 | 728 | 4 | 25 | 7,5 |
| D. majalis | 2 | bot | gyn | 35 | 0,595 | 20 | 728 | 4 | 25 | 7,5 |
| D. majalis | 2 | bot | pol | 48 | 0,85 | 15 | 728 | 4 | 30 | 7 |
| D. majalis | 2 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7,25 |
| D. majalis | 2 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7,25 |
| D. majalis | 2 | top | pol | 48 | 0,85 | 15 | 728 | 4 | 25 | 7,5 |
| D. majalis | 2 | top+ | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7 |
| D. majalis | 2 | top+ | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7 |
| D. majalis | 2 | top+ | pol | 48 | 0,85 | 15 | 728 | 4 | 28 | 6,5 |
| D. majalis | 3 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 19 | 8 |
| D. majalis | 3 | bot | gyn | 35 | 0,595 | 15 | 728 | 4 | 19 | 8 |
| D. majalis | 3 | bot | pol | 48 | 0,85 | 15 | 728 | 4 | 25 | 6,75 |
| D. majalis | 3 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7,5 |
| D. majalis | 3 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7,5 |
| D. majalis | 3 | mid | pol | 48 | 0,85 | 15 | 728 | 4 | 25 | 6,75 |
| D. majalis | 5 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,25 | 7,5 |
| D. majalis | 5 | mid | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,25 | 7,5 |
| D. majalis | 5 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,25 | 8 |
| D. majalis | 5 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,25 | 8 |
| D. majalis | 4 | top | gyn | 35 | 0,595 | 22 | 728 | 4 | 25 | 7 |

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|---|---|---|---|---|---|---|---|---|---|---|
| D. majalis | 4 | top | gyn | 35 | 0,595 | 22 | 728 | 4 | 25 | 7 |
| D. majalis | 4 | top | pol | 48 | 0,85 | 15 | 728 | 4 | 25 | 6,75 |
| D. majalis | 3 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 6,25 |
| D. majalis | 3 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 6,25 |
| D. majalis | 3 | top | pol | 48 | 0,85 | 15 | 728 | 4 | 18 | 6,25 |
| **D. majalis** | **3** | **top** | **pol** | **80** | **1,84** | **80** | **728** | **20** | **18** | **6,75** |
| O. militaris | 3 | bot | gyn | 50 | 1,8 | 10 | 728 | 4 | 22,5 | 11,5 |
| O. militaris | 3 | bot | gyn | 50 | 1,8 | 10 | 728 | 4 | 22,5 | 11,5 |
| O. militaris | 3 | bot | pol | 50 | 1,8 | 10 | 728 | 4 | 22,5 | 11,5 |
| O. militaris | 4 | top | gyn | 50 | 1,8 | 10 | 728 | 4 | 22,5 | 11,5 |
| O. militaris | 4 | top | gyn | 50 | 1,8 | 10 | 728 | 4 | 22,5 | 11,5 |
| O. militaris | 4 | top | pol | 50 | 1,8 | 10 | 728 | 4 | 22,5 | 11,5 |
| O. militaris | 4 | bot | gyn | 50 | 1,8 | 10 | 728 | 4 | 30 | 8,5 |
| O. militaris | 4 | bot | gyn | 50 | 1,8 | 10 | 728 | 4 | 30 | 8,5 |
| O. militaris | 4 | bot | pol | 50 | 1,8 | 10 | 728 | 4 | 22,5 | 10,5 |
| O. militaris | 2 | mid | gyn | 50 | 1,8 | 10 | 728 | 4 | 22,5 | 11,5 |
| O. militaris | 2 | mid | gyn | 50 | 1,8 | 10 | 728 | 4 | 22,5 | 11,5 |
| O. militaris | 2 | mid | pol | 50 | 1,8 | 10 | 728 | 4 | 22,5 | 11,5 |
| O. militaris | 3 | top | gyn | 50 | 1,8 | 10 | 728 | 4 | 26 | 10,5 |
| O. militaris | 3 | top | pol | 50 | 1,8 | 10 | 728 | 4 | 26 | 10,5 |
| O. militaris | 2 | top | gyn | 50 | 1,8 | 10 | 728 | 4 | 23 | 11,5 |
| O. militaris | 2 | top | gyn | 50 | 1,8 | 10 | 728 | 4 | 23 | 11,5 |
| O. militaris | 2 | top | pol | 50 | 1,8 | 10 | 728 | 4 | 23 | 11,5 |
| O. militaris | 2 | bot | gyn | 50 | 1,7 | 10 | 728 | 4 | 20 | 9 |
| O. militaris | 2 | bot | gyn | 50 | 1,7 | 10 | 728 | 4 | 20 | 9 |

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|---|---|---|---|---|---|---|---|---|---|---|
| O. militaris | 2 | bot | gyn | 50 | 1,7 | 10 | 728 | 4 | 20 | 9 |
| O. militaris | 2 | bot | pol | 50 | 1,7 | 10 | 728 | 4 | 20 | 9 |
| O. militaris | 2 | mid | fruit | 40 | 8 | 5 | 728 | 4 | 20 | 8 |
| **O. militaris** | **2** | **bot** | **pol** | **85** | **2,72** | **90** | **728** | **20** | **18** | **7,1** |
| G. conopsea | 2 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 7,75 |
| G. conopsea | 2 | top | pol | 50 | 0,825 | 15 | 728 | 4 | 25 | 7,75 |
| G. conopsea | 2 | top+ | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 6,75 |
| G. conopsea | 2 | top+ | pol | 50 | 0,825 | 15 | 728 | 4 | 25 | 7,75 |
| G. conopsea | 1 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7 |
| G. conopsea | 1 | top | pol | 50 | 0,825 | 15 | 728 | 4 | 25 | 7,75 |
| **G. conopsea** | **2** | **top+** | **pol** | **80** | **1,76** | **80** | **728** | **20** | **18** | **7** |
| G. conopsea | 3 | midtop | gyn | 35 | 0,595 | 15 | 728 | 4 | 22 | 7,25 |
| G. conopsea | 3 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 18,5 | 7,5 |
| G. conopsea | 3 | top | pol | 50 | 0,825 | 15 | 728 | 4 | 25 | 7,75 |
| G. conopsea | 4 | top | gyn | 35 | 0,595 | 15 | 728 | 4 | 18 | 7,25 |
| G. conopsea | 4 | top | pol | 50 | 0,825 | 15 | 728 | 4 | 25 | 7,75 |
| G. conopsea | 4 | mid | gyn | 35 | 0,525 | 15 | 728 | 4 | 18,75 | 8,25 |
| G. conopsea | 4 | mid | gyn | 35 | 0,525 | 15 | 728 | 4 | 18,75 | 8,25 |
| G. conopsea | 3 | bot | gyn | 35 | 0,525 | 15 | 728 | 4 | 18,5 | 7,25 |
| G. conopsea | 3 | bot | gyn | 35 | 0,525 | 15 | 728 | 4 | 18,5 | 7,25 |
| G. conopsea | 4 | bot | gyn | 35 | 0,525 | 15 | 728 | 4 | 18 | 7,5 |
| G. conopsea | 4 | bot | gyn | 35 | 0,525 | 15 | 728 | 4 | 18 | 7,5 |
| E. palustris | 3 | top(bud) | gyn | 50 | 2 | 10 | 728 | 4 | 31 | 10,16 |
| E. palustris | 3 | top(bud) | gyn | 50 | 2 | 10 | 728 | 4 | 31 | 10,16 |
| E. palustris | 3 | top(bud) | pol | 50 | 2 | 10 | 728 | 4 | 24 | 9 |

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|---|---|---|---|---|---|---|---|---|---|---|
| E. palustris | 4 | top(bud) | gyn | 50 | 2 | 10 | 728 | 4 | 24 | 9 |
| E. palustris | 4 | top(bud) | gyn | 50 | 2 | 10 | 728 | 4 | 24 | 9 |
| E. palustris | 4 | top(bud) | pol | 50 | 2 | 10 | 728 | 4 | 24 | 9 |
| E. palustris | 2 | mid(bud) | gyn | 50 | 2 | 10 | 728 | 4 | 24 | 9 |
| E. palustris | 2 | mid(bud) | gyn | 50 | 2 | 10 | 728 | 4 | 24 | 9 |
| E. palustris | 2 | mid(bud) | pol | 50 | 2 | 10 | 728 | 4 | 24 | 9 |
| E. palustris | 6 | top(bud) | gyn | 50 | 1,95 | 10 | 728 | 4 | 33 | 10 |
| E. palustris | 6 | top(bud) | gyn | 50 | 1,95 | 10 | 728 | 4 | 33 | 10 |
| E. palustris | 6 | top(bud) | pol | 50 | 1,95 | 10 | 728 | 4 | 23 | 10 |
| E. palustris | 1 | top(bud) | gyn | 50 | 1,95 | 10 | 728 | 4 | 23 | 10 |
| E. palustris | 1 | top(bud) | gyn | 50 | 1,95 | 10 | 728 | 4 | 23 | 10 |
| E. palustris | 1 | top(bud) | pol | 50 | 1,95 | 10 | 728 | 4 | 23 | 10 |
| E. palustris | 2 | midtop(bud) | gyn | 50 | 1,95 | 10 | 728 | 4 | 23 | 10 |
| E. palustris | 2 | midtop(bud) | gyn | 50 | 1,95 | 10 | 728 | 4 | 23 | 10 |
| E. palustris | 2 | midtop(bud) | pol | 50 | 1,95 | 10 | 728 | 4 | 23 | 10 |
| E. palustris | 2 | mid(late bud) | gyn | 50 | 2 | 10 | 728 | 4 | 31 | 10 |
| E. palustris | 2 | mid(late bud) | gyn | 50 | 2 | 10 | 728 | 4 | 31 | 10 |
| E. palustris | 2 | mid(late bud) | pol | 50 | 2 | 10 | 728 | 4 | 31 | 10 |
| E. palustris | 1 | mid(early flower) | gyn | 50 | 2 | 10 | 728 | 4 | 23 | 10 |
| E. palustris | 1 | mid(early flower) | gyn | 50 | 2 | 10 | 728 | 4 | 23 | 10 |
| E. palustris | 1 | mid(early flower) | pol | 50 | 2 | 10 | 728 | 4 | 23 | 10 |
| E. palustris | 5 | top(bud) | gyn | 50 | 2 | 10 | 728 | 4 | 23 | 10 |
| E. palustris | 5 | top(bud) | gyn | 50 | 2 | 10 | 728 | 4 | 23 | 10 |
| E. palustris | 5 | top(bud) | pol | 50 | 2 | 10 | 728 | 4 | 23 | 10 |
| E. palustris | 2 | mid(late bud) | gyn | 50 | 1,9 | 10 | 728 | 4 | 21,5 | 11 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| E. palustris | 2 | mid(late bud) | gyn | 50 | 1,9 | 10 | 728 | 4 | 21,5 | 11 |
| E. palustris | 2 | mid(late bud) | pol | 50 | 1,9 | 10 | 728 | 4 | 21,5 | 11 |
| E. palustris | 6 | top(bud) | gyn | 50 | 1,9 | 10 | 728 | 4 | 21,5 | 11 |
| E. palustris | 6 | top(bud) | gyn | 50 | 1,9 | 10 | 728 | 4 | 21,5 | 11 |
| E. palustris | 6 | top(bud) | pol | 50 | 1,9 | 10 | 728 | 4 | 21,5 | 11 |
| E. palustris | 6 | top(early bud) | gyn | 50 | 2 | 10 | 728 | 4 | 22,5 | 11 |
| E. palustris | 6 | top(early bud) | gyn | 50 | 2 | 10 | 728 | 4 | 22,5 | 11 |
| E. palustris | 6 | top(early bud) | pol | 50 | 2 | 10 | 728 | 4 | 22,5 | 11 |
| E. palustris | 5 | top(early bud) | gyn | 50 | 2 | 10 | 728 | 4 | 22,5 | 11 |
| E. palustris | 5 | top(early bud) | pol | 50 | 2 | 10 | 728 | 4 | 22,5 | 11 |
| E. palustris | 6 | top(early bud) | gyn | 50 | 2 | 10 | 728 | 4 | 22,5 | 11 |
| E. palustris | 6 | top(early bud) | gyn | 50 | 2 | 10 | 728 | 4 | 22,5 | 11 |
| E. palustris | 6 | top(early bud) | pol | 50 | 2 | 10 | 728 | 4 | 22,5 | 11 |
| ~~E. palustris~~ | ~~test flower~~ | ~~top~~ | ~~pol~~ | ~~50~~ | ~~1,8~~ | ~~10~~ | ~~728~~ | ~~4~~ | ~~23~~ | ~~11~~ |
| **~~E. palustris~~** | **~~test flower~~** | **~~top~~** | **~~pol~~** | **~~50~~** | **~~2,05~~** | **~~23~~** | **~~728~~** | **~~10~~** | **~~20~~** | **~~9,45~~** |
| **~~E. palustris~~** | **~~test flower~~** | **~~top~~** | **~~pol~~** | **~~50~~** | **~~2,05~~** | **~~23~~** | **~~728~~** | **~~10~~** | **~~20~~** | **~~9,45~~** |
| **E. palustris** | **test flower** | **top** | **gyn** | **50** | **2,05** | **20** | **728** | **10** | **20** | **8** |
| **E. palustris** | **test flower** | **top** | **gyn** | **50** | **2,05** | **20** | **728** | **10** | **20** | **8** |
| **E. palustris** | **test flower** | **top** | **gyn** | **50** | **2,05** | **20** | **728** | **10** | **20** | **8** |
| **E. palustris** | **test flower** | **top** | **gyn** | **50** | **2,05** | **20** | **728** | **10** | **20** | **8** |
| ~~E. palustris~~ | ~~test flower~~ | ~~top~~ | ~~pol~~ | ~~40~~ | ~~1,48~~ | ~~8~~ | ~~728~~ | ~~4~~ | ~~20~~ | ~~9,5~~ |
| E. palustris | test flower | top | gyn | 40 | 1,48 | 8 | 728 | 4 | 20 | 9,5 |
| E. palustris | test flower | top | gyn | 40 | 1,48 | 8 | 728 | 4 | 20 | 9,5 |
| E. palustris | test flower | top | gyn | 40 | 1,48 | 8 | 728 | 4 | 20 | 9,5 |
| **E. palustris** | **test flower** | **top** | **pol** | **55** | **2,31** | **50** | **1300** | **10** | **19,5** | **8,5** |

## Lebenslauf

### Schulische Ausbildung

| | |
|---|---|
| *1994 – 1998* | Volksschule Mauerkirchen |
| *1998 – 2006* | Bundesgymnasium/ Bundesrealgymnasium Braunau am Inn |
| *14.06.2006* | Matura (ausgezeichneter Erfolg) |

### Grundwehrdienst/Zivildienst

| | |
|---|---|
| *2006 – 2007* | Zivildienst im Diakoniewerk Gallneukirchen, Werkstätte Mauerkirchen (Behindertenbetreuung) |

### Studium

| | |
|---|---|
| *WS[*] 2007/08 – WS 2014/15* | Universität Wien<br>- Lehramtsstudium Mathematik<br>- Lehramtsstudium Biologie und Umweltkunde |
| *SS 2011* | Erasmus-Auslandsaufenthalt in Luleå (Schweden) an der Luleå University of Technology |
| *WS 2012/13 – SS 2014* | DaF-DaZ Ausbildung im Zuge des DaF-DaZ Moduls für Diplomstudierende |

[*] WS = Wintersemester, SS = Sommersemester