

Outcrossing, hybridization, pollen quantity, and the evolution of deceptive pollination in *Dactylorhiza incarnata*

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We conducted two hand-pollination experiments in the nectarless orchid *Dactylorhiza incarnata* to test two hypotheses explaining the evolution of deceptive pollination in orchids. In the first experiment testing the “outcrossing” hypothesis, we pollinated flowers with pollinia from different crossing distances, and with interspecific pollinia (*Dactylorhiza maculata*). In the second experiment testing the “pollinia” hypothesis, we used only conspecific pollinia, but varied the number of pollinia received per stigma. Pollen source (self-pollination, cross-pollination within population, cross-pollination between populations, and interspecific pollination) did not affect female reproductive success of flowers. Increase in the number of pollinia received per stigma had a positive effect on reproductive success. Thus, a plant could benefit from repeated pollinator visits. Our results suggest that pollinator visitation rates may be more important for reproductive success than the pollinia source, and that neither the “outcrossing” nor the “pollinia” hypothesis was clearly supported.

Key words: crossing distance, deceptive pollination, orchids, pollination biology, pollinia, reproductive success

Introduction

Orchids are famous for their variable ways of rewarding but also deceiving their pollinators. The deceptive orchids exploit pollinator foraging behaviour by attracting insects with a variety of dummy signals, and nectarless or pollenless structures (Dafni 1984, Ackerman 1986). Orchids deceive their pollinators by manipulating several pollinator behaviours associated with

territorial defence, sexual response, brood-site selection and, most commonly, food-foraging (Little 1983, Ackerman 1986, Dafni & Bernhardt 1990, Nilsson 1992).

How this deceptive pollination system has evolved is enigmatic, and there are several hypotheses. All of these hypotheses agree that the lack of pollinator reward reduces pollinator activity and that the reduction in the pollinator visitation rate must be outweighed by other

advantages (Ackerman & Montalvo 1990, Nilsson 1992). The most common hypothesis is that deceptive orchids rely on naive pollinators whose visits are sufficient to pollination (Little 1983, Ackerman 1986). In this paper we address two additional hypotheses: the “outcrossing” hypothesis and the “pollinia” hypothesis.

The “outcrossing” hypothesis for the evolution of deceptive pollination proposes that greater outcrossing is achieved by longer flight distances of deceived pollinators between successively visited flowers (Gill 1989, Nilsson 1992, Peakall & Beattie 1996). This situation is analogous for species producing nectar, in which the flight distances of pollinators are longer after visiting a plant with low nectar reward (e.g. Pyke 1984, Marden 1984). The “outcrossing” hypothesis emphasizes the importance of pollen source and genetic quality for reproductive success. The hypothesis is based on the idea that inbreeding depression is always occurring either through selfing or through biparental inbreeding, and it ignores the possibility of outbreeding depression over the range of flight distances of deceived pollinators. The hypothesis also assumes that genetic distance between plants is related to physical distance (Sobrevila 1988, Waser & Price 1993).

In the deceptive orchid species, *Dactylorhiza incarnata*, crossing distance is related to hybridization as well. Populations of *D. incarnata* are usually located in small, open and mesotrophic mires surrounded by herb-rich swamp woods where also the closely related nectarless species, *D. maculata*, occurs (Lammi & Kuitunen 1995). These species have similar floral display and there is often a hybrid zone around open mires in which hybrids and plants from both species coexist. Thus, hybridization could threaten the less abundant species through the process of genetic assimilation, despite the positive aspects of hybridization such as adaptation to new environments (e.g. Rieseberg 1995). Actually, hybridization should be likely to occur, because orchids in general (Gill 1989) and the genus *Dactylorhiza* in particular are well-known for hybridization (e.g. Heslop-Harrison 1968, Dafni & Bernhardt 1990). Even species having different chromosome numbers may hybridize (Gill 1989, Hedrén 1996a). Terms like “population complex” and “evolutionary unit” (Nilsson 1981a) and “habitat

racess” (Heslop-Harrison 1968) have been used to describe this situation in orchids. Thus, the benefit of greater crossing distance could be lost if the probability of hybridization increases with increasing crossing distance.

The evolution of deceptive pollination has also been explained by the “pollinia” hypothesis, which proposes that pollinia (the packaging of pollen into a few compact units) allow such a great pollination success from single visits that no pollinator reward is needed (Nilsson 1992). In orchids, the pollinia are dispensed intact or as subunits onto the stigma of flowers, an “all-or-nothing” event that can lead to the production of numerous tiny seeds (Nilsson 1992). The “pollinia” hypothesis assumes absence of pollinator limitation i.e. hand-pollinations should not increase fruit set as compared with natural pollination levels. Thus, even one fruit with numerous seeds could assure high fitness for the plant. However, in deceptive orchids hand-pollination does usually increase fruit set substantially compared to natural levels (e.g. Nilsson 1992, Mattila & Kuitunen 2000). The hypothesis also assumes that the amount of pollen received per stigma does not increase seed or fruit set, because one pollinium per stigma can sire all the seeds. In other words, the plant is not supposed to be dependent on repeated pollinator visits for reproductive success.

The aim of this study is to determine the validity of the “outcrossing” and the “pollinia” hypotheses in the nectarless and self-compatible orchid, *D. incarnata*, based on the early phases of the life cycle. However, one should notice that later in the life cycle different patterns may emerge. Other hypotheses explaining the evolution of deceptive pollination are based on frequency dependence or density-attractiveness interactions (Ackerman 1986, Dafni & Bernhardt 1990, Peakall & Beattie 1996, Smithson & Macnair 1997) and on the tendency to save long-term costs of reproduction by giving up nectar production (e.g. Nilsson 1992, Golubov et al. 1999) are not considered in this study. We addressed the following specific questions: (1) Do self- and outcross pollen yield different female reproductive success (as assumed in the “outcrossing” hypothesis)? (2) Does increasing crossing distance increase female reproduc-

tive success (as assumed in the “outcrossing” hypothesis)? (3) Do intra- and interspecific pollination lead to equivalent seed and fruit set? (4) Does female reproductive success remain constant as the number of pollinia received per stigma increases (as assumed in the “pollinia” hypothesis)?

Materials and methods

The study species, *Dactylorhiza incarnata*, has a disjunct distribution in northern Europe extending up to the 70° latitude (Hultén 1971). In Finland it is an endangered species, often occurring in small and patchy populations (Rassi *et al.* 1992). Each plant produces on average fifteen bright pink, nectarless (Lammi & Kuitunen 1995), and scentless (Nilsson 1981a) flowers, borne in a spike. Each flower has two pollinia. The species is self-compatible, but spontaneous autogamy is absent or very rare in nature (Lammi & Kuitunen 1995). Fruit set is typically 20%–50% in natural populations and the species is pollinator limited within a season, because after hand-pollination fruit set may rise to 100% (Lammi 1991, Mattila & Kuitunen 2000). In pollination, *D. incarnata* exploits short visits by inexperienced or unconditioned bumble bee workers (Nilsson 1981a, Fritz & Nilsson 1994), which find their first food-flowers solely by optical cues (Kugler 1935) and attractive floral display is needed to achieve a good pollination success. Deception occurs throughout anthesis, because fresh bumble bee workers emerge from their nests during the summer (Nilsson 1980).

We studied the “outcrossing” hypothesis and hybridization in June 1994 at the Katajaneva mire, situated in Toivakka, central Finland (62°30'N, 26°12'E). In the year of study, the number of flowering *D. incarnata* individuals at Katajaneva was approximately 200; since 1989 the number of flowering plants has varied between 100 and 200 individuals (A. Lammi, unpubl. data). *Dactylorhiza maculata* occurs in herb-rich swamp woods surrounding Katajaneva mire approximately 50–400 m away from *D. incarnata* and it is much more abundant than *D. incarnata*. The “pollinia” hypothesis was studied in June 1993 at the Paanasenneva mire

situated in Pihtipudas, central Finland (63°31'N, 25°32'E). The number of flowering plants at Paanasenneva in 1993 was 150. In both areas *D. incarnata* occurs in mesotrophic sedge fens or *Sphagnum papillosum* fens. The vegetation consisted mainly of *Carex lasiocarpa*, *C. rostrata*, *Menyanthes trifoliata*, *Scheuchzeria palustris*, *Drosera rotundifolia*, *D. anglica*, *Vaccinium oxycoccos*, and *Andromeda polifolia*. However, during the flowering of *D. incarnata*, pollinator populations are sustained by other flowering species in adjacent habitats. Both study areas were relatively homogenous in light and moisture conditions.

In the first hand-pollination experiment, testing the “outcrossing” hypothesis and hybridization, we pollinated the experimental flowers with an equal amount of pollen (two pollinia) from different sources: self-pollination (within a plant), outcrossing (20 m, within population), outcrossing (16 km, between populations), and interspecific pollination (*D. maculata*, occurring near the study population). In these nectarless orchids, pollinators usually remove two pollinia from a flower on a single visit (e.g. Fritz & Nilsson 1994), and therefore two pollinia were used for the pollination of an individual flower. All four pollination treatments were performed within an individual plant (18 experimental plants). In each inflorescence, two or three random flowers were pollinated with the pollen from the same source. Altogether, about 60% of the flowers on each plant were hand-pollinated to elicit reproductive effort on the maternal plants equally. During the two hour transfer from the other population (Leivonmäki, Haapasuo 61°52'N, 26°40'E, population size 30 plants), the pollinia were kept in a shaded box to avoid damage by the sun. In each treatment, the storage time between pollinia-collecting and hand-pollinations was similar. We covered the inflorescences with nylon bags to exclude pollinators before and during flowering. We used a toothpick to withdraw a pollinium and to place it on the stigma of an experimental flower marked individually by a small plastic ring. Only newly opened flowers were used as pollinia sources and recipients. At the same time we removed pollinia of receipt flowers to exclude any possibilities for spontaneous autogamy.

In the second hand-pollination experiment, testing the “pollinia” hypothesis, we studied the effect of additional pollinia received per plant. Results of the same experimental treatment were gained from the first experiment as well. We also studied the effect of number of pollinia received per stigma (a half, two or three pollinia) on reproductive success. In several nectarless orchid species, pollinators usually remove two pollinia per flower (e.g. Fritz & Nilsson 1994) and we used that treatment to compare natural and artificial pollination success. The scale of pollinia added per stigma is biologically relevant since *Dactylorhiza* pollinia may break apart while attached to pollinators (Nilsson 1980, Johnson & Nilsson 1999). We took pollinia from *D. incarnata* individuals situated about five meters away from the experimental plants. In the first treatment, one pollinium was gently cut into half by pressing on it with a sharp toothpick. In this experiment, we did only one type of treatment for each experimental plant (ten plants per treatment). In each inflorescence we pollinated 4–11 random flowers with the same number of pollinia. The treatments were randomized over maternal plants of different size and we observed no difference in the viability of the maternal plants among the pollination-treatment groups. About 60% of the flowers on each plant were hand-pollinated to elicit reproductive effort on the maternal plants equally. We covered the experimental inflorescences with nylon bags before and after the treatment to exclude pollinators. Supplementary hand-pollinations were carried out as described earlier.

In the first experiment, we estimated female reproductive success as: (1) relative fruit set, (2) seed set per fruit (dimensions of a capsule), and (3) proportion of seeds with well-developed

embryo. In the second experiment, seed set per fruit was not estimated. In both experiments, relative fruit set was measured as the proportion of experimental flowers producing fruits per plant in each treatment. We measured fruit set 6–8 weeks after the end of flowering, when the fruits were fully swollen. Because of the enormous number of seeds per fruit the seed set was not counted, but instead the length and width of a fruit, which are correlated to seed set, were used as an estimate of seed set. To estimate the proportion of well-developed seeds, a random sample of approximately 100 seeds from each fruit was placed on a microscope slide and examined with 120× magnification. We counted the proportion of seeds containing well-developed embryos (1/3 the width of the testa) to estimate seed fertility in both experiments (Nilsson 1981b). In the first experiment, we analysed the data of seed set and production of seeds with well-developed embryos by using means of two or three fruits per plant (18 plants) to test differences among treatments. In the second experiment, we analysed the data of seeds with well-developed embryos by using means of 4–11 fruits per plant (ten plants) to test differences among treatments. In outcrossing species, like orchids, inbreeding and outbreeding depression can be expressed early in the life cycle e.g. during embryo development (Husband & Schemske 1996), and consequently early life stages could be used as a rather valid measure of reproductive success.

Statistical analyses were performed with the computer program SPSS for Windows (Norusis 1992). If the assumptions of parametric tests were not met, we applied non-parametric tests e.g. Friedman test in the first experiment. In the second experiment we used two-way ANOVA to

Table 1. Relative fruit set, mean length and width of fruits (mm), and proportion of seeds with well-developed embryo (means with SD) in *Dactylorhiza incarnata* plants following hand-pollination with pollinia from different sources. *N* is the number of experimental plants.

Pollen source:	<i>N</i>	Fruit set (%)		Caps. length		Caps. width		Embr. seeds (%)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Within plant	18	95.39	13.72	15.35	1.31	4.03	3.2	57.92	11.15
Outcross (20 m)	18	95.39	13.72	15.61	1.49	4.14	3.2	64.36	11.45
Outcross (16 km)	18	94.44	16.17	15.66	1.45	3.96	2.9	59.11	10.17
<i>D. maculata</i>	18	92.59	17.25	15.75	1.56	4.14	2.2	59.27	10.25

test the effect of additional pollinia on reproductive success and maternal plants of similar size were used as a block.

Results

The first experiment tested the “outcrossing” hypothesis and the effect of hybridization on female reproductive success. Pollinia source did not affect fruit set (Friedman ANOVA, $\chi^2 = 0.10$, $df = 3$, $p = 0.992$), seed set measured as fruit width and length (width: $\chi^2 = 3.08$, $df = 3$, $p = 0.379$, length: $\chi^2 = 5.92$, $df = 3$, $p = 0.116$) or proportion of seeds with well-developed embryos ($\chi^2 = 4.55$, $df = 3$, $p = 0.208$) (Table 1).

Plants used in the second experiment were equal in size. The number of flowers (Kruskal-Wallis test, $H = 1.84$, $df = 2$, $p = 0.397$), height of individual plants ($H = 4.11$, $df = 2$, $p = 0.128$) and length of inflorescence ($H = 3.16$, $df = 2$, $p = 0.206$) did not vary significantly among the treatment groups. The number of pollinia received per stigma affected the proportion of flowers setting fruit (two-way ANOVA, treatment: $F = 3.94$, $df = 2$, $p = 0.038$, block: $F = 1.02$, $df = 9$, $p = 0.462$) and proportion of seeds with well developed embryo (treatment: $F = 4.62$, $df = 2$, $p = 0.024$, block: $F = 0.67$, $df = 9$, $p = 0.726$) (Fig. 1). Increase in the number of pollinia used in pollination had a positive effect on reproductive success. The significant results in fruit set and proportion of seeds with well-developed embryo were due to differences between treatments using a half pollinium and three pollinia (Tukey test, $p < 0.05$). There were no differences in reproductive success measured as fruit set and proportion of seeds with well-developed embryo between treatments using a half pollinium and two pollinia or between two and three pollinia used (Tukey test, $p > 0.05$).

Discussion

The “outcrossing” hypothesis

Avoidance of selfing is commonly accepted as an explanation for the evolution of deceptive pollination (Nilsson 1992). However, our results of

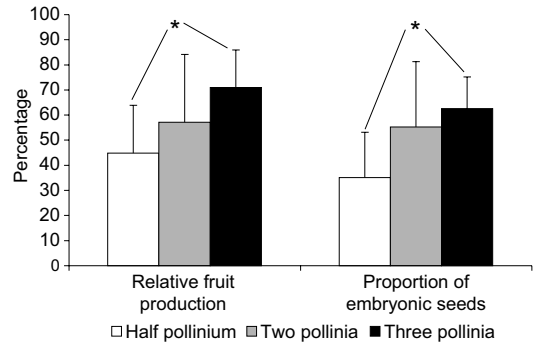


Fig. 1. Relative fruit set and the proportion of seeds with well-developed embryo (means with standard deviations) in *Dactylorhiza incarnata* following pollinations with different number of pollinia per stigma. A half, two or three pollinia per stigma were used for pollination of ten experimental plants per treatment. Means marked with * differ significantly from each other ($p < 0.05$).

the early phases of the life cycle did not clearly support the idea, because there were no differences in reproductive success between the outcrossed and the selfed plants. Actually, a review of hand-pollination experiments conducted with entomophilous, self-compatible and nectarless orchids showed that only three species (*Dactylorhiza sambucina*, *Orchis mascula*, *O. spitzelii*) studied had reduced fruit or seed set after artificial selfing (Nilsson 1980, 1983, Fritz 1990). In most orchid species, there were practically no differences in reproductive success between selfed and cross-pollinated plants (e.g. Firmage & Cole 1988, Ackerman & Montalvo 1990, Fritz & Nilsson 1995). Johnson and Nilsson (1999) found also no evidence for the “outcrossing” hypothesis. Despite the added nectar and consequently increased pollinator activity, the pollinators left the plants before the pollinia had enough time to bend to a position which would have caused geitonogamy. Thus, there seems to be little evidence to date for the benefit of outcrossing compared to selfing, although this benefit is commonly invoked (Gill 1989). Negative effects of self-pollination may, however, be seen after seed germination, as found in the orchid, *Caladenia tentaculata* (Peakall & Beattie 1996).

We were also unable to detect inbreeding depression within and outbreeding depression within or between populations in relation to crossing distances. Inbreeding depression was defined

as the reduction in fitness of progeny derived from inbreeding relative to those derived from outcrossing. Mehrhoff (1983) found also no clear difference in reproductive success in the terrestrial, nectarless orchid *Isotria verticillata*, as he artificially moved pollen between clones in the same population and between separate populations.

Evidently the “outcrossing” hypothesis may have limited application, because genetic structure may vary markedly among populations (e.g. Waser & Price 1983). In our study, it is possible that the purging of lethal recessive alleles could have removed the negative effects of inbreeding (Barrett & Charlesworth 1991, Byers & Waller 1999). Our study populations have most likely been isolated for centuries, because suitable mesotrophic mires have always been scarce and patchily distributed in the area. Moreover, during this century peatland drainage has destroyed 67% of the populations (Välivaara et al. 1991). Swedish populations of *D. incarnata* from a similar-sized geographical area show no genetic variation at seven enzymes screened for electrophoresis (Hedrén 1996a, 1996b). This suggests that populations may be highly isolated, inbreeding and purging may be possible, and therefore selfing may not reduce female reproductive success. Thus, the benefits of cross-pollen and longer crossing distances may not be self-evident in deceptive orchids.

Hybridization

Our results suggest that hybridization may commonly occur between *D. incarnata* and *D. maculata*, at least when *D. incarnata* is used as maternal plant. However, this conclusion is based on results concerning only the early phases of life cycle (see Waser & Price 1994). Consequently, increased crossing distance could be associated with hybridization which could counteract the assumed benefit of increased crossing distance. Flowering time, habitat (Lammi & Kuitunen 1995), and most likely pollinator fauna may not provide mechanisms for reproductive isolation. However, pre-pollination mechanisms such as floral morphology, known to prevent hybridization in other orchids (Gill 1989, Dafni & Bernhardt 1990), were not studied here.

The harmful consequences of hybridization include demographic effects, genetic assimilation of a rare taxon by a numerically larger one, loss of locally adapted populations, and outbreeding depression (e.g. Rieseberg 1991, Levin et al. 1996). Genetic and demographic processes may interact at the expense of pure species, affecting more severely the less abundant species (e.g. Levin et al. 1996), in this case *D. incarnata*. The process of genetic assimilation is reinforced, if hybrids are more vigorous and superior in competition (Rieseberg 1995, Levin et al. 1996, Burke et al. 1998). Our results indicate that *D. incarnata* produced seeds also after interspecific pollination. This suggests that genetic assimilation between species could be common and effective in the mixed populations of *D. incarnata* and *D. maculata*, as observed by Wang and Cruzan (1998) in *Piriqueta caroliniana* and *P. viridis*. Based on our morphological comparisons, there are numerous individuals in the populations which appear to be hybrids.

However, hybridization may not be completely negative for the species. The benefit of hybridization could come from increased viability after heterosis and adaptation to new environments (e.g. Rieseberg 1991, Levin et al. 1996). In the case of deceptive orchids, hybrids may gain more pollinator visits, because they increase morphological variation within a population, and could prolong the learning process of pollinators (Ackerman 1981).

Pollinator limitation and pollen quantity

Most deceptive orchids are pollinator-limited with low natural fruit set, and after additional hand-pollinations fruit set may rise to 100% (e.g. Nilsson 1992). Consequently, repeated pollinator visits should give higher total reproductive success. Fruit sets up to 100% may be achieved in *D. incarnata* after hand-pollination (Lammi 1991, Mattila & Kuitunen 2000) and fruit set is typically 20%–50% in natural populations (Lammi & Kuitunen 1995). The current reproductive success might partly be determined by the previous reproductive effort of the individuals (Ackerman & Montalvo 1990, Mattila & Kuitunen 2000). If so, pollinator limitation and

the “pollinia” hypothesis could only be verified by a long-term study monitoring the balance between reproductive effort and fitness of individuals during the life time (see Waser & Price 1994), but this is very difficult to accomplish in orchids (Calvo & Horvitz 1990).

At the flower level, increasing the number of pollinia received per stigma had a positive effect on reproductive success in *D. incarnata*, suggesting that the quantity of pollen received per flower is important. Usually the seed production of an angiosperm flower depends on the quantity of pollen reaching its stigma (e.g. Niesenbaum 1999). Pollinium of *Dactylorhiza* may fall into pieces especially while the pollinator tries to scrape it off, which further enhances possibilities for multiple pollinations (Nilsson 1980). Previously, Proctor and Harder (1994) also demonstrated in the nectarless *Calypso bulbosa* that increase in the number of pollinia received per flower enhanced seed set. However, the number of pollinia received per flower did not affect fruit or seed set in epiphytic orchids (Ackerman & Montalvo 1990). Thus, the importance of pollen quantity received per flower seems to vary among deceptive species.

Based on the early phases of the life cycle, we conclude that the “outcrossing” hypothesis may have limited application, because populations may differ in their genetic structure. At the same time hybridization may complicate reproduction. The importance of pollen quantity seems to vary among species and possibly even among populations. These results could give a new prospect to understand the evolution of deceptive pollination and reproductive success in orchids and show important hypotheses to be tested in the future. Moreover, in order to address the validity of different hypotheses explaining the evolution of deceptive pollination, we especially need long-term experiments (e.g. Waser & Price 1994).

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