Evidence for Inbreeding Depression in the Food-Deceptive Colour-Dimorphic Orchid *Dactylorhiza sambucina* (L.) Soò

N. Juillet[1], S. Dunand-Martin[2], and L. D. B. Gigord[1]

[1] Department of Ecology and Evolution, Biophore Building, University of Lausanne, 1015 Lausanne, Switzerland
[2] Laboratoire de culture in vitro, Conservatoire et Jardins Botaniques – Ville de Genève, 1 chemin de l'Impératrice, 1292 Chambésy, Switzerland

Received: May 2, 2006; Accepted: May 23, 2006

Abstract: **About one third of all orchid species are deceptive, i.e., not providing any reward to their pollinator. Such species often have lower visitation rates compared to rewarding relatives. This could result in lower levels of geitonogamous selfing and thus would provide an advantage in term of progeny fitness through inbreeding avoidance. This hypothesis could be tested by comparing the level of inbreeding depression between deceptive and rewarding orchids. However, due to the difficulty to raise orchids from seeds, few studies of inbreeding depression are available, and most are focused on very early life stages, such as seed mass or embryo viability. Here, we present the results from an experimental investigation of inbreeding depression in the deceptive flower-colour dimorphic *Dactylorhiza sambucina,* from *in vitro* cultivation to greenhouse soil transplantation. We found strong inbreeding depression at all recorded stages (i.e., germination and survival), with estimates ranging from 0.47 to 0.75. Our study finally proposes a simple and suitable experimental protocol to raise orchids from seeds with high germination rates.**

Key words: **Inbreeding depression, *in vitro* cultivation, deceptive orchid, seed germination, *Dactylorhiza sambucina*.**

Introduction

Inbreeding depression, the relative reduction in fitness of selfed versus outcrossed progeny, is recognized as a primary factor affecting evolution of plant mating systems (Charlesworth and Charlesworth, 1987; Keller and Waller, 2002). Generally, selfing rates and inbreeding depression are negatively correlated, i.e., plant species with high selfing rates should show low levels of inbreeding depression (Husband and Schemske, 1996). Beyond the evolution of mating system *per se,* inbreeding depression has been hypothesized to favour the emergence of specific life history traits that directly or indirectly tend to decrease selfing events (Barrett, 2002). For instance, inbreeding depression is seen as a potential mechanism favouring the evolution and maintenance of food deception in orchid species (Dressler, 1981; Nilsson, 1992; Johnson and Nilsson, 1999). In food deceiving orchids, pollinators are not rewarded during a visit to a plant because of a lack of nectar production and the clumping of pollen into specific structures called pollinia, from which pollinators generally cannot extract and use the pollen grains as reward. It has been suggested that food deception causes pollinators to probe fewer flowers per plant, thus reducing geitonogamous selfing. Experimental evidence shows that pollinators visit fewer flowers on deceptive inflorescences compared to artificially nectar-supplemented ones, but the subsequent reduction in geitonogamy is still unclear (Smithson, 2002; Johnson et al., 2004). If selfing causes strong inbreeding depression among offspring, the advantages of reduced geitonogamous selfing may outweigh the cost of reduced visitation rate and fruit set (Gill, 1989; Neiland and Wilcock, 1998), and being deception may become advantageous. In this sense, nectar-producing species should be less prone to inbreeding depression than deceptive species because repeated geitonogamous selfing could purge the genetic load. However, to date few studies have investigated inbreeding depression in nectar producing and nectarless orchids (but see Smithson, 2006).

The germination of orchid seeds is complex because seeds are minute, lack endosperm and rely on an obligate mycorrhizal association to germinate (Arditti and Abdul Ghani, 2000; Rasmussen, 1995). Because the germination process is slow and difficult to follow *in situ* (but see Rasmussen and Whigham's [1993] study technique), *in vitro* propagation protocols have been developed, especially for horticultural purposes. By providing the seeds with a substrate containing suitable nutrients, it is possible to germinate seeds in the lab, even without a symbiotic fungus (Rasmussen, 1995). Although these methods considerably accelerate germination and growth, relatively few studies have employed such techniques to study early life history stages in orchids (Rasmussen and Whigham, 1993). For example, a recent bibliographic survey shows that studies investigating inbreeding depression in orchids often use fruit set, seed mass, or seed viability (Tremblay et al., 2005) but rarely germination rates as fitness measurements (Peakall and Beattie, 1996; Ferdy et al., 2001; Jersáková et al., 2006; Smithson, 2006). To our knowledge, later development stages such as seedling survival rate, growth rate, time before first flowering, or lifetime reproductive success have never been used.

Plant Biol. 9 (2007): 147 – 151

DOI 10.1055/s-2006-924310 · Published online August 1, 2006
ISSN 1435-8603

In this study, we investigated inbreeding depression in the deceptive orchid *Dactylorhiza sambucina* (L.) Soò, using *in vitro* micropropagation techniques to germinate and grow the seeds from controlled crosses. Because this species is dimorphic for corolla colour (purple and yellow coloured individuals), we used both colour morphs as pollen donor and receiver for the reciprocal controlled crosses. The culture conditions, both *in vitro* and in the greenhouse, are described. Using these methods, we investigated the influence 1) of corolla colour morph and 2) artificial pollination treatments on progeny germination and survival rates in order to 3) estimate the magnitude of inbreeding depression in *D. sambucina*.

Materials and Methods

Plant species

Dactylorhiza sambucina Soò is a widespread European food deceptive orchid, found from Spain and Sicily to southern Scandinavia, mainly in open meadows, from sea level up to 2600 m in altitude. This species exhibits strong colour dimorphism, with yellow and purple flowered individuals frequently co-occurring in variable frequencies throughout the species range (Smithson et al., in press).

Pollination treatments

In a *D. sambucina* natural population of about 500 plants (yellow morph biased; Valais, Switzerland), 12 plants of each colour morph bearing a minimum of 10 buds (13.7 ± 3.7 flowers per plant) were randomly selected at bud stage and protected from pollinators using insect-proof mesh bags. When all flowers were open, six randomly selected flowers on each plant were pollinated: two were self-pollinated, two were cross-pollinated with the pollen of a similarly coloured plant (intramorph crossing), and two were cross-pollinated with the pollen of a differently coloured plant (intermorph crossing). The position of treatments along the inflorescence were randomized to avoid potential position effects linked to resource limitation (Vallius, 2000). Pollen donor and receiver were randomly selected, ensuring that each plant contributed equally to the crossing design through both male and female function. After manual pollinations, the plants were bagged to prevent any further natural pollination or predation. All capsules were harvested just before dehiscence (about 35 days after pollination), and kept in wet cotton in a fridge until sowing.

In vitro *and greenhouse culture*

The seeds were sown using the "packet technique" (McKendrick, 2000). Seeds were removed from the capsule and put into a filter paper packet, closed with a staple and specifically labelled. The packets were placed in a 5% bleach solution for 20 min, sonicated first for 3 min, and subsequently given magnetic agitation. The sterilized packets were rinsed twice in sterile water jars under an airflow bench, carefully opened and directly dabbed onto medium plates (2 – 5 plates per seed packet). The seeds were sown asymbiotically in Petri dishes filled with KewA medium (Mitchell, 1989). Myo-inositol concentration was 0.1 mg L^{-1}, instead of 0.1 g L^{-1} as originally published (R. Mitchell, personal communication). The Petri dishes were sealed with Parafilm tape (Pechiney, Menasha, Wisconsin) and kept in an unlit growth chamber at 20 °C. After 5 months of asymbiotical culture, 40 protocorms from each capsule were randomly selected and transplanted on Petri dishes (5 per dish) filled with BOM medium (Mitchell, 1989) and inoculated with B1 fungal strain (strain available on request). The dishes were kept in a growth chamber with 12 h dark at 16 °C, and 12 h light at 20 °C for 2 months. The protocorms were then transplanted twice with a 2-month interval into larger boxes (ref. 71022, 500 mL, Polylabo, each contained 10 plantlets) filled with BOM medium and B1 fungal strain. The boxes were kept in a growth chamber with the same light and temperature conditions for 6 months. After 15 months of *in vitro* culture, the plantlets were individually potted in sandy soil (2/5 sand, 1/5 peat, 2/5 leaf compost), kept in a greenhouse and watered regularly.

Data collection and statistical analysis

Seed germination was recorded 65 and 130 days after sowing by counting at least 100 seeds in randomly selected grid squares on two Petri dishes per capsule. Seeds were considered germinated if rhizoids were present and average germination rate per capsule was noted. Survival rates were recorded in September 2005 (1 year after greenhouse transplantation) by counting the proportion of dead and live plantlets per capsule.

The effect of pollen recipient's plant corolla colour and pollination treatment on germination rates after 65 and 130 days of culture were analyzed, as were survival rates after a year in the greenhouse, with a two-way ANOVAs using permutation tests on mean squares (Manly, 1997), given that no data transformation were found to improve normality and homoscedasticity. Tukey tests were then used to compare pollination treatments.

Inbreeding depression coefficients for each family were calculated as $\delta = (wo - ws)/wmax$, where *wo* and *ws* were the fitness estimates of outcrossed and selfed progenies, respectively and *wmax* was the maximum of both (Ågren and Schemske, 1993). Only families that were monitored until greenhouse planting out and that were balanced with respect to pollination treatments were used. The statistical package R was used for all data analyses (R Development Core Team, 2004).

Results

Out of 144 hand-pollinated flowers, 122 produced a capsule; all others failed to initiate fruit production or aborted. The number of fruit set did not differ significantly between crossing treatments (39 selfed, 41 intramorph outcrossed, 42 intermorph outcrossed, X-squared = 0.11, df = 2, $p = 0.94$), or between colour morphs (68 yellow, 54 purple, X-squared = 1.61, df = 1, $p = 0.21$). The lower total number of fruit set by purple plants can mainly be explained by the wilting of two purple inflorescences just after pollination. Due to wilting, fruit abortion, manipulation errors and contamination during culture, some capsules were lost. 16 balanced families were retained for analyses of germination and survival rates (6 yellow and 10 purple).

Germination rates were not significantly different between seeds from yellow and purple families (Table **1**). There was a marginally significant difference in survival rate between

seedlings from purple and yellow families (lower survival rates for purple families), especially between selfed progenies (0.02 ± 0.04 and 0.16 ± 0.13 for purple and yellow selfed progenies, respectively).

Pollination treatment effect was highly significant for the three fitness estimates. Multiple comparison tests showed that progenies from intramorph and intermorph crosses have comparable germination and survival rates, which are significantly higher than those of selfed crosses (Fig. **1**). No significant interaction terms between morph and pollination treatments were found (Table **1**).

When colour factor was removed (not significant), progenies from selfing had lower germination and survival rates than outcrossed progenies (Wilcoxon rank sum test: $p < 0.001$ for germination rate after 65 and 130 days and survival rate).

Inbreeding depression for germination rates was not different between purple and yellow families (Wilcoxon rank sum test: $p = 0.63$ and $p = 0.43$ for germination rate after 65 and 130 days respectively). Inbreeding depression for survival rates was significantly higher for purple ($\delta = 0.88 \pm 0.24$) than yellow families ($\delta = 0.54 \pm 0.40$, Wilcoxon rank sum test: $p = 0.047$). Overall, inbreeding depression coefficients were high for all three fitness estimates ($\delta = 0.46 \pm 0.22$, $\delta = 0.60 \pm 0.20$, and $\delta = 0.75 \pm 0.35$ for germination rates at 65 and 130 days and survival rates, respectively).

Table 1 Results of two-way ANOVA testing for maternal colour morphs (purple or yellow) and pollination treatment effects (selfing, intramorph crossing, and intermorph crossing) on three indices of fitness: **a** and **b** germination rates measured 65 and 130 days after sowing, respectively, and **c** survival rates after one year in the greenhouse in *D. sambucina*

	Variable	df	Mean squares	*p*
a	**% germination after 65 days**			
	Colour	1	0.00002	0.970
	Treatment	2	0.21888	< 0.001
	Colour × Treatment	2	0.00644	0.630
	Residuals	42	0.00484	
b	**% germination after 130 days**			
	Colour	1	0.00002	0.983
	Treatment	2	1.28747	< 0.001
	Colour × Treatment	2	0.01972	0.778
	Residuals	42	0.02073	
c	**% survival after one year in the greenhouse**			
	Colour	1	0.10617	0.071
	Treatment	2	0.25898	< 0.001
	Colour × Treatment	2	0.00481	0.870
	Residuals	42	0.02316	

Discussion

In this study, germination and survival rates between seeds from hand-pollinated *Dactylorhiza sambucina* plants was investigated. No difference in fitness estimates between yellow and purple plants, or between intramorph and intermorph crosses were detected. However, a highly significant difference in fitness between selfed and outcrossed progenies was found, resulting in a strong inbreeding depression in this species.

In *D. sambucina,* Nilsson (1980) found inbreeding depression ($\delta = 0.42$) by measuring the relative percentage of embryos formed between selfed and outcrossed fruits. More recently, Jersáková et al. (2006) also found inbreeding depression ($\delta = 0.63$) for seed viability, but the results were inconclusive for *in vitro* germination rates because of very low overall germination success. Including our results on the expression of the genetic load for germination and survival rates of this species, there are now three independent studies indicating that inbreeding depression is common and strong in *D. sambucina*.

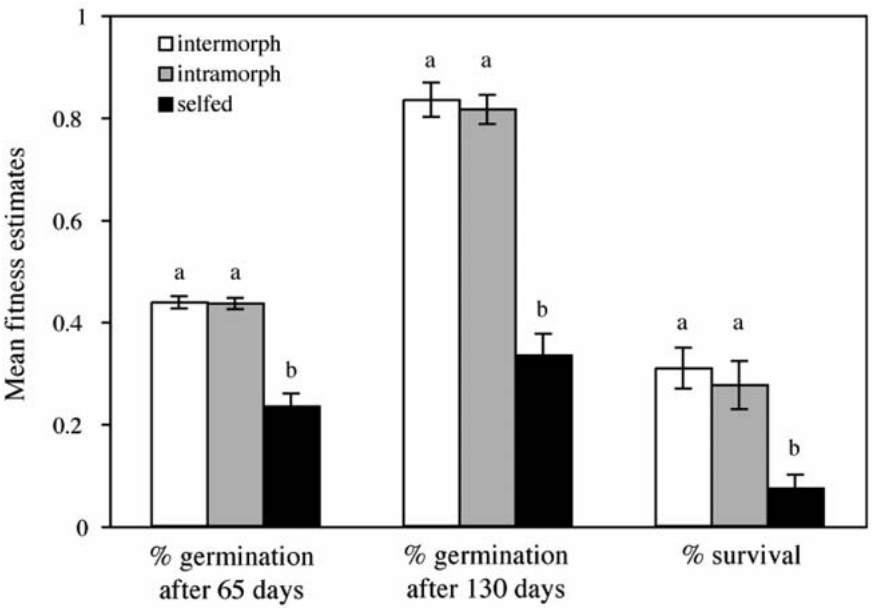

Fig. 1 Mean (± SE) fitness estimates (germination rates after 65 and 130 days, and survival rates) as a function of pollination treatment in *D. sambucina*. Different letters denote significant differences between treatment means within fitness. Data were pooled across colour morphs.

This study also provides an example of inbreeding depression in orchids, and to our knowledge, is the first example of its expression beyond the stage of germination in this plant family, i.e., on survival rate in semi-natural environmental conditions. For conservation, this finding indicates that seed quality is an important parameter to take into account before propagating orchids from seeds. The large difference in germination and survival rate between selfed and outcrossed seeds should encourage orchid growers to use artificial cross-pollination to produce seeds. The protocol proposed here allowed fast and significant germination (44% of the outcrossed seeds had rhizoids after 2 months).

Dactylorhiza sambucina is dimorphic for corolla colour and has been used to study the evolution of colour polymorphism (Gigord et al., 2001, 2002). Gigord et al. (2001) experimentally demonstrated that pollinator-mediated negative frequency-dependent selection (NFDS) accounts for the maintenance of this colour polymorphism. However, several ecological factors might explain local deviations from a 1 : 1 morph ratio (Jersáková et al., 2006; Smithson et al., in press; Gigord et al., in prep.).

Interestingly, there was a tendency for purple plant progenies to have lower survival rates than yellow ones due to higher inbreeding depression coefficients for purple families compared to yellow ones. This fitness difference between colour morphs could have an impact on population morph ratio, and might induce a bias in NFDS equilibrium frequency towards higher frequency of the fittest morph. Jersáková et al. (2006) also found higher seed mass and seed viability for purple plant progenies compared to yellow ones in two purple biased Czech populations. Thus, fitness difference between morphs clearly deserves further detailed studies, particularly in relation to population corolla colour morph ratio.

Acknowledgements

We thank Stéphane Voeffray and François Fulleman for help in the lab, and Mailyn Gonzalez, Antonina Internicola, Vincent Herpailler, and Jean-Marie Robert-Nicoud for their help in transplanting and rearing the seedlings in the greenhouse. We also thank Mélanie Glaettli and Antonina Internicola for their comments on a previous version of the manuscript. This research was supported by the Swiss National Science Foundation research grant referenced 3100A0-100754/1 to Luc D. B. Gigord.

References

Ågren, J. and Schemske, D. W. (1993) Outcrossing rate and inbreeding depression in two annual monoecious herbs, *Begonia hirsuta* and *B. semiovata*. Evolution 47, 125 – 135.

Arditti, J. and Abdul Ghani, A. K. (2000) Numerical and physical properties of orchid seeds and their biological implications. New Phytologist 145, 367 – 421.

Barrett, S. C. H. (2002) The evolution of plant sexual diversity. Nature Reviews Genetics 3, 274 – 284.

Charlesworth, D. and Charlesworth, B. (1987) Inbreeding depression and its evolutionary consequences. Annual Reviews of Ecology and Systematics 18, 237 – 268.

Dressler, R. L. (1981) The Orchids: Natural History and Classification. Cambridge, Massachussets: Harvard University Press.

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

Gigord, L., Macnair, M., and Smithson, A. (2001) Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.) Soò. Proceedings of the National Academy of Sciences of the USA 98, 6253 – 6255.

Gigord, L. D. B., Macnair, M. R., Stritesky, M., and Smithson, A. (2002) Experimental evidence for floral mimicry in a rewardless orchid. Proceedings of the Royal Society London B 269, 1389 – 1395.

Gill, D. E. (1989) Fruiting failure, pollinator inefficiency, and speciation in orchids. In Speciation and its Consequences (Otte, D. and Endler, J. A., eds.), Sunderland, Massachussets: Sinauer, pp. 458 – 481.

Husband, B. C. and Schemske, D. W. (1996) Evolution and timing of inbreeding depression in plants. Evolution 50, 54 – 70.

Jersáková, J., Kindlmann, P., and Renner, S. S. (2006) Is the colour dimorphism in *Dactylorhiza sambucina* maintained by differential seed viability instead of frequency-dependent selection? Folia Geobotanica 41, 61 – 76.

Johnson, S. D. and 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., and Ågren, J. (2004) The effects of nectar addition on pollen removal and geitonogamy in the non-rewarding orchid *Anacamptis morio*. Proceedings of the Royal Society of London Series B 271, 803 – 809.

Keller, L. F. and Waller, D. M. (2002) Inbreeding effects in wild populations. Trends in Ecology and Evolution 17, 230 – 241.

Manly, B. F. J. (1997) Randomization, Bootstrap and Monte Carlo Methods in Biology. London: Chapman and Hall.

McKendrick, S. L. (2000) *In vitro* germination of orchids : a manual. Available from http://www.ceiba.org/documents/CFTCpropman.pdf.

Mitchell, R. B. (1989) Growing hardy orchids from seed at Kew. The Plantman 11, 152 – 169.

Neiland, M. R. M. and Wilcock, C. C. (1998) Fruit set, nectar reward, and rarity in the Orchidaceae. American Journal of Botany 85, 1657 – 1671.

Nilsson, L. A. (1980) The pollination ecology of *Dactylorhiza sambucina* (Orchidaceae). Nordic Journal of Botany 1, 461 – 480.

Nilsson, L. A. (1992) Orchid pollination biology. Trends in Ecology and Evolution 7, 255 – 258.

Peakall, R. and Beattie, A. J. (1996) Ecological and genetic consequences of pollination by sexual deception in the orchid *Caladenia tentaculata*. Ecology 50, 2207 – 2220.

Rasmussen, H. N. and Whigham, D. F. (1993) Seed ecology of dust seeds *in-situ* – a new study technique and its application in terrestrial orchids. American Journal of Botany 80, 1374 – 1378.

Rasmussen, H. N. (1995) Terrestrial Orchids: From Seed to Mycotrophic Plant. Cambridge: Cambridge University Press.

R Development Core Team (2004) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-00-3. http://www.R-project.org.

Smithson, A. (2002) The consequences of rewardlessness in orchids: reward supplementation experiments with *Anacamptis morio* (Orchidaceae). American Journal of Botany 89, 1579 – 1587.

Smithson, A. (2006) Pollinator limitation and inbreeding depression in orchid species with and without nectar rewards. New Phytologist 169, 419 – 430.

Smithson, A., Juillet, N., Macnair, M. R., and Gigord, L. D. B. (2006) Is there a positive relationship between phenotypic diversity and reproductive success in rewardless orchids? Ecology, in press.

Tremblay, R. L., Ackerman, J. D., Zimmerman, J. K., and Calvo, R. N. (2005) Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. Biological Journal of the Linnean Society 84, 1 – 54.

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

N. Juillet

Department of Ecology and Evolution
University of Lausanne
1015 Lausanne
Switzerland

E-mail: nicolas.juillet@unil.ch

Editor: F. Salamini