UPPSALA
UNIVERSITET

Population size, pollination intensity and reproductive success in the deceptive bumble-bee pollinated orchid *Dactylorhiza incarnata*

Sofia Larsson

Avdelningen för växtekologi
Evolutionsbiologiskt centrum
Uppsala universitet
Villavägen 14
752 36 Uppsala

Population size, pollination intensity and reproductive success in the deceptive bumble-bee pollinated orchid Dactylorhiza incarnata

Abstract

The reproductive success experienced by an animal-pollinated plant may be influenced by population characteristics such as density, size and degree of isolation. In deceptive species the reproductive success of individual plants has been suggested to decrease with increasing population size. If a pollinator learns to avoid a deceptive species after some visits, plants in large populations may become less frequently visited and have lower reproductive success than plants in small populations. To examine the correlation between reproductive success and population size and density, I quantified pollinia removal, pollen receipt and female reproductive success in 11 populations of the deceptive orchid species *Dactylorhiza incarnata* on the island of Öland. Supplemental hand-pollination was performed to test for variation in pollen limitation among populations. I further examined whether the reproductive success of the deceptive orchid was influenced by the local abundance of reward-producing plants. Three subspecies of *D. incarnata* occurs on Öland: *D. incarnata* ssp. *incarnata, D. incarnata* ssp. *cruenta* and *D. incarnata* ssp. *ochroléuca. D. incarnata* ssp. *incarnata* and ssp. *cruenta* have red flowers and occur more frequently than *D. incarnata* ssp. *ochroléuca,* whose flowers are whitish-yellowish. Rare morphs of deceptive species without mimicry may experience a selective advantage caused by the behaviour of the pollinator. The subspecies differed in reproductive success, but *D. incarnata* ssp. *incarnata* consistently had higher reproductive success than the rare *D. incarnata* ssp. *ochroléuca.* The density of reward-producing plants was low and did not correlate with the reproductive success of *D. incarnate.* The number of pollinia removed per flower was negatively correlated with population size, and the proportion of flowers that received pollen tended to be negatively correlated to population size. Pollen limitation, fruit and seed production varied among populations, but were not related to population size or density. The results suggest that the risk of pollination failure may increase with increasing population size, but that that variation in resource availability and other factors considerably influence among-population differences in pollen limitation and fruit output in the deceptive *D. incarnata.*

Introduction

The pollinator visitation rate experienced by an animal-pollinated plant may be influenced by the density, total size and degree of isolation of the population (Ågren 1996, Kéry et al.. 2000). In reward-producing species a smaller population size may reduce the reproductive success (Ågren 1996) and the attractiveness to pollinators (Sih & Baltus1987), whereas in deceptive species the reproductive success of individual plants has been suggested to decrease with increasing population size (Fritz & Nilsson 1994). If a pollinator learns to avoid deceptive species after some visits, plants in a large population may be less frequently visited than plants in a small population (Alexandersson & Ågren 1996).

In addition the pollinator visitation rate of a deceptive species may be affected by the composition of the surrounding reward-producing flowering species (Johnson et al. 2003). Pollination success of neighbouring plants may be diminished by competition, if the reward-producing plants attract the pollinator away from the deceptive species (Lammi & Kuitunen 1995), and by reproductive interference i.e. negative effects of receipt of heterospecific pollen or export of pollen to a heterospecific stigma (Johnson et al. 2003). In some studies, reward-producing species have been shown to increase the local abundance of pollinators and enhance the pollination success of deceptive neighbouring plants (Laverty 1992, Johnson et al. 2003).

Colour polymorphism in a species may be retained by a balance between mutation and selection, heterozygote advantage or negative frequency dependent selection (Smithson & Macnair 1997). Rare morphs of deceptive species without mimicry may experience a selective advantage caused by the behaviour of the pollinator. In a deceptive species with dissimilar frequency of morphs, the rare morph will be proportionally more visited than the common, if the pollinators show a tendency to visit dissimilar morphs sequentially (Smithson & Macnair 1997). Bumblebees have been found to switch from a common morph preference when the flowers are rewarding to rare morph preference when the flowers are deceptive (Smithson & Macnair 1997). Negative frequency-dependent selection apparently contributes to the maintenance of the flower colour polymorphism in *Dactylorhiza sambucina,* which is a deceptive orchid with two colour morphs, red and yellow. It has been found that the male and female reproductive success of the yellow morph was negatively correlated to the frequency of the morph in the local populations (Gigord et al. 2001).

Floral display has been found to influence the reproductive success of reward-producing species (Mitchell 1994, Brody & Mitchell 1997, Maad 2000, Ehlers et al.. 2002, Maad & Alexandersson 2004). In some studies, flower number was positively correlated to the pollinator visitation rate (Mitchell 1994, Brody & Mitchell 1997), whereas in other studies no correlation between flower number of a plant and pollinator visitation rate were found, instead the pollinator visitation rate was positively correlated to the total number of open flowers in the population (Thompson 2001). A larger number of flowers per inflorescence may increase the total fruit production of a plant (Mitchell 1994, Brody & Mitchell 1997, Maad 2000), and have an impact on male reproductive success (Brody & Mitchell 1997, Ehlers et al. 2002, Maad & Alexandersson 2004). Moreover, plants with large floral displays are more likely to be visited first, and the first visited flowers in a foraging sequence may have a higher probability of receiving cross pollen from more distant neighbours and a large number of mates (Brody & Mitchell 1997).

Deception in many orchid species may reduce geitonogamy i.e. self pollination between flowers, by causing fewer flowers on an inflorescence to be probed by each pollinator.

3

Another mechanism found to reduce the geitonogamy is the pollinarium bending. After attached to the pollinator, pollinaria of many orchid species undergo a bending movement, which results in a time delay before the pollinium assumes a position from which it can strike a stigma. The time delay may be enough to allow the pollinator to leave the inflorescence before the pollinium has bent into position (Johnson et al. 2004).

In this study, I investigate population and plant characteristics that may influence the reproductive success of the deceptive self-compatible orchid *Dactylorhiza incarnata*. *Dactylorhiza incarnata* occurs in two colour morphs, *D. incarnata* ssp. *incarnata* and *D. incarnata* ssp. *cruenta* (red) and *D. incarnata* ssp. *ochroléuca* (white/yellow). *D. incarnata* is found in rich fens where nectar-producing species are scarce. An experimental study by Lammi & Kuitunen (1995) indicated interspecific exploitation competition in populations of *D. incarnata*. The reproductive success of the deceptive orchid was negatively affected by the introduction of a reward-producing *Viola* cultivar. Positive effects of surrounding reward-producing species may be associated with certain reward-producing species. Similarity in the shape and colour of flowers between reward-producing and deceptive species increase the probability that the pollinator temporally will switch from reward-producing to a deceptive plant (Johnson et al. 2003). On the Island of Öland, *D. incarnata* is found in habitats containing reward-producing species, which may act as magnet species and increase the local abundance of pollinators.

The aims of this study were 1) to examine whether the reproductive success of *D. incarnata* is related to population size and/or density 2) to determine whether the reproductive success is enhanced or diminished by the presence of reward-producing plants 3) to estimate whether the relative reproductive success of the two colour morphs is related to population size and/or the frequency of *D. incarnata* ssp. *ochroléuca* 4) to examine whether the floral display affects the reproductive success 5) to investigate whether the degree of pollen limitation is related to population size or morph frequency and 6) to test whether it is possible to track pollen dispersal in *D. incarnata* by staining pollinia.

Study species

Dactylorhiza incarnata is a perennial herb found in wet meadows and fens in calcareous areas. In Sweden, the plant has a flowering period from early June to mid July. The pollen of *D. incarnata* is packed in two pollinia per flower, which attach to the pollinator by a sticky viscidium. There is large intraspecific variation in morphology in *D. incarnata*, and three subspecies are recognised; *D. incarnata* ssp. *incarnata*, *D. incarnata* ssp. *cruenta* and *D. incarnata* ssp. *ochroléuca*. *D. incarnata* ssp. *ochroléuca* often occur together with *D. incarnata* ssp. *cruenta*. The flower of *D. incarnata* ssp. *ochroléuca* is whitish-yellowish (Sterner 1986). *D. incarnata* ssp. *incarnata* occurs in a range of rich fen habitats, *D. incarnata* ssp. *cruenta* and *D. incarnata* ssp. *ochroléuca* are restricted to calcareous fens (Sterner 1986 and Hedrén et al. 2001).

An explanation for the intraspecific differentiation in the species may be that the subspecies are adapted to particular habitats. *D. incarnata* ssp. *ochroléuca* seems to prefer slightly wetter and shadier habitats than *D. incarnata* ssp. *cruenta* (Hedrén et al. 2001). All forms of *D. incarnata* can be found on the island of Öland (Sterner 1986). Intermediate plants seem to be rare (Hedrén 1996).

D. incarnata usually produces one inflorescence, which contains about 20 flowers (Mossberg 1997). The number of flowers is related to age and reproductive history of the plant, and the quality of the site (Mattila & Kuitunen 2000).

Study populations

The studied populations of *D. incarnata* were located on the island of Öland in the Baltic Sea.
Eleven populations of different size were chosen (Fig.1.). They were all situated in fens or wet meadows, surrounded by deciduous forest. The composition of reward-producing species varied among study sites. The following nectar-producing species were recorded at the study sites: *Tetragonolobus maritimus*, *Trifolium pratense*, *Trifolium repens*, *Geum rivale*, *Lotus corniculatus* and *Allium schoenoprasum*. The grazing pressure varied among populations.
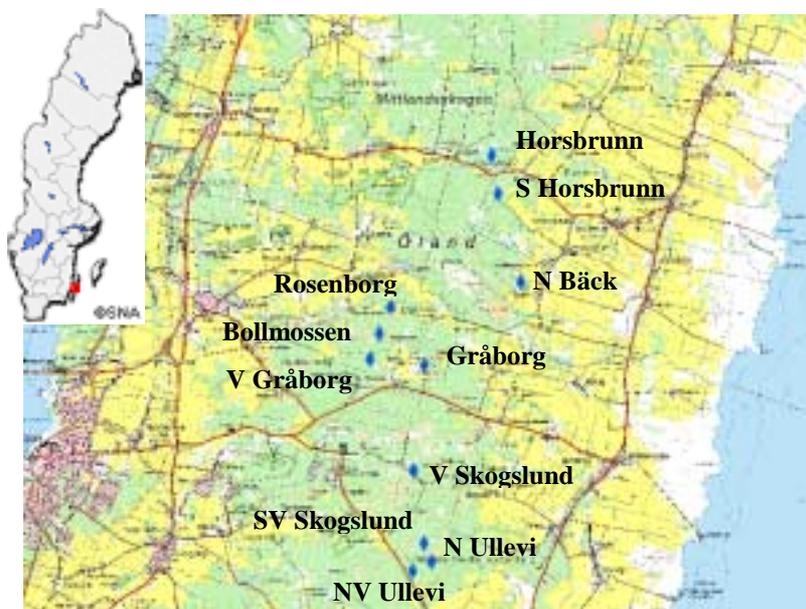

Fig .1. Map of Öland with locations of study populations of *Dactylorhiza incarnata* in 2004.

Table 1. Population size (number of flowering plants), population density (mean distance to nearest neighbour), proportion of the white flowering morph ssp. ochroléuca in the population and flower number per plant in 11 *Dactylorhiza incarnata* populations. The study was conducted on Öland in 2004.

Population	Population size	Proportion of ssp. ochroléuca	Population density (cm)	Flowers/plant ssp. *incarnata*	Flowers/plant ssp. *ochroléuca*
Gråborg	2000	0.13	73	24.0	22.2
Horsbrunn	1610	0.32	55	22.9	23.8
Bollmossen	1344	0.06	68	21.9	19.1
Rosenborg	300	0	109	18.4	-
SV Skogslund	278	0.22	85	21.3	20.2
N Bäck	206	0	103	21.1	-
N Ullevi	160	0	23	20.4	-
V Gråborg	115	0.06	113	16.1	19.6
NV Ullevi	65	0	80	20.0	-
S Horsbrunn	45	0.18	84	22.0	20.3
V Skogslund	27	0.04	402	24.2	-

Methods

Population characteristics
For each population, the total number of flowering plants was counted, and the proportion of D. incarnata ssp. ochroléuca in the populations was recorded. In this study

the two subspecies D. incarnata ssp. incarnata and D. incarnata ssp. cruenta were not separated. Population density was estimated as the mean distance to the nearest plant. The subspecies of the nearest neighbouring plant was recorded (measured for plants whose reproductive success were recorded, see below).

Reproductive success
The number of flowers per plant, male pollination success (number of removed pollinia) and female pollination success (the number of flowers with pollen deposited on their stigma) were recorded for 20 to 40 randomly chosen plants per population. The randomly chosen plants were marked, to be able to quantify female reproductive success as the number of fruits and seeds produced. The inflorescences were collected the first week of August, except for one population (S Horsbrunn) that flowered early, which was collected the third week of July. The large number of seeds per fruit made it impossible to count the seeds, instead width and length of the capsule were measured and from these measures fruit volume and seed production were estimated. The volume was quantified as length x radius^2 x π. The volume of the capsule has been shown to be correlated to the numbers of seeds produced in *D. incarnata* (Nasarov 1988). In a study by Nazarov (1988), the relationship between number of seed and volume of the capsule was found to be y = - 0.46+38.1x, where x is the volume of a capsule (cm^3) and y is thousands of seeds. The volume per fruit and per plant were estimated and used to calculate the number of seeds produced per fruit and per plant. I calculated the proportion of flowers from which pollinia were removed or deposited, and the proportion of flowers producing fruits.

Floral display
For each individual, plant height and the length of the inflorescence was recorded (distance from lowermost to uppermost flower).

Hand pollination
To determine whether the plants were pollen limited, supplemental hand-pollination was performed on about one third of the randomly chosen plants (between eight and fifteen plants per population). Experimental plants were pollinated with one pollinium per flower. The pollinium originated from plants of the same population and subspecies. In three of eleven populations, five plants of *D. incarnata* ssp. *ochroléuca* were hand-pollinated.

Nectar-producing plants
The abundance of nectar-producing plants in the populations was estimated in plots of 1 m^2. Parallel transects were laid out across each population at 10 m intervals and the occurrence of nectar-producing species was recorded in 1m^2 quadrates spaced at 5 m intervals. The number of quadrates ranged from 37 to 278 plots per population. In populations that did not contain any nectar-producing plants, no plots were established. The estimation of the density of nectar-producing plants in Gråborg and SV Skogslund populations were restricted to areas containing control and/or hand-pollinated plants. The outermost quadrates were placed out at least 20 m from the nearest control or hand-pollinated plant.

Pollinia movement
To estimate pollen dispersal and geitonogamy, pollinia of every second flower of four respectively three plants were stained in two populations. Four and three plants of *D. incarnata* ssp. *incarnata* were randomly chosen in the Rosenborg and Norra Bäck populations respectively. The number of flowers and buds were counted on the stained plants. To be able to determine the origin of the massulae the plants were stained with different colours (green, purple, pink and red). The plants were collected four days later. Every plant with receptive stigmas within 15 m from the stained plants was recorded for deposition of stained pollen. The number of massulae deposited and the position (counted from the lowermost flower of an inflorescence) of the flower receiving pollen were documented.

Statistical analysis
Minitab (release 14.1, Minitab Inc) was used to analyse the data statistically.

Reproductive success
Reproductive success (RS) was quantified as male pollination success (MPS; number of pollinia removed), female pollination success (FPS; number of flowers with pollen deposition on the stigma), female reproductive success (FRS; number of fruits produced) and number of seeds produced. Reproductive success was estimated per flower and per plant**.** The mean reproductive success of each morph of each population was tested against population density, population size and frequency of ssp. *ochroléuca* in the populations. Grazed control and hand-pollinated plants were excluded from analyses of female reproductive success and number of seeds. The data were transformed prior to statistical analysis. Population size and density were log-transformed, proportions were arcsine square-root transformed and numbers of flowers, number of fruits produced per plant and seeds and capsule volume produced per plant and fruit were square-root transformed. Regression analyses were used to determine the relationship between variables.

The correlation among independent variables population density (range, 23-402 cm), population size (27-2000 plants), mean display (18.6-30 cm), mean inflorescence height (4.5-7.5 cm), mean number of flowers (16.1-24.2 flower per plant) and the frequencies of ssp. *ochroléuca* (0.04-0.32) were quantified as Pearson correlation coefficients.

The number of flowers was positively correlated with inflorescence height in both morphs (*D. incarnata* ssp. *incarnata* $R^2 = 0.49$ *P* < 0.001 *D. incarnata* ssp. *ochroleuca* $R^2 = 0.58$ *P* < 0.001). No other significant correlations was detected among independent variables. Inflorescence height differed significantly between subspecies (t = 6.3, Df = 177, *P* < 0.001). No other significant differences in independent variables were detected between subspecies.

Pollen limitation
ANCOVA´s and ANOVA were conducted to investigate if pollen limitation was correlated to population size or density. Nine populations were included in the test. Two populations were excluded, Gråborg and Norra Bäck, due to high grazing pressure (large amount of control and hand-pollinated plants were grazed). A pollen limitation index ($L = 1 - (P_o/P_s)$) was calculated for each population, where P_o is the percent fruits produced per flower by control plants and P_s is the percent fruits produced per flower that of hand-pollinated plants. $L = 0$ indicates no pollen limitation in the population studied (Larson & Barrett 2000).

Analysis of difference between subspecies
Differences between subspecies in measures of reproductive and pollination success and numbers of flowers were tested with ANOVA and ANCOVA.

Table 2. Independent and dependent variables in *D. incarnata ssp. incarnata* and *D. incarnata ssp. ochroléuca* from all study populations pooled. The study was conducted on Öland in 2004.

	D. incarnata ssp. incarnata		*D. incarnata ssp. Ochroléuca*	
	Mean ± SE /Median(Range)	Sample size	Mean ± SE /Median(Range)	Sample size
Male pollination success	0.64 (0.0 –1.0)	242	0.44 (0.025-1.0)	83
Female reproductive success (number of fruits produced per plant)	13 (0-36)	182	8 (1-21)	60
Female pollination success	0.51 (0.0-1.0)	241	0.24 (0.0-0.87)	83
Seed production (thousands of seeds per fruit)	8.36(1.59-17.75)	179	8.79 (1.33-20.79)	60
Number of flowers	20 (7-42)	242	21 (7-41)	83
Inflorescence height (cm)	6.3 ± 0.098	242	5.2 ± 0.13	82
Display (cm)	25.0 ± 0.39	242	24.7 ± 0.6	82

Results

The average number of flowers per plant ranged from 16.1 to 24.2 (11 populations). The reproductive success ranged from 7.9 to 6.9 fruits per plant (female 10 populations) and from 0.27 to 0.82 numbers of pollinia removed per flower (male; 11 populations). The female pollination success (proportion of flowers on a plant which received pollen) ranged from 0.22 to 0.67 (11 populations), and seed production ranged from 2.6 to 13.3 thousands of seeds per fruit (10 population).

Reproductive success
Male pollination success per flower (MPS; population mean value) was negatively related to the population size ($y = 1.46 - 0.236x$, $R^2 = 0.54$, $P < 0.05$), and female pollination success (FPS; population mean value) tended to be negatively correlated to population size ($y = 1.142 - 0.1585x$, $R^2 = 0.34$, $P = 0.058$; Fig.2.). The MPS ($P = 0.376$, $P = 0.167$) and FPS ($P = 0.462$, $P = 0.4$) were not correlated to the morph frequency or population density. Female reproductive success (population mean value)($P = 0.295$, $P = 0.658$, $P = 0.508$) and seed number per fruit (population mean value) ($P = 0.105$, $P = 0.141$, $P = 0.212$) were not related to population size, population density or frequencies of *Dactylorhiza* ssp. *ochroléuca*. The female reproductive success (fruits per flower) and MPS of *Dactylorhiza* ssp. *incarnata* was negatively correlate with number of flowers ($y = 1,221 - 0,05791x$, $R^2 = 0.02$, $P < 0.05$; $y = 1,190 - 0,05849x$, $R^2 = 0.02$, $P < 0.05$). The fruit per flower and male pollination success ratio did not follow a normal distribution, which have to be considered when the results are interpreted.

- ● Female pollination success
- ■ Male pollination success
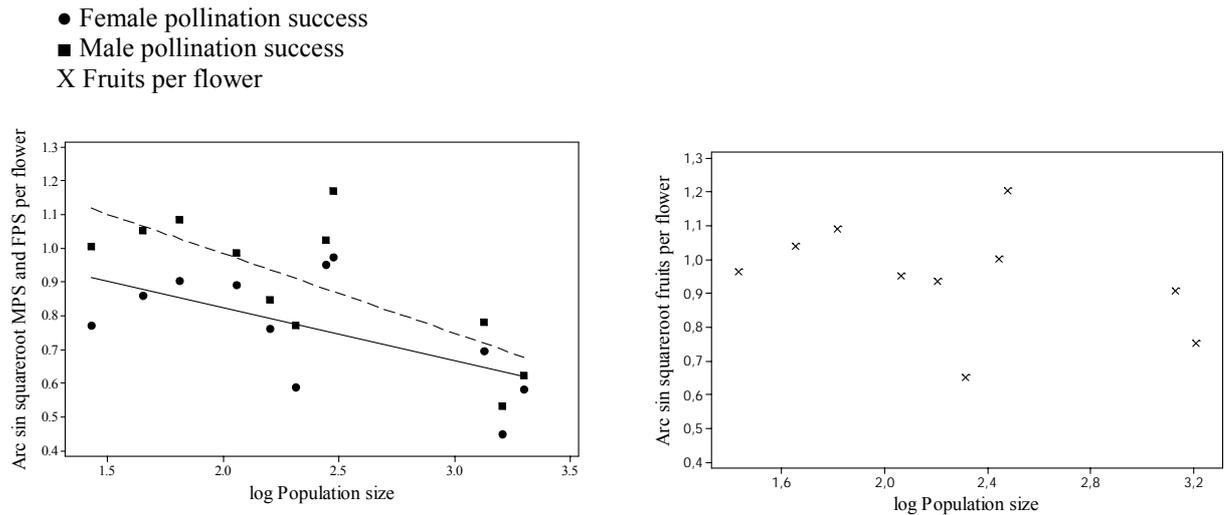- X Fruits per flower

Fig.2. Male and female pollination success per flower vs. population size and Fruits per flower vs. population size. Eleven populations were included in the regression analysis. The study was conducted on Öland in 2004.

Multiple regressions were performed to establish if morph frequency, density of the population and/or population size together could explain variation in reproductive success (fruits per plant) between populations. No combination of these factors could explain the variation in reproductive success, i.e. no regression was significant ($P > 0.05$).

Pollen limitation
The level of pollen limitation varied among populations (Table 3, Figure 3), but was not related to population size (y = 0.107 + 0.0809x, R^2 = 0.1 P = 0.374), population density (y = 0,368 – 0.041x, R^2 = 0.06, P = 0.825) or morph frequency (y = 0.219 + 0.5x, R^2 = 0.27, P = 0.289). Hand-pollinated plants did not produce more fruits per plant, number of fruit per flower or capsule volume than control plants in all populations (Table 4). The number of flowers did not differ between hand-pollinated plants and control plants, except in the Rosenborg population, where the number of flowers per plant was larger for control than hand-pollinated plants ($P < 0.05$). The treatment by number of flowers interaction was not significant for the pollen limitation (P = 0.05). The fruit per flower ratio did not follow a normal distribution, which have to be considered when the results are interpreted.

Table 3. Results of ANCOVA´s and ANOVA tests of number of fruits per plant (Fruit production, number of flowers included as a covariate), capsule volume per fruit (Capsule production, number of flowers included as a covariate), and number of fruit per flower (FRS) in nine populations of *D. incarnata*. The study was conducted on Öland in 2004.

Source	Fruit production				Capsule production				FRS			
	Df	F	P		Df	F	P		Df	F	P	
Treatment (Control vs. hand-pollinated)	1	64.65	0.000	***	1	5.3	0.022	*	1	120.79	0.000	***
Population	8	1.8	0.077	n.s	8	17.03	0.000	***	8	2.32	0.021	*
Treatment x Population	8	2.76	0.006	**	8	2.45	0.015	*	8	2.14	0.033	*
Number of flowers	1	186.23	0.000	***	1	5.71	0.018	*				
Error	232				231				231			

* *P* < 0.05, ** *P* < 0.01***, *P* < 0.001

Table 4. Fruits per plant (Fruit production), capsule volume per fruit (Capsule production) and number of fruit per flower (FRS) of hand-pollinated and control plants in nine populations were tested with unpaired t-tests. The study was conducted on Öland in 2004.

Population	Fruit production				Capsule production				FRS			
	Df	t- value	P		Df	t- value	P		Df	t-value	P	
Horsbrunn	20	2.3	0.028	*	9	2.54	0.032	*	23	8.19	0.000	***
Bollmossen	19	2.37	0.033	*	23	4.80	0.000	***	13	2.66	0.020	*
Rosenborg	23	- 1.2	0.244	n.s	30	2.31	0.028	*	18	1.68	0.110	n.s
SV Skogslund	20	0.5	0.621	n.s	13	1.48	0.162	n.s	11	3.99	0.002	**
N Ullevi	24	2.21	0.037	*	13	-0.77	0.455	n.s	21	3.70	0.001	***
V Gråborg	23	2.14	0.043	*	14	-1.55	0.144	n.s	17	2.88	0.010	**
NV Ullevi	11	2.24	0.046	*	22	-1.82	0.082	n.s	23	3.97	0.001	***
S Horsbrunn	24	0.22	0.824	n.s	17	1.75	0.098	n.s	16	3.23	0.005	**
V Skogslund	13	1.64	0.125	n.s	11	1.17	0.265	n.s	10	5.08	0.000	***

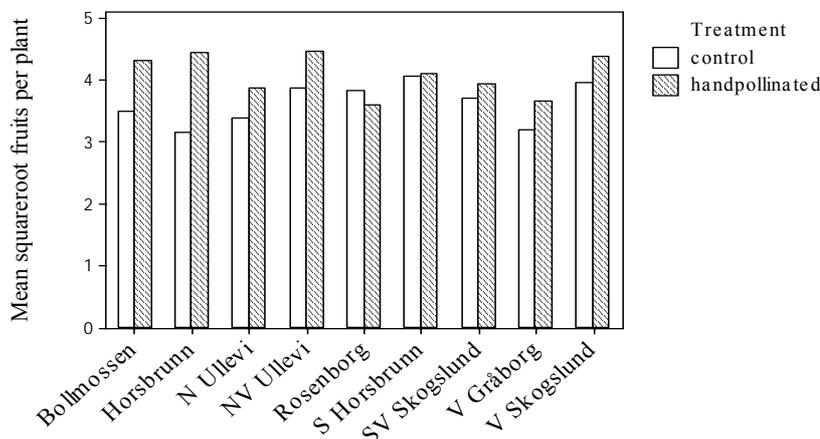* *P* < 0.05, ** *P* < 0.01***, *P* < 0.001.

Fig. 3. Fruits per plant of hand-pollinated and control plants of nine populations. The study was conducted on Öland in 2004.

Analysis of difference between subspecies
Female pollination success, female reproductive success and seed production varied between subspecies and among populations (female pollination success tended to differ between populations (*P* = 0.056); Table 5.).

Table 5. Effects of subspecies (*D. incarnata* ssp. *ochroléuca* vs. *D. incarnata* ssp. *incarnata*) on male and female pollination success (population means; n=6) were analysed with ANOVA-tests. Differences in female reproductive success (population means; n=5) and seed production (population means; n=5) were examined with ANCOVA ,which included number of flowers as a covariate. The study was conducted on Öland in 2004.

Variable	Source of variation	Df	F-value	P	
Male pollination success	Subspecies	1	1.57	0.266	n.s
	Population	1	9.65	0.013	*
	Error	5			
Female reproductive success	Subspecies	1	62.97	0.004	**
(number of fruits produced per plant)	Population	4	25.74	0.012	*
	Number of flowers	1	2.65	0.202	n.s
	Error	3			
Female pollination success	Subspecies	1	18.78	0.007	**
	Population	1	4.75	0.056	n.s.
	Error	5			
Seed production (thousands of seeds per plant)	Subspecies	1	12.8	0.037	*
	Population	4	41.25	0.006	**
	Number of flowers	1	0.32	0.612	n.s
	Error	3			

*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Reward-producing plants

Reward-producing plants were scarce in the study populations (Table 6) and the density of nectar-producing plant was not correlated with the reproductive success of *D. incarnata*.

Table 6. Density of nectar-producing plants expressed as number of flowering shoots per plots in study populations on Öland in 2004.

Population	*Geum rivale*	*Lotus corniculatus*	*Tetragonolobus maritimus*	*Trifolium pratense*	*Trifolium repens*	*Allium Schoenoprasum*	Number of plots (m^2)
Gråborg	1.43	0.29	0	1.88	5.24	0	138
Horsbrunn	0	0	0	0	0	0	0
Bollmossen	0.34	1.90	0	0.81	0	37.1	248
Rosenborg	0	0	0	0	0	0	0
SV Skogslund	0.01	0	1.32	0	0	0	167
N Bäck	5	19.9	1.32	0.02	2.40	0	237
N Ullevi	0.35	0	5.6	0	0	0	37
V Gråborg	0	0	0	0	0	0	0
NV Ullevi	0.03	0	0	0	0	0	68
S Horsbrunn	0	0	0	0	0	0	0
V Skogslund	0	0	0	0	0	0	0

Pollinia movement

The number of flowers (and buds) and the proportion of pollinia stained and removed per inflorescence varied between experimental plants. In the Rosenborg population, 36 plants with receptive stigmas were found within 15 m of the stained plants, in Norra Bäck, 42 plants were controlled. Dispersal was detected in a range from 0 to 26 m from the stained plant (Table 7). Geitonogamy was observed in three flowers of three different plants (Table 7).

Table 7. Numbers of flowers, buds, stained and removed pollinia per flower of *Dactylorhiza incarnata* in the Rosenborg and Norra Bäck populations. The study was conducted on Öland in 2004.

Population	Stain	Number of flowers	Number of buds	Stained pollinia	Removed stained pollinia
Rosenborg	Green	3	7	4	4
Rosenborg	Purple	15	7	14	6
Rosenborg	Pink	28	5	12	7
Rosenborg	Red	12	4	9	2
Norra Bäck	Green	17	0	6	1
Norra Bäck	Purple	17	0	10	2
Norra Bäck	Pink	10	0	8	4

Table 8. The number of massulae deposited per flower of *Dactylorhiza incarnata* and the position (counted from the lowermost flower of an inflorescence) of the flower that received stained massulae. The study was conducted on Öland in 2004.

Population	Plant received stained massulae	Number of stained massulae received	Plant exported stained massulae	Distance from source (cm)
Rosenborg	Green	4	Green	0
Rosenborg	Purple	10	Green	11
Rosenborg	Purple	7	Green	11
Rosenborg	Purple	5	Green	11
Rosenborg	Purple	7	Green	11
Rosenborg	Non-stained	4	Green	2600
Norra Bäck	Green	3	Green	0
Norra Bäck	Pink	1	Pink	0
Norra Bäck	Non-stained	1	Green	532

Discussion

The male and female pollination success of *D. incarnata* ssp. *incarnata* tended to decrease with increasing population size (Fig. 2). This may be explained by a negative effect of population size on the pollinator visitation rate. A large population of deceptive plants is more conspicuous and easier to detect than a small population. If the pollinator learns to avoid the non-rewarding species after some visits, the plants in larger populations will be less frequently visited than plants in smaller populations (Alexandersson & Ågren 1996). The number of populations included in the present study was limited. To increase the statistical power, and to more determine more accurately the effect of population characteristics on pollination success a larger sample size would be required.

The degree of pollen limitation of fruit production, capsule production and number of fruit per flower (FRS) in *Dactylorhiza incarnata* varied among populations, but was not related to population size, population density or morph frequency (Table 3, 4; Fig. 3). The number of fruits per flower differed significantly in all populations, except Rosenborg population ($P > 0.05$), whereas the capsule production differed between treatments in tree populations out of nine. However, there was no evidence that pollen limitation would increase with increasing population size in this deceptive orchid. The tendency for a decrease in pollen receipt with increasing population size did not translate into an increase in the degree of pollen limitation. This suggests that among-population variation in fruit output and pollen limitation is influenced considerably by other factors. Both resource availability and inbreeding may vary among populations and thus influence patterns of fruit production (cf. Ferdy et al. 2001).

Reproduction in a given year may reduce future growth and reproduction in several orchid species (Zimmerman and Aide 1989, Mattila & Kuitunen 2000). In a study by Mattila & Kuitunen (2000), plants of *Dactylorhiza incarnata* that produced many fruits had a lower probability of flowering the following year. On the other hand Calvo (1990) found that fruit set of the nectar-less orchid *Cyclopogon cranichoides* did not affect growth or reproduction the following year. In another test of resource limitation, Vallius (2001) found evidence for resource allocation trade-offs within a season, but not between years in the deceptive orchid *Dactylorhiza maculata*. A decreased photosynthetic activity reduced seed production, whereas mycorrhiza proved to be less important and dependent on site conditions (Vallius 2001). Lowered photosynthetic activity, reduction of mycorrhiza and defoliation did not lower the probability of *D. maculata* to flower the next year (Vallius 2001).

Pollinator availability may vary between years and therefore it may be favourable for deceptive, pollen-limited plants to reallocate resources to reproduction in years with high abundance of pollinators (Mattila & Kuitunen 2000). The pollinator visitation rate has been shown to be positively related to number of flowers per inflorescence (Brody & Mitchell 1997), and few-flowered plants may be neglected by the pollinator (Johnson & Nilsson 1999). Both pollinator visitation and fruit set may therefore increase with number of flowers per inflorescence (Mitchell 1994, Brody & Mitchell 1997, Maad 2000). However, in this study the degree of pollen limitation did not seem to decreased with increased number of flowers ($P = 0.05$). Because the cost of producing a flower should be lower in a deceptive than in a rewarding species, deceptive species may be able to produce more flowers per inflorescence than rewarding species (Johnson & Nilsson 1999). An increase in the number of flowers of an inflorescence has been found to lower the allocation of resources to the latest flowering flowers, and the capsule weight of uppermost flowers was found to decrease with increasing number of fruits produced per flower (Vallius 2000).

This may indicate that plants with large inflorescence may suffer more from resource limitation than small plant in years with high pollinator visitation rate. In this study pollen limitation was found to vary among populations, and if resource limitation may increase with increased pollinator visitation rate, the resource limitation possibly may vary among populations.

In a study by Brody & Mitchell (1997) it was found that plants with larger floral display received more total flower visits and were more likely to be first visited by the pollinator in any given foraging bout. The number of pollinator visits a flower receives may affect the reproductive success, since increased number of pollinia deposited on the stigma may enhance seed production (Lammi et al. 2003). The total number of visits of plants with larger display will be greater than for small plants, but few-flowered plants may experience that a higher proportion of the flowers being visited, which may result in a higher reproductive success per flower in plants with few flowers (Brody & Mitchell 1997). In this study plants with tall many-flowered inflorescences seem to produce less fruits per flower and to have lower male pollination success than plants with low, few-flowered inflorescences in *D. incarnata* ssp. *incarnata*. The inflorescence height and the number of flowers per plant of *D. incarnata* were found to be correlated, and in this study it was not possible to separate the effects of the two factors. It seems likely that both number of flowers and inflorescence height contributed to floral display. The number of flowers, which defines the potential fruit production of the plant, and the floral display will influence the reproductive success.

The movement of stained pollinia in this study indicates occurrence of geitonogamy, despite the mechanism of pollinium bending, which occurs after the pollinium has attached to the pollinator (Johnson et al. 2004). Self pollination in *Orchis mascula* has been found to reduce the percentage fertile embryos produced by each fruit (Johnson & Nilsson 1999). The majority of the removed stained pollinia were not deposited on plants within 15 m from the source plant. Possible explanation may be that the pollen is transported over longer distances than the area searched for stained pollinia. Indeed, one pollen transport was documented between two plants separated by 26 m in the Rosenborg population (from one stained plant to an unstained plant close to another stained plant; Table 8). Another possible explanation may be that a substantial fraction of pollinia are lost in transport between plants. In a study by Johnson et al (2004) pollen transfer efficiency (percentage of removed pollen that was deposited on stigmas) for the deceptive orchid *Anacamptis* morio was 8.1 %.

In the present study *D. incarnata* ssp. *incarnata* was found to have a higher female reproductive success, female pollination success and inflorescence height than *D. incarnata* ssp. *ochroléuca*. Bumble bee workers show a tendency to visit dissimilar morphs of reward-less plants sequentially (Smithson &.Macnair 1997). However, there was no evidence for frequency-dependent pollination success of the two subspecies. If pollinators would visit dissimilar morphs of *D. incarnata* sequentially, this should result in hybrid formation, but few intermediate plants of the subspecies of *D. incarnata* are recognized (Hedrén et al. 2001). The results of the present study imply that other factors than negative frequency-dependence of reproductive success maintains the flower colour polymorphism in *D. incarnata*. Hedrén et al. (2001) suggested that the differentiation in the species may be due to particular habitat preferences of the subspecies and that the formation of the subspecies may have occurred several times at different locations. On Öland the two *D. incarnata* ssp. included in the study were not spatially isolated and did not differ in flowering times. No effective pre-pollinated barrier has been demonstrated between the colour morphs (Pedersen 1998).

The reproductive success of *D. incarnata* was not related to the local abundance of nectar-producing plants. The populations of *D. incarnata* were located in areas that

contained few nectar-producing species at low densities, and the composition of reward-producing species differed among populations. The N Bäck, Gråborg and N Ullevi populations were located in wet meadows with the highest densities of nectar-producing plants and the highest grazing pressure. In the Gråborg population, 31 out of 46 marked plants were grazed. To estimate the effect of nectar-producing plants on the reproductive success of *D. incarnata* a larger sample of populations with similar composition of nectar-producing species would be needed. Also populations with high densities of nectar-producing plants without high grazing pressure should be investigated to be able to distinguish the effects of the grazing pressure and density of nectar-producing plants. Often nectar-producing plants had a clumped distribution in the study population, which may have influenced the results.

The number of massulae deposited by the pollinator on the stigma may vary among flowers. The volume of capsules produced by *D. incarnata* has been shown to be enhanced by an increased number of massulae deposited on the stigma (Lammi et al. 2003). An increased seed production may indicate a positive response to the number of visits per flower and/or a positive effect of visits by pollinators carrying a large amount of pollinia. To evaluate the importance of these factors on the reproductive success of the deceptive orchid *Dactylorhiza incarnata* further studies will be needed.

References

Alexandersson, R., Ågren, J. 1996. Population size, pollinator visitation and fruit production in the deceptive orchid *Calypso bulbosa*. Oecologia 107:533-540.

Brody, A.K., Mitchell, R.J. 1997. Effects of experimental manipulation of inflorescence size on pollination and pre-dispersal seed predation in the hummingbird-pollinated plant *Ipomopsis aggregata*. Oecologia 110:86-93.

Brys, R., Jacquemyn, H., Endels, P., van Rossum, R., Hermy, M., Triest, L., de Bruyn, L., Blust, G. D. E. 2004. Reduced reproductive success in small populations of the self-incompatible *Primula vulgaris*. Journal of ecology 92:5-14.

Calvo, R.N. 1990. Four-year growth and reproduction of *Cyclopogon cranichoides* (Orchidaceae) in south Florida. American Journal of Botany 77:736-741.

Ehlers, B.K., Olesen, J.M., Ågren, J. 2002. Floral morphology and reproductive success in the orchid *Epipactis helleborine*: regional and local across-habitat variation. Plant Systematics and Evolution 236:19-32.

Ferdy, J-B., Loriot, S., Sandmeier, M., Lefranc, M., Raquin, C. 2001. Inbreeding depression in a rare deceptive orchid. Canadian Journal of Botany 79:1181-1188.

Fritz, A-L., Andersson, L.A. 1994. How pollinator-mediated mating varies with population size in plants. Oecologia 100:451-462.

Gigord, L.D.B., Macnair, M.R., Smithson, A. 2001. Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.) Soo. Proceedings of the National Academy of Sciences of the United States of America. 98: 6253-6255.

Hedrén, M. 1996a. Notes on the esterase variation in swedish Dactylorhiza incarnata s.l (Orchidaceae). Nordic Journal of Botany 16:253-256.

Hedrén, M. 1996b. Genetic differentiation, polyploidization and hybridization in northern European *Dactylorhiza (*Orchidaceae): evidence from allozyme markers. Plant systematics and evolution 201:31-55.

Hedrén, M., Fay., M.F., Chase, M.W. 2001. Amplified fragment length polymorphism (AFPL)reveals details of polyploid evolution in *Dactylorhiza* (Orchidaceae). American Journal of botany 88:1868-1880.

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

Johnson, S.D., Peter, C.I., Nilsson, L.A., Ågren, J. 2003. Pollination success in a deceptive orchid is enhanced by co-occurring rewarding magnet plant. Ecology 84:2919-2927.

Johnson, S.D., Peter, C.I., Ågren, J. 2004. The effect of nectar addition on pollen removal and geitonogamy in the non-rewarding orchid *Anacamptis morio*. Proceedings of the Royal Society of London. B 271:803-809.

Knight, T.M. 2003. Floral density, pollen limitation, and reproductive success in *Trillium grandiflorum*. Oecologia 137:557-563

Larson, B.M.H., Barrett, S.C.H. 2000. A comparative analysis of pollen limitation in flowering plants. Biological Journal of the Linnean Society 69:503-520.

Lammi, A., Kuitunen, M. 1995. Deceptive pollination of *Dactylorhiza incarnata*: an experimental test of the magnet species hypothesis. Oecologia 101:500-503.

Lammi, A., Vallius, E., Vauhkonen, T., Kuitunen, M. 2003. Outcrossing, hybridization, pollen quantity, and the evolution of deceptive pollination in *Dactylorhiza incarnata*. Annales botanici fennici 40: 331-338.

Laverty, T.M. 1992. Plant interaction for pollinator visits: a test of the magnet species effect. Oecologia 89:502-508.

Maad, J. 2000. Phenotypic selection in hawkmoth-pollinated *Platanthera bifolia:* targets and fitness surfaces. Evolution 54:112-123.

Maad, J., Alexandersson, R. 2004. Variable selection in *Platanthera bifolia* (Orchidaceae): phenotypic selection differed between sex functions in a drought year. Journal of Evolutionary Biology 17:642-650.

Mattila, E., Kuitunen, T. 2000. Nutrient versus pollen limitation in *Platanthera bifolia* and *Dactylorhiza incarnata* (Orchidaceae). Oikos 89: 360-366.

Mitchell, R.J., 1994. Effects of floral traits, pollinator visitation and plant size on *Ipomopsis aggregata* fruit production. American naturalist 143:870-889.

Mustajärvi, K., Siikamääki, P., Rytkönen, S., Lammi, A. 2001. Consequences of plant population size and density for plant-pollinator interactions and plant performance. Journal of ecology 89:80-87.

Nazarov, N. 1988. Determination of the actual seed productivity in *Dactylorhiza romana* and *Dactylorhiza incarnata* Orchidaceae. Botanicheskii Zhurnal 73:231-233.

Pedersen, H.Æ. 1998. Allozyme variation and genetic integrity of *Dactylorhiza incarnata* (Orchidaceae). Nordic Journal of Botany 18:15-21.

Proctor, H.C., Harder, L.D. 1994. Pollen load , capsule weight, and seed production in three orchid species. Canadian Journal of Botany 72:249-255.

Smithson, A., Macnair, M.R. 1997. Negative frequency-dependent selection by pollinators on artificial flowers without rewards. Evolution 51:715-723.

Sterner R. 1986. Ölands kärlväxtflora pp84, 197-198. 2: nd uppl. Svensk Botanisk Tidsskrift. Lund

Thompson, J.D. 2001. How do visitation pattern vary among pollinators in relation to floral display and floral design in a generalist pollination system? Oecologia 126:386-394.

Vallius, E. 2001. Factors affecting fruit and seed production in *Dactylorhiza maculata* (Orchidaceae). Botanical Journal of Linnean Society 135:89-95.

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