Master of Science Thesis

The Role of Geitonogamy in the Reproduction Success of a Nectarless *Dactylorhiza maculata* (Orchidaceae)

Jonna Niiniaho

University of Jyväskylä

Department of Biological and Environmental Science

Ecology and Environmental Management

18.09.2011

UNIVERSITY OF JYVÄSKYLÄ, Faculty of Mathematics and Science

Department of Biological and Environmental Science

Ecology and Environmental Management

NIINIAHO, J.: The Role of Geitonogamy in the Reproduction Success of a Nectarless *Dactylorhiza maculata* (Orchidaceae)

Master of Science Thesis: 18 p.
Supervisors: PhD Elisa Vallius
Inspectors: Dr. Minna-Maarit Kytöviita, Dr. Janne Kotiaho
September 2011

Key Words: deceptive pollination, geitonogamy, hand-pollination, *in vitro* germination, Orchidaceae, beetle pollination, reward

ABSTRACT

Dactylorhiza maculata is a common terrestrial orchid in Finland. It is nectarless and the seed production is relatively high for a deceptive species. The pollinators of *D. maculata* do not have frequent visits to the flowers, but the species is capable of over 50 % seed capsule production. Geitonogamy is the transfer of self pollen between flowers on the same individual. It is typical that the pollinators of deceptive inflorescences visit only few flowers and have short stays in them, and therefore it is commonly thought that geitonogamy is infrequent among deceptive species. I examined the role of geitonogamy in the seed production of *D. maculata*. I prevented geitonogamy in male-sterilization experiment by removing the pollinaria in the experiment group, and in hand-pollination experiment I conducted cross-fertilization and self-fertilization (in the form of both geitonogamy and autogamy). When the seeds had matured I calculated the relative seed capsule production in male-sterilization experiment, and estimated the proportions of embryonic seeds in both experiments. From this data I analyzed the differences in fertilization success and seed quality. The seed quality in both experiments was verified with *in vitro* germination of the seeds. I found that geitonogamy is not a significant reproduction strategy for *D. maculata*, because self-pollination is a disadvantage that reduces the seed set and the seed quality. One mechanism to prevent geitonogamy is pollinaria bending, a time delay before the freshly withdrawn pollinaria are able to conduct fertilization. In *D. maculata* this delay is approximately 50 s which is rather long for a deceptive species. The explanation for the high natural seed capsule production of *D. maculata* might be a fact that probably the species is not deceptive in all means. Longhorn beetles (Cerambycidae) were found to act as beetle pollinators in the study population. The beetles were found to bear large pollinaria loads of up to approximately 30 pollinaria.

JYVÄSKYLÄN YLIOPISTO, Matemaattis-luonnontieteellinen tiedekunta
Bio- ja ympäristötieteiden laitos
Ekologia ja ympäristönhoito

NIINIAHO, J. : Geitonogamian merkitys medettömän kämmekkäkasvin maariankämmekän (*Dactylorhiza maculata*) lisääntymismenestyksessä

Pro Gradu –tutkielma: 18 s.
Työn ohjaajat: FT Elisa Vallius
Tarkastajat: Prof. Minna-Maarit Kytöviita, Prof. Janne Kotiaho
Syyskuu 2011

Hakusanat: geitonogamia, *in vitro* idätys, kovakuoriaispölytys, käsipölytys, Orchidaceae, palkkio, petospölytys

TIIVISTELMÄ

Maariankämmekkä (*Dactylorhiza maculata*) on Suomessa yleinen medetön kämmekkäkasvi. Sen siementuotto on verrattain korkea lajiksi, jonka pölytys perustuu petokseen. Vaikka maariankämmekän pölyttäjät vierailevat kukissa harvoin, laji kykenee yli 50 % kotatuottoon. Geitonogamia on siitepölyn siirtymistä saman kukinnon eri kukkien välillä. Yleensä pölyttäjät vierailevat ainoastaan muutamissa pettävissä kukissa ja vierailut ovat lyhyitä. Tämän vuoksi uskotaan, että geitonogamia on harvinaista pettävien lajien joukossa. Tutkimuksessani selvitin geitonogamian merkitystä maariankämmekän siementuotossa. Koirassterilisaatiokokeessa estin geitonogamian mahdollisuuden poistamalla koeryhmän yksilöiltä siitepölymyhkyt, ja käsipölytyskokeessa tein ristipölytteisiä ja itsepölytteisiä (sekä autogamisia että geitonogamisia) hedelmöityksiä. Siementen kypsyttyä laskin kotaprosentit koirassterilisaatiokokeessa, ja molemmissa kokeissa arvioin alkiollisten siementen osuudet. Tämän aineiston perusteella analysoin erot hedelmöitysmenestyksessä ja siementen laadussa. Molemmissa kokeissa tuotettujen siementen laatu varmistettiin *in vitro* idätyksellä. Tulosten mukaan geitonogamia ei ole maariankämmekällä merkittävä lisääntymisstrategia, koska itsepölytys on haitta, joka alentaa siementen määrää ja laatua. Yksi tapa välttää geitonogamiaa on siitepölymyhkyn taipuminen, joka tarkoittaa viivettä ennen kuin juuri irronnut siitepölymyhky kykenee suorittamaan hedelmöityksen. Maariankämmekällä tämä taipumisaika on noin 50 s, mikä on pettävälle lajille melko pitkä viive. Näyttäisi kuitenkin siltä, että maariankämmekkä ei ole kaikilta osin pettävä, mikä voisi selittää myös lajin suuren luonnollisen kotatuoton. Tutkimusalueelta löydettiin kukkajääriä (Cerambycidae), jotka toimivat kovakuoriaispölyttäjinä tutkimuspopulaatiossa. Jäärät kantoivat suuria siitepölymyhkyjen kimppuja, joissa oli jopa noin 30 siitepölymyhkyä.

Contents

1. INTRODUCTION

In sexual reproduction of a seed-producing plant the flowers need to be pollinated (Salonen 2006). The great majority of seed-producing plants need an animal as a vector for pollen transfer. In the remainder, the pollination is mediated by abiotic vectors such as wind and water. Most of the seed-producing plants are hermaphrodites that have both male and female functions in the same flower. Hermaphroditic plants may be pollinated both with cross pollen and self pollen (Darwin 1877; Salonen 2006). There are two kinds of self-pollination: pollination within a single flower (autogamy) and within the flowers of the same individual (geitonogamy) (de Jong et al. 1993). Usually self-pollination addresses expenses to the plant: reduced seed set and offspring fitness indicate inbreeding depression (Darwin 1877; Waser & Price 1989). Cross-fertilization usually increases the average fitness of the zygotes and provides new genetic material for evolution (Lloyd 1979; Salonen 2006).

To attract pollinators, flowers usually produce rewards for them (Johnson & Nilsson 1999). Nectar is the most common reward. Other rewards include pollen, fragrances, oils and resins (Salonen 2006; Doetterl & Schaeffler 2007; Pemberton & Liu 2008). Larger amounts of rewards increase the number of flowers the pollinators visit within an inflorescence, and the duration of these probes (Johnson & Nilsson 1999). Both of these lead to greater levels of pollen removal and deposition. This in turn should increase the plants' reproductive success, unless the selfing increases and leads to inbreeding depression.

Providing pollinator reward may require a large amount of resources (Johnson & Nilsson 1999). During flowering, nectar production may take several percents of the daily energy the plant has produced (Salonen 2006). It is not useful to produce excessive amounts of rewards. In some species the evolution has led to giving up nectar production and to the development of other deceptive strategies. Though, it seems unlikely that the energy saved by producing no nectar is important enough to explain the evolution of a nectarless strategy (Dressler 1981). Selection may have favored the production of less or no nectar if it increases the probability of outbreeding by encouraging pollinators to stay a shorter time in single flowers and leave the inflorescense fairly quickly (Dressler 1981; Hodges 1995). Deceptive species may also have greater pollination distances, which encourages outbreeding (Dressler 1981). Deceptive species are supposed to be pollinated by naive pollinators that are commencing to explore their foraging environment without previous experience of the empty inflorescences (Nilsson 1980; Ferdy & Smithson 2002). The majority of deceptive species belong to Orchidaceae (Johnson & Nilsson 1999).

In this study, I examine the geitonogamy rates of a nectarless orchid *Dactylorhiza maculata* and the possible costs of self-pollination to the seed set. Specifically I addressed the following questions: 1) What is the proportion of geitonogamous fertilization in *D. maculata* natural seed production? 2) Does self-pollination reduce the seed set or the seed quality?

2. BACKGROUND OF THE STUDY

2.1. Geitonogamy

Geitonogamy is the transfer of self pollen between flowers on the same individual (Ferdy & Smithson 2002). Geitonogamy has noticeable importance in determining selfing rates,

and it plays a major role in pollen dispersal patterns that influence the spatial genetic structure of populations (de Jong et al. 1993; Ferdy & Smithson 2002). In self-compatible plants geitonogamy leads to increased selfing and possible inbreeding depression (de Jong et al. 1993; Harder & Wilson 1998; Johnson & Nilsson 1999). Even in self-incompatible plants the seed set can suffer, and less pollen is available for cross-pollination. Only if the selfed offspring are equal in viability and fecundity to outbred offspring, geitonogamy has no effect on reproductive success (Lloyd 1979).

Because self-pollination addresses expenses to the plant, the plants have several mechanisms to prevent it (Waser & Price 1989; de Jong et al. 1993). These mechanisms include floral traits that decrease the probability of self pollen striking the stigma, and self-incompatibility that halts the seed production (de Jong et al. 1993; Salonen 2006). The occurrence of autogamy is more dependent on floral traits, whereas that of geitonogamy is strongly affected by pollinator behavior (Takebayashi & Delphi 2000; Ferdy & Smithson 2002). The factors related to pollinator behaviour that influence the rate of geitonogamous pollinations include the duration of pollinator visit in an inflorescence, the number of flowers available on a plant and the availability of rewards (de Jong et al. 1993; Johnson & Nilsson 1999). Other factors include structural mechanisms of a flower and the extent of pollen carryover.

Attractive rewarding inflorescences usually have many pollinator visits and many flowers visited, which may lead to high geitonogamous selfing if the same pollinators stay long in the inflorescence (Ferdy & Smithson 2002). In unrewarding inflorescences pollinators visit only few flowers and geitonogamy is usually infrequent (Nilsson 1983; Ferdy & Smithson 2002). Given that the pollinator arrives with pollen, the geitonogamy rates may even decrease when the pollinator visits many unrewarding inflorescences of the same species (Ferdy & Smithson 2002). Usually pollinators fly longer distances between unrewarding inflorescences than the rewarding ones, which should enhance the outbreeding of the fraud species (Peakall & Beattie 1996).

2.2. Orchid reproduction

The orchid family, Orchidaceae, is a group characterized with specialized flowers, abundant dust-like seeds and a high degree of specialization (Chase 2001). Orchidaceae is the most species-rich plant family with approximately 25 000 species and they are found on every continent except Antarctica (Dressler 1981; Nilsson 1992).

Orchid pollen is packaged to pollinaria (pollinarium is the singular) (Johnson & Nilsson 1999). Pollinia are the uppermost parts of pollinaria (Grimshaw 2005). Each pollinium is a mass of pollen grains. Because of the pollen packaging, orchids are a well suited group for studies of plant evolution (Nilsson 1992). Pollinia can be marked, removed and placed on fertile destinations. At the base of the pollinarium is the viscidium that is sticky and adheres most effectively to smooth surfaces, such as the insect eyes and mouthparts, and bird beaks (Johnson & Edwards 2000; Grimshaw 2005). After touching the viscidium the pollinator removes the whole pollinaria (Grimshaw 2005). The sticky glue of viscidium dries soon, usually within minutes (Johnson & Edwards 2000). Orchids have been reported to have high pollen viability. This is not surprising, as orchids often grow at low densities and their pollinators usually visit flowers of other species. It is likely that several days may pass between pollinaria removal and deposition.

There are two main categories of pollinarium types among orchids (Johnson & Nilsson 1999). 1) Solid pollinaria are deposited onto a stigma as a single unit (there is no carryover), and 2) sectile pollinaria consist of numerous individual pollen subunits called massulae. The massulae progressively break away from pollinaria attached to the pollinator, and can be deposited to several destinations. In other words, several flowers

may be pollinated from a single pollinarium (Johnson & Edwards 2000). The pollen load deposited on each sequential flower differs by orchid species (Johnson & Nilsson 1999). It is not before pollen massulae attach to a sticky surface such as stigma that they separate from the rest of the pollinaria carried by the pollinator.

Pollinaria ensure large pollen loads to the stigma, a phenomenon which enables the fertilization of a huge number of ovules found in each ovary (Johnson & Edwards 2000). The orchid pollination is an 'all-or-nothing' event that can lead to the production of over two million tiny, wind-dispersed seeds (Nilsson 1992). Even one visit may lead to a successful pollination. Pollinaria also ensure efficient pollen removal from the anther, minimal pollen wastage during transportation between flowers, and a high probability of deposition on stigmas within the same species (Johnson & Edwards 2000).

The fruit set of orchids is commonly pollinator limited and hand-pollination usually increases the seed production (Nilsson 1992; Johnson & Nilsson 1999). It is commonly thought that each orchid species is adapted to attract precisely one pollinator species or a small group of pollinators, though this is an exaggeration (Dressler 1981; Grimshaw 2005). There exists a continuum between plants pollinated by many different pollinators and those pollinated by one species or a group of closely related species, and orchids are on the specialist end of this continuum (Johnson & Steiner 2000). The specificity is usually one-sided, as the pollinators of highly specialized orchids are not strictly limited to the pollination of a single plant species (Dressler 1981).

About one-third of orchid species are frauds that provide no reward to floral visitors (Boyden 1982). In deception, the flowers utilize the behavior of the insects in various ways by attracting the insect to copulate, to search for food, a hiding place, or a mate, to lay eggs or to defend territories (van der Cingel 2007). As in other angiosperms, the original and principal floral reward of orchids has been assumed to be nectar (Nilsson 1992). There are also speculations whether the ancestral condition in orchids was the absence of nectar (Neiland & Wilcock 1998). The origin of orchids has been speculated to date before the end of Cretaceous period, an era with no highly advanced subgroups of Hymenoptera and Lepidoptera (Schmid & Schmid 1977; Endress 1990; Neiland & Wilcock 1998). In the absence of bees and butterflies, the flowers would have been visited by unspecialized insects like beetles and flies that were feeding on floral and vegetative parts of the flowers they were pollinating (Endress 1990). In the evolution of Orchidaceae, shifts between deceptive and rewarding pollination systems have evolved many times (Dressler 1981).

Most fraud orchid species are food-deceptive and they are pollinated by nectar-seeking insects (Nilsson 1992; Neiland & Wilcock 1998). They usually function without a mimicry of a rewarding species (Ferdy & Smithson 2002). Most pollinators of deceptive orchids appear to carry low numbers of pollinia (Johnson & Edwards 2000). Fraud orchid species usually have low pollination success and low fruit set in all geographic areas, while rewarding orchid species in the same habitats often have high levels of fruit set (Neiland & Wilcock 1998; Peter & Johnson 2009). In natural populations, many deceptive plants do not set any fruit (Johnson & Nilsson 1999).

In orchids, the most important mechanisms that prevent self-pollination are extensive pollen carryover and structural mechanisms such as pollinaria bending (Johnson & Nilsson 1999). The extent of pollen carryover is the fraction of the pollen load carried from one flower to the next ones in the sequence (Geber 1985; Johnson & Nilsson 1999). In orchids this fraction is high, as the pollen is packaged to pollinaria that are removed as whole and include thousands of pollen grains, and a single visit may ensure a successful pollination (Nilsson 1992; Johnson & Nilsson 1999; Johnson & Edwards 2000; Chase 2001) Pollinaria bending is a term for a time delay of freshly withdrawn pollinaria to bend into the correct orientation to strike the stigma (Johnson & Nilsson 1999). Geitonogamy will not happen in an

inflorescence before the bending of the first withdrawn pollinarium. Pollinators usually leave the inflorescence before the pollinaria have bended. The time taken to complete the bending movement varies between orchid species, from 20 s to several hours (Dressler 1981). Because nectariferous orchids usually have longer pollinator probes, their natural selection has favored longer delays in pollinaria bending and extensive pollen carryover (Johnson & Nilsson 1999). Self-incompatibility is unusual in orchids (Neiland & Wilcock 1998). Where it occurs, very low fruit set levels have been reported.

The geitonogamy rates differ between orchid species. The rates depend on many factors, such as the reward, the pollinarium type and the population size and density (Johnson & Nilsson 1999; Peter & Johnson 2009). Rewarding orchids are more prone to geitonogamy, just as are the plants in a small sparse population. Sectile, bending pollinaria are widespread among temperate European orchid genera (Johnson & Nilsson 1999). Orchids with this type of pollinaria do not often face geitonogamy. The majority of the world's orchids have solid pollinaria that are deposited onto the stigmas as single units. The orchid species with solid bending pollinaria can delay the commence of geitonogamy. The orchid species with solid, non-bending pollinarium type are most likely to encounter geitonogamy.

3. MATERIALS AND METHODS

3.1. The study species and site

Dactylorhiza maculata shows considerable morphological variation, and the species is divided to several separate subspecies (Foley 2005). *D. maculata* ssp. *maculata* is a common terrestrial orchid taxa in Finland (Kuitunen & Kuitunen 1994; Hämet-Ahti et al. 1998). It inhabits oxygen-rich mesotrophic mires and peat-covered forests. The flowers are nectarless and the pollination is based on non-mimic food deception (Nilsson 1981; Dafni 1987). Sometimes *D. maculata* may have a gentle, sweet scent (Vallius & Salonen 2000). The flowers do not have frequent pollinator visits, and only ~24 % of the visits result in removal of pollinia (Koivisto et al. 2002). The main pollinators are naive bumble bee workers (*Bombus*, Apidae) (Nilsson 1981). Other important pollinators include species from Empididae, Syrphidae, Cerambycidae and Halictidae. If not pollinated, pollinia stay fresh and stigma is receptive for several days, approximately 10 days (Vallius 2000). After self-pollination capsules are formed, but spontaneous autogamy does not occur in nature (Mattila 2000).

D. maculata is a long-lived, polycarpic species which stores resources in a corm (Vallius & Salonen 2000; Vallius 2001). Reproductive plants usually have a 30 cm high stalk with on average 15 acropetally opening flowers in a single spike (Vallius 2001). Among different individuals the colour of the inflorescence varies from pink to white, with purple markings (Vallius & Salonen 2000; Vallius 2001). The seed capsule production of *D. maculata* is dependent on the available pollen (Vallius 2000). The natural relative seed capsule production may vary from 0 to 100 % (Vallius & Salonen 2000). At least in some populations in Finland the capsule production is on average over 50 % which is high compared to most deceptive species that average globally 22.2% and in Europe 27.7 % (Neiland & Wilcock 1998; Vallius & Salonen 2000).

The field experiments and observations were carried out in a *D. maculata* population on a small, open minerotrophic fen Härkösuo (62^{o} 11´ N; 25^{o} 40´ W) in Jyväskylä, central Finland. This population is rather large with approximately 200 flowering plants annually. Other plant species that flower at about the same time as *D. maculata* include *Melampyrum*

pratense, *Vaccinium oxycoccos* and *Dactylorhiza incarnata. Menyanthes trifoliata* is finishing its flowering at the commence of *D. maculata* flowering period.

3.2. Experimental design

3.2.1. Pollinaria removal experiment

There were 20 experiment - control plant pairs in the pollinaria removal treatment. In each pair, the plants were chosen to be approximately the same size near each other and starting their flowering around the same time. This was to ensure the pairs and their location in the population were most alike. In the experiment group, the mature pollinaria were removed with a toothpick. The pollinaria removal was conducted every second day from the first open flowers until the end of the flowering period. The pollinaria are usually adhesive on the second day after the flower has opened.

The control plants were not treated in other ways than touching the flowers and gently bending the stem. This kind of control treatment was chosen to prevent the impact of human activity *per se* to affect the results. Additionally, 10 pairs had a treatment-control plant (t-control). The t-control plants were not treated in any way after placing the identification number with a treasury tag. All the plants were marked and identified the same way. The t-control plants were chosen to be near their experiment-control pair and approximately the same size relative to them. The pollinaria removal period lasted for 17 days until the last flowers were open. The flowering of the plants in the experiment took place in the latter part relative to the whole population flowering period.

After the seeds had matured, the flower stems were collected from the site. It was planned to weigh the whole seed capsules, but due to an extraordinary hot and dry summer with peak temperatures of 35 ^{o}C, the seed maturation had been faster than usual and some of the seed capsules had already opened and the seeds partially dispersed when they were collected. The stems were air-dried at room temperature in paper envelopes and the relative seed capsule production (capsules/flowers) was calculated. This identifies whether or not the fertilization has succeeded and the capsule has been formed. The relative seed capsule production is a robust method for estimating the pollination success because it is possible to calculate it from the stem even if the capsule has fell off or been removed.

In addition, approximately 200 seeds from every plant were studied with a light microscope and the proportion of embryonic seeds from all seeds was calculated. This identifies the success of the fertilization by appointing the proportion of viable seeds that are capable to germinate. The relative seed capsule production and the proportion of embryonic seeds between different treatment groups were tested with Paired-Samples T-Test on SPSS/PASW statistics.

The pollinaria removal treatment acted as male sterilization and inhibited geitonogamous self pollination within the inflorescence. If there is a significant difference in seed production between experiment and control plants, that should be due to geitonogamy. The relative seed capsule production shows the pollination success that is verified by the proportion of embryonic seeds from all seeds.

3.2.1. Hand-pollination experiment

The hand-pollination treatments were carried out concurrent with the pollinaria removal treatments. In the hand-pollination treatments there were 15 plants which were covered with light mesh bags prior to flowering in order to prevent pollinator visits. In each experimental plant, flowers were hand-pollinated with one pollinium from i) another *D. maculata* plant (cross-pollination), ii) from other flowers of the same inflorescence

(geitonogamy) and iii) from the same flower (autogamy). The pollinium was removed using a toothpick and placed on a stigma with tweezers. Each treatment was repeated in two-three flowers in each inflorescence and individual flowers were marked using light plastic rings with different colours indicating the treatment. The treatments were carried out on two separate days, and every inflorescence was treated only once. Approximately half of the flowers in an inflorescence were included in the experiment. All the flowers were pollinated when the stigmas were receptive.

The mature seed capsules were collected from the site and air-dried at room temperature. After this the proportion of embryonic seeds from all seeds was calculated in order to identify the seed quality. The proportion of embryonic seeds between different treatment groups was tested with Paired-Samples Wilcoxon test on SPSS/PASW statistics.

3.2.2. Pollinaria bending

The time for pollinaria bending was measured from 6 inflorescences with a total of 20 pollinarium. The inflorescences were chosen randomly from the population.

3.2.3. Pollinator activity

At the study site while conducting the pollinaria removal and hand pollination experiments, the pollinator activity was observed. These observations were not systematic. On a few days I confronted pollinators that were collected as specimens. Insects were also collected with net bags, window traps and colour traps. Salt water was used in the traps. The specimens were later studied with a microscope for possible pollinaria adhesions.

3.3. Seed germination

3.3.1. Culture medium

The culture medium was prepared to a volume of 500 ml sterile water. Other substances were 10 g TGZ medium for *Dactylorhiza*, 10 ml fresh pressed pineapple juice and 2.5 ml antibiotic solution. The TGZ medium for *Dactylorhiza* is manufactured by Manfred Meyer. It includes i. a. activated carbon, sucrose and agar-agar.

The mix of sterile water and TGZ medium was autoclaved. The pineapple juice and the antibiotic solution were added after the medium had cooled but still warm (Bruns & Read 2000). The solution was kept warm in a water bath until poured on petri dishes. The petri dishes with the medium were left to set and let the water evaporate. After this, they were sealed and stored for approximately one week before placing the seeds on them. This was to ensure that the medium itself was not contaminated.

The pineapple juice was acting as a complex organic substance that is an important ingredient in the culture medium (Malmgren & Nyström 2011). The complex organic substance contains plant hormones and unknown growth factors that benefit the orchid seed germination. It is mentioned in the instructions of TGZ medium for *Dactylorhiza*. The pineapple was sterilized with alcohol from the outside for one hour before opening it. 10 ml of juice was transferred with a pipette. In the final culture medium there was 2 % pineapple juice.

The antibiotic solution was used to prevent fungal development. The antibiotic solution included penicillin 10 000 unit/ml and streptomycin 10 mg/ml. 2.5 ml of this antibiotic solution was added to the medium. The final culture medium included penicillin 50 units/ml and streptomycin 50 μg/ml.

3.3.2. Seed sterilization and sowing

Orchid seeds have a thin seed coat that is impermeable to water (Malmgren & Nyström 2011). The coat has to be broken down chemically to enable the seed to germinate. This can be done with chlorine in tandem with the seed sterilization. The chlorine solution was made of Klorite that is a commercial bleach manufactured by KiiltoClean. Klorite includes approximately 1 % active chlorine.

The seeds were sterilized with a packet technique in 0.05 % active chlorine solution before placing them on the culture medium (McKendrick 2000; Phyto Technology Laboratories 2008). The seeds were set inside a filter paper packet and sterilized for 4-5 minutes. After this they were put to sterile water for some minutes to rinse and wait to be placed on the culture medium. The seeds were sterilized in a group of approximately five filter paper packets. Each packet was cut open with alcohol sterilized instruments before placing only the seeds on the culture medium on a petri dish. The dish was sealed with cling-film. The dishes were stored in room temperature for three weeks before the determination of germination.

3.3.3. Determination of germination

The germination was determined after three weeks of germination time. Most *Dactylorhiza* species germinate within two weeks (Malmgren & Nyström 2011). The seed was counted to have been germinated if the embryo was white and it had swallowed to a ball-shape. Most of these seeds had already produced a tuber-like seedling called a protocorm (Tatarenko 2007). Protocorm is a fairly undifferentiated mass of cells that later on develop to roots and leaves (McKendrick 2000).

The proportion of germinated seeds indicates the seed quality. The proportion of germinated seeds was counted of the seeds having the embryo, in other words the seeds without an embryo were excluded from the total amount of seeds. The proportion of germinated seeds was tested with Paired-Samples T-Test on SPSS/PASW statistics. If there were contaminated media, those were discarded and the experiment was repeated to those pairs or groups by sowing new seed sets on a new culture medium.

4. RESULTS

In the pollinaria removal experiment, there was no difference in relative capsule production between treatment and control plants ($t = 0.637$, $df = 16$, $P = 0.533$) (Fig. 1). Instead, the relative seed capsule production was significantly higher in treatment control plants (t-control) than in control plants ($t = 2.351$, $df = 8$, $P = 0.047$) (Fig. 1). There were no significant differences in the proportion of embryonic seeds between different treatments (Treatment – Control $t = 0.440$, $df = 16$, $P = 0.666$; Control – T-Control $t = 1.443$, $df = 8$, $P = 0.187$) (Fig. 1). The proportion of successfully germinated seeds did not differ between treatment and control plants (mean = 4.88, SD = 18.65, $t = 0.827$, $df = 9$, $P = 0.430$).

There were significant differences in the proportion of embryonic seeds between different hand-pollination treatments (Friedman: $\chi^2 = 13.0$, $df = 2$, $P = 0.002$) (Fig. 2). The proportion of embryonic seeds was highest in flowers pollinated with cross-pollen and lowest in those pollinated with autogamous self-pollen (Fig. 2). Differences in the proportion of embryonic seed production were significant between cross-pollen and self-pollen (cross-pollen – geitonogamy $Z = -2.73$, $P = 0.006$; cross-pollen – autogamy $Z = -3.04$, $P = 0.002$), and there was also a trend that the proportion of embryonic seeds was lower in autogamous fertilization compared to geitonogamy ($Z = -1.85$, $P = 0.064$).

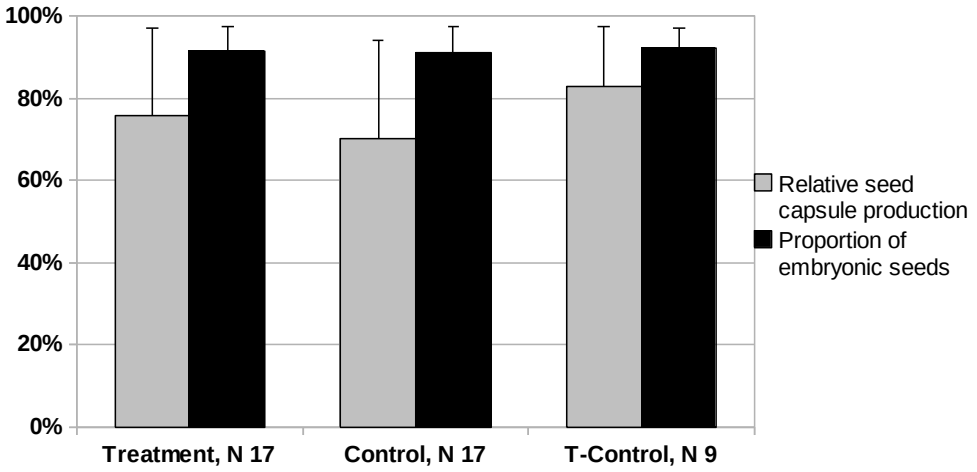

Fig. 1. Relative seed capsule production and proportion of embryonic seeds (mean + SD) by treatment in pollinaria removal experiment. Treatment = pollinaria removed, Control = pollinaria not removed but the inflorescence treated by touching, T-Control = inflorescence not treated in any way.

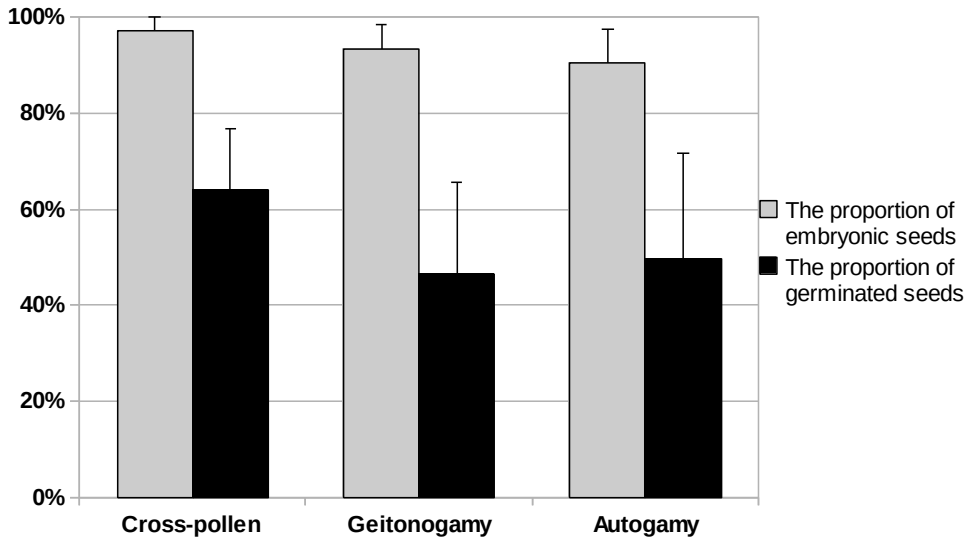

Fig. 2. The proportion of embryonic seeds and the proportion of germinated seeds (mean + SD) by treatment in hand-pollination experiment. Cross-pollen, geitonogamy and autogamy indicate the source of the pollen.

In hand-pollination experiment, the proportion of successfully germinated seeds of the cross-pollinated plants was higher than that of geitonogamous plants ($t = 3.533$, $df = 7$, $P = 0.010$) and autogamous plants ($t = 3.974$, $df = 6$, $P = 0.007$) (Fig. 2). No difference was found in the proportion of successfully germinated seeds between geitonogamous and autogamous plants ($t = -0.092$, $df = 6$, $P = 0.930$).

The time before the pollinarium had undergone its full angle of movement varied between 33.97 s and 89.10 s (mean=53.32, SD=13.08). The bending speed and direction differed and thus the margin of error might be a few seconds. The bending started when approximately 2/3 of the total bending time was elapsed.

The insects collected with window traps and colour traps did not have pollinaria adhesions. A few insects captured with net bags did carry pollinaria. From these the individuals of *Anoplodera sanguinolenta* (Coleoptera: Cerambycidae) were regarded as

certain pollinators. These beetles had accumulated large clumps of pollinaria on their head, forming large masses of up to approximately 30 pollinaria (Fig. 3).

Fig. 3. *A. sanguinolenta* can carry substantial loads of pollinaria on their head. This individual has 16 pollinaria adhesions. (Photo: J. Haimi)

5. DISCUSSION

The male sterilization treatment did not affect the relative seed capsule production, the proportion of embryonic seeds from all seeds or the proportion of successfully germinated seeds. Therefore I can conclude that geitonogamous fertilization does not have a significant role in the natural seed production of *Dactylorhiza maculata*.

The hand-pollination results in this study show that self-pollination as such is a disadvantage to the plant. The seed quality identified by the proportion of embryonic seeds and the proportion of successfully germinated seeds was significantly lower in plants pollinated with self pollen compared to plants pollinated with cross pollen. Because of the disadvantages of self-pollination, mechanisms to prevent it have evolved (de Jong et al 1993; Johnson & Nilsson 1999). Johnson & Nilsson (1999) concluded that at least within their temperate study populations the most important of these mechanisms were extensive pollen carryover and pollinaria bending.

D. maculata has a mixed set of visitors (Dressler 1981; Nilsson 1981). *A. sanguinolenta* (Coleoptera: Cerambycidae) act as pollinators in the study population, though there might be other species involved as well. Nilsson (1981) has reported at least 11 beetle (Coleoptera) species to pollinate *D. maculata*, of which Cerambycidae account for 8 species. Gutowski (1990) has reported Cerambycidae as the main pollinators in a population of a closely-related species, *Dactylorhiza fuchsii.* Beetle pollination occurs on orchids usually in plants that are pollinated by a variety of insects, either within the same population or in other populations of the same species (Steiner et al. 1994; Steiner 1998).

The pollinaria loads collected by *Anoplodera sanguinolenta* were large. High pollinaria loads indicate long sequences of flowers visited, which is typical of a rewarding visit (Dafni 1987). Deceptive inflorescences usually receive shorter visits to one or a few flowers. Therefore I suggest that *D. maculata* is not deceptive in all means, even though nectar is missing. My research does not provide information on what the reward is.

The orchid stigmatic surface is sticky with an exudation of sugars and amino acids that the pollinators might feed on (Grimshaw 2005). At least honeybees have been observed to lick the stigmatic liquid (Dafni 1987). In a closely-related *D. fuchsii* this fluid occurs in very small amounts, but still it has been suggested to act as a reward. Dafni

(1987) claims that *D. maculata* would not have surplus stigmatic liquid. In the deceptive *D. sambucina* (with no stigmatic fluid) the capsules are concentrated in the basal part of the inflorescence (Nilsson 1981). The capsules of *D. fuchsii* (with stigmatic liquid) and *D. maculata* (claimed to be deceptive) are produced over the entire inflorescence. I suggest that *D. maculata* either has some stigmatic fluid (at least in my study population), or the flowers bear some other reward. This suggestion might hold true, as *D. maculata* is not anthecologically an uniform taxon, and beetles are generalist pollinators that feed for example on the floral parts of the flowers they successfully pollinate (Dafni 1987; Bernhardt 2000). The pollinated flowers on the field site appeared undestroyed, which indicates that the pollinators did not carry out heavy harvesting.

Most probably *D. maculata* pollen does not act as a reward. According to Dressler (1981), orchid pollen is very rarely used as food by insects. It has been recorded to be available as food only in the subfamily Apostasioideae, and in Vanilloideae occasional pollen collecting has been observed. (Gregg 1991; Pansarin & Estanislau do Amaral 2008). The orchid pollen is clumped into pollinia and attach to the pollinator, which make it unaccessible to most pollinators (Johnson & Edwards 2000). In the study population, the pollinators tried to get rid of the adhesions and once I encountered a clump of pollinaria on the posterior side of a petal, which indicates that the pollinator had succeeded in removing the cluster from its head. The rubbed-off untouched pollinaria clump supports the hypothesis that *A. sanguinolenta* does not use pollen as alimentation.

The natural seed capsule production of *D. maculata* is high compared to most deceptive species (Vallius & Salonen 2000). I find this as another prove that *D. maculata* might not be deceptive. Deceptive species have low visitation numbers and shorter pollinator stays that usually lead to reduced seed set (Dafni 1987; Neiland & Wilcock 1998). The most successful nectarless orchids with over 50 % capsule sets are usually found to provide an alternative reward, successfully mimic a prey species or provide a sleeping place for the pollinator (Neiland & Wilcock 1998).

Pollinaria bending is an effective mechanism for an orchid to prevent geitonogamy because the pollinators usually leave the inflorescence before the pollinaria are in a correct orientation to reach the stigma (Dressler 1981; Johnson & Nilsson 1999). Johnson & Nilsson (1999) measured pollinaria bending times of 30 s for *Orchis morio* and 40 s for *O. mascula*, both of which are deceptive species. The pollinaria bending time of a nectariferous *Platanthera chlorantha* was 80 s. I measured the pollinaria bending time for *D. maculata* to be ~50 s. This bending time is rather long and falls in between the bending times of deceptive and nectariferous plants. It does not prove that *D. maculata* was a rewarding species, but the long bending time supports the conclusion of effective mechanisms to prevent geitonogamous fertilization.

The relative seed capsule production and the proportion of embryonic seeds from all seeds were slightly higher in treatment plants than in control plants, though no statistical difference was found. This could be due to a fact that the male-sterilized plants were free of pollen, which could have made them more appealing to the pollinators on a harvesting-flight. The pollinators may favour inflorescences with less pollen and have longer probes in them, as the disadvantages of the adhesive pollinaria are not present. The observed trend could also be due to the method of pollinaria removal. Autogamous self-pollination might have taken place while carrying out the treatments, though it is unlikely. Autogamy should decrease the seed quality, but the total amount of fertilized seeds might increase.

The control plants had significantly lower relative seed capsule production and proportion of embryonic seeds from all seeds compared to the treatment-control plants (t-control). This means that the human influence has an effect on the plants' reproductive success. The t-control plants were not treated in the experiment, whereas the control plants

were touched and stem was gently bended the same time as the pollinaria removal treatments were conducted. Different factors may cause the human impact. One of these factors may be that the pollinators avoid these plants due to the smell of human presence. Regardless the mechanism behind the human impact, the impact must be considered when planning experiments.

The most important relapse this study faced was the seed capsule maturation and opening before they were collected from the site. Due to the early opening of some of the seed capsules in experimental plants, I could not weigh the capsules and this way some information was lost. It remains unsolved whether this information had added value to the results. Fortunately the relative seed capsule percentage is a robust method that measures the proportion of flowers with successful pollination. The uppermost capsules may have less embryonic seeds compared to the capsules lower on the stem. This may affect the results of the proportion of embryonic seeds from all seeds. The random sample of seeds, especially when a proportion of the seeds have already been naturally dispersed, may not be a reliable source of information.

The method used in this study should be a reliable method to measure geitonogamous fertilization on *D. maculata*, though the pollinator behaviour (possible preference for pollen-free inflorescences) was not taken in account when designing the experiment. When the pollinaria are removed in the male-sterilization treatment, no geitonogamy may occur in these plants. If geitonogamous fertilization had an importance in *D. maculata* natural seed production, it would have been shown in this experiment.

6. CONCLUSIONS

My experiment proves that geitonogamous fertilization does not have a significant role in *Dactylorhiza maculata* natural seed production, because self-pollination is a disadvantage that reduces the seed-set and seed-quality. Long pollinaria bending time acts as an effective mechanism to prevent geitonogamous fertilization.

A. sanguinolenta (Coleoptera: Cerambycidae) act as pollinators in the study population, and the pollinaria loads are large. High pollinaria loads indicate that the visits are rewarding in some means. The pollinators might prefer rewarding inflorescences without aggravating pollinaria, a phenomenon which does not get statistical support from my experiment, but which may be biologically significant and could be shown if N was higher.

D. maculata is commonly referred to as a deceptive species. If it will be confirmed that the flowers bear a reward, may it be for example stigmatic fluid or floral parts, I would also wish to see a research in which the total number of deceptive orchid species on a global scale is estimated once more. I would as well suggest a new definition to deceptiveness. Way too often nectarless species are referred to as deceptive, though the flowers may serve another reward such as pollen, fragrance or floral parts. Being deceptive to one pollinator species does not mean being deceptive to all the pollinating species. The pollination strategies are more complex than commonly thought.

The most intriguing study topic that my research may have provoked is: What is the reward that *D. maculata* is offering to its pollinators? It would be also nice to know the population size of *A. sanguinolenta* on the field site, as the pollination proves to be successful but the pollinators were encountered only occasionally.

ACKNOWLEDGMENTS

I thank my supervisor E. Vallius for her knowledge and support for the process of this MSc thesis. Together with R. Jokisuu they provided valuable material for my use. Special thanks go to J. Haimi for his skills with insects, T. Ketola for his instructions in the laboratory work, and my dear friend A. Vankka for her peer support. I want to thank Suomen biologian seura Vanamo for the financial support and trust in my topic, and Metsähallitus for the permission to carry out the experiments on Härkösuo. Last but not least, a big hug to my mum, who pushed me to complete this task.

LITERATURE

Bernhardt, P. (2000) Convergent evolution and adaptive radiation of beetle-pollinated angiosperms. Plant Syst. Evol. 222:293-320.

Boyden, T. (1982) The pollination biology of *Calypso bulbosa* var. *americana* (Orchidaceae): Initial deception of bumblebee visitors. Oecologia 55:178-184.

Bruns, T. & Read, D. (2000) *In vitro* germination of nonphotosynthetic, myco-heterotrophic plants stimulated by fungi isolated from the adult plants. New Phytol. 148:335-342.

Chase, M. (2001) The origin and biogeography of Orchidaceae. In: Pridgeon, A., Crinn, P., Chase, M. & Rasmussen, F. (2001) Genera Orchidacearum. Volume 2. Orchidoideae (Part one). Oxford University Press.

Dafni, A. (1987) Pollination in *Orchis* and Related Genera: Evolution from Reward to Deception. In: Arditti, J. (ed) Orchid Biology. Reviews and Perspectives, IV. Cornell University Press.

Darwin, C. (1877) The Different Forms of Flowers on Plants of the Same Species. John Murray, London.

de Jong, T., Waser, N. & Klinkhamer, P. (1993) Geitonogamy: the neglected side of selfing. TREE 8:321-325.

Doetterl, S. & Schaeffler, I. (2007) Flower scent of floral oil-producing *Lysimachia punctata* as attractant for the oil-bee *Macropis fulvipes*. J. Chem. Ecol. 33:441-445.

Dressler, R. (1981) Orchids – natural history and classification. Harvard University Press, Cambridge, Massachusetts.

Endress, P. (1990) Evolution of reproductive structures and functions in primitive angiosperms (Magnoliidae). Mem. N. Y. Bot. Gard. 55:5-34.

Ferdy, J. & Smithson, A. (2002) Geitonogamy in rewarding and unrewarding inflorescences: modelling pollen transfer on actual foraging sequences. Evol. Ecol. 16:155-175.

Foley, M. (2005) Species Accounts – The Orchids of the British Isles. In: Foley, M. & Clarke, S. (2005). Orchids of the British Isles. Griffin Press Publishing Limited.

Geber, M. (1985) The relationship of plant size to self-pollination in *Mertensia ciliata*. Ecology 66:762-772

Gregg, K. (1991) Reproductive Strategy of *Cleistes divaricata* (Orchidaceae). Am. J. Bot. 78: 350-360.

Grimshaw, J. (2005) The orchid plant. In: Foley, M. & Clarke, S. (2005) Orchids of the British isles. Griffin Press Publishing Limited.

Gutowski, J. (1990) Pollination of the Orchid *Dactylorhiza fuchsii* by Longhorn Beetles in Primeval Forests of Notheastern Poland. Biol. Conserv. 51:287-297.

Harder, L.D. & Wilson, W.G. (1998) A clarification of pollen discounting and its joint effects with inbreeding depression on mating system evolution. Am. Nat. 152:684-695.

Hodges, S. (1995) The influence of nectar production on hawkmoth behavior, self pollination, and seed production in *Mirabilis multiflora* (Nyctaginaceae). Am. J. Bot. 82:197-204.

Hämet-Ahti, L., Suominen, J., Ulvinen, T. & Uotila, P. (ed.) (1998) Retkeilykasvio. Luonnontieteellinen keskusmuseo, Kasvimuseo.

Johnson, S. & Edwards,T. (2000) The structure and function of orchid pollinaria. Plant Syst. Evol. 222:243-269.

Johnson, S. & Nilsson, L. (1999) Pollen carryover, geitonogamy, and the evolution of deceptive pollination systems in orchids. Ecology 80:2607-2619.

Johnson, S. & Steiner, K. (2000) Generalization versus specialization in plant pollination systems. Trends Ecol. Evol. 15:140-143.

Koivisto, A.-M., Vallius, E. & Salonen, V. (2002) Pollination and reproductive success of two color variants of a deceptive orchid, *Dactylorhiza maculata* (Orchidaceae). Nord. J. Bot. 22:53-58.

Kuitunen, M. & Kuitunen, T. (1994) Ecological comparison of a threatened and an abundant orchid species (*Dactylorhiza incarnata* and *D. maculata*) in Finland. Aquilo Ser. Botanica 33:63–68.

Lloyd, D. (1979) Some reproductive factors affecting the selection of self-fertilization in plants. Am. Nat. 113:67-79.

Malmgren, S. & Nyström H. (2011) Orchid propagation. http://www.lidaforsgarden.com/Orchids/engelsk.htm Read: 15.1.2011

Mattila, E. (2000) Sources of resources for seed production in *Dactylorhiza maculata* (Orchidaceae). In: Mattila, E. (2000) Factors limiting Reproductive Success in Terrestrial Orchids. Jyväskylä Studies in Biological and Environmental Science 82.

McKendrick, S. (2000) *In vitro* germination of orchids : a manual. Ceiba Foundation for Tropical Conservation.

Neiland, M. & Wilcock, C. (1998) Fruit set, nectar reward, and rarity in the Orchidaceae. Am. J. Bot. 85:1657–1671.

Nilsson, L. (1980) The pollination ecology of *Dactylorhiza sambucina* (Orchidaceae). Bot. Notiser 133:367-385.

Nilsson, L. (1981) Pollination ecology and evolutionary process in six species of orchids. Abstracts of Uppsala Dissertations from the Faculty of Science and Technology 593:1–40.

Nilsson, L. (1983) Anthecology of *Orchis mascula* (Orchidaceae) Nord. J. Bot. 3:157-179.

Nilsson, L. (1992) Orchid pollination biology. TREE 7:255-259.

Pansarin, E. & Estanislau do Amaral, M. (2008) Pollen and nectar as a reward in the basal epidendroid *Psilochilus modestus* (Orchidaceae: Triphoreae): A study of floral morphology, reproductive biology and pollination strategy. Flora 203:474-483.

Peakall, R. & Beattie, A. (1996) Ecological and genetic consequences of pollination by sexual deception in the orchid *Caladenia tentactulata*. Evolution 50:2207-2220.

Pemberton, R. & Liu, H. (2008) Naturalized orchid bee pollinates resin-reward flowers in Florida: Novel and known mutualisms. Biotropica 40:714-718.

Peter, C. & Johnson, S. (2009) Reproductive biology of *Acrolophia cochlearis* (Orchidaceae): estimating rates of cross-pollination in epidendroid orchids. Ann. Bot. 104:573–581.

Phyto Technology Laboratories LLC (2008) Technical Support Information, Orchid Seed Germination.

Salonen, V. (2006) Kasviekologia. Millaista on luonnonkasvien elämä? WSOY.

Schmid, R. & Schmid, M. (1977) Fossil History of the Orchidaceae. In: Arditti, J. (ed.) (1977) Orchid Biology. Reviews and perspectives, I. Cornell University Press.

Steiner, K. (1998) The Evolution of Beetle Pollination in a South African Orchid. Am. J. Bot. 85: 1180-1193.

Steiner, K., Whitehead, V. & Johnson, S. (1994) Floral and Pollinator Divergence in Two Sexually Deceptive South African Orchids. Am. J. Bot. 81: 185-194.

Takebayashi, N. & Delphi, L. (2000) An association between a floral trait and inbreeding depression. Evolution 54:840-846.

Tatarenko, I. (2007) Growth Habits of Temperate Terrestrial Orchids. In: Cameron, K., Arditti, J. & Kull, T. (ed.) (2007) Orchid biology. Reviews and Perspectives, IX. The New York Botanical Garden Press.

Vallius, E. (2000) Position-dependent reproductive success of flowers in *Dactylorhiza maculata* (Orchidaceae*)* Funct. EcoI. 14:573-579.

Vallius, E. (2001) Factors affecting fruit and seed production in *Dactylorhiza maculata* (Orchidaceae). Bot. J. Linn. Soc. 135:89-95.

Vallius, E. & Salonen, V. (2000) Effects of defoliation on male and female reproductive traits of a perennial orchid, *Dactylorhiza maculata*. Funct. Ecol. 14:668–674.

van der Cingel, N. (2007) Pollination of Orchids by Lepidoptera: Outcrossing by Long Distance Transport. In: Cameron, K., Arditti, J. & Kull, T. (ed.) (2007) Orchid biology. Reviews and Perspectives, IX. The New York Botanical Garden Press.

Waser, N. & Price, M. (1989) Optimal Outcrossing in Ipomopsis aggregata: Seed Set and Offspring Fitness. Evolution 43:1097-1109.