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ORIGINAL ARTICLE

Pollen flow and post-pollination barriers in two varieties of *Dactylorhiza incarnata* s.l. (Orchidaceae)

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Abstract *Dactylorhiza* orchids are known for their high variation in morphology and distinct varieties have been named in *D. incarnata* s.l. However, it is not known how these varieties interact in mixed populations and why they remain stable. We conducted three field experiments in West-Estonian populations of *D. incarnata* to examine if the two most common varieties co-occurring these are separated from each other either by pre-pollination or post-pollination reproductive barriers. We found that pollinia were far more frequently transferred between the purple-flowered var. *incarnata* and the pale-flowered var. *ochroleuca* than between plants of the same variety. Furthermore, in hand-pollination and germination experiments concerning the same two varieties, we found that pollen source (self-pollination, within- or between-varieties pollination) did not affect seed production or probability of fungus infection of the germinating seeds. These two varieties of *D. incarnata* thus had no pre-pollination or early functioning post-pollination reproductive barriers. Post-pollination barriers may, however, act later in seedling or adult stage.

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Introduction

Orchids are known for their high morphological variation and abundance of species, which have been thought to be a result of a deceptive pollination system in one-third of the species (Cozzolino and Widmer 2005a). As visiting deceptive flowers is not advantageous for insects, no flower constancy develops, and pollinators quickly abandon the non-rewarding plants and move on to other species in the plant community (Waddington 1983; Dafni 1987; Goulson 1999; Smithson and Gigord 2003). Neiland and Wilcock (1999) found that stigmas of the nectarless orchid species were contaminated with heterospecific pollen more often than those of the nectariferous species. These findings indicate that pollinators of deceptive orchids frequently move from one inflorescence type to another. Consequently, pollinator movements in a mixed population of deceptive taxa would be more frequent between morphologically distinct taxa (e.g. species, subspecies or varieties) than within similar ones. Because of this shifting from one form to another, individuals representing the rare form may benefit as they have relatively more visits compared to the more common one (Smithson and Macnair 1997). This negative frequency dependence of pollinator visits has been first confirmed experimentally with artificial flowers (Smithson and Macnair 1997) and later with *Dactylorhiza sambucina* (L.) Soó morphs (Gigord et al. 2001). However, in our previous study conducted with Estonian *Dactylorhiza incarnata* (L.) Soó populations, we failed to find such negative frequency dependence in reproductive success (Vallius et al. 2004). On the contrary, the less common variety usually had lower capsule set in mixed populations.

Pollination biology of deceptive species, especially orchids, has been studied using several different methods. Smithson and her colleagues chose to use artificial flowers instead of natural plants (e.g. Smithson and Macnair 1997). There are several advantages related to the use of artificial flowers and laboratory-raised bumblebees to control the variation in flowers (physical condition, previous visits, scents, etc.) and in pollinators (species, previous experiences from other flowers). Theories formed with the help of these experiments are worth testing, but they cannot be taken as sole answers to the questions in pollination ecology. Nilsson (1980) observed insects visiting deceptive orchids in the field, which has been found to be not only a useful but also a laborious method to collect data on pollinator behaviour (see also Koivisto et al. 2002). Pollen flow has also been studied using differences in the morphology of pollen (Neiland and Wilcock 1999) or molecular methods (e.g. Cozzolino et al. 2005) to identify pollen found on stigmas or attached to the pollinating insects. Tracking the transfer of individual pollinia from plant to plant was conducted by Nilsson et al. (1992) who used micro-tags to examine pollen flow in *Aerangis ellisii* (Rchb. f.) Schltr. Micro-tagging is, however, a very laborious method, and the exact faith of each pollinium is not always important. An easier way to examine pollen flow was introduced by Peakall (1989) who used differently coloured histochemical stains to mark orchid pollinia. This method has been found not to affect the probability of pollinia removal in orchids (e.g. Johnson et al. 2004). Despite its good properties, this method has been very seldom used in studies on orchid pollination.

Before molecular methods became more widely used, plants were classified mainly by morphological characters. High number of different taxa found in orchids has been explained by pollinators' speciation (e.g. Tremblay 1992). More advanced methods have helped to see the orchid taxonomy from a different angle. In his study using allozyme markers to classify *Dactylorhiza* Necker ex Nevski species, Hedrén (1996a) found that *D. incarnata* var. *incarnata* (L.) Soó, var. *ochroleuca* (Wüstnei ex Boll) P.F. Hunt & Summerh. and var. *cruenta* (O.F. Müll.) P.D. Sell had very little variation and were identical in alloenzymes, even though *D. incarnata* s.l. differed clearly from *D. maculata* (L. Soó). This means that despite distinctive differences in morphology the varieties of *D. incarnata* are very closely related, which was also shown for var. *incarnata* and var. *ochroleuca* in the study of Pillon et al. (2006) using molecular analyses (plastid and nuclear ITS ribosomal DNA markers). High variation in morphology found in nectarless orchid species may be a result of crossing between morphologically different forms or caused by environmental conditions (phenotypic plasticity). Different varieties of *D. incarnata* s.l. are often found in mixed populations (Hedrén 1996b). Neiland and Wilcock (2000) found inter-specific pollination to be frequent in mixed populations of orchids in Scotland. Pedersen (1998) also argued that no effective pre-pollination barrier had been demonstrated between *D. incarnata* var. *incarnata* and the white-flowered var. *ochrantha* Landwehr, which was also supported by allozyme data with no diagnostic alleles in *D. incarnata* s.l. These studies thus show that variation in morphology of *D. incarnata* s.l. cannot be explained by genetic diversification.

In our previous study in Estonian *Dactylorhiza incarnata* s.l. populations (Vallius et al. 2004), we failed to find a satisfactory explanation to the co-occurrence of var. *incarnata*, var. *ochroleuca* and var. *cruenta* in mixed populations. We found var. *cruenta* to differ morphologically from the other two varieties only in one population and differences in flowering times and natural seed production were controversial. The varieties thus seem to differ only in pigmentation of leaves and flowers, which probably has no strong selection effect. If functioning pre-pollination reproductive barriers are absent between co-occurring varieties, pollen flow between differently coloured plants is probably frequent, especially in deceptive species. Diversification of different varieties to new species would thus be hampered by continuous gene flow if there is no strong post-pollination barrier present to prevent the production of hybrid progeny.

To examine the role of pollinator behaviour and reproductive barriers in the maintenance of mixed populations, we studied pollen flow and post-pollination reproductive barriers between two varieties of *D. incarnata* s.l. (var. *incarnata* and var. *ochroleuca*) in the island of Hiiumaa in western Estonia. Using hand-pollination experiments, in situ germination method, and by observing pollinia transfer we examined (1) if pollinia movements are restricted between varieties (pre-pollination reproductive barrier) and (2) if there is reproductive isolation functioning during seed production (post-pollination reproductive barrier). To our knowledge, this is the first study in which dyed pollen was used to examine pollen flow between orchid morphs or varieties.

Materials and methods

Study species and sites

Dactylorhiza incarnata s.l. has many varieties, three of which are found in Estonia (Kuusk 1984; Kuusk et al. 2003). The most common of these varieties is *D. incarnata* var. *incarnata*, characterised by its light green, erect, broad leaves and purple flowers; occurring in wide range of areas from mesotrophic fens to alvar meadows. *D. incarnata* var. *ochroleuca*, occurs only in calcareous areas. This variety greatly resembles *D. incarnata* var. *incarnata*, but the flowers are creamy white with yellowish centre of the lip. According to our previous study in Estonia, flowering times of the two varieties overlap (Vallius et al. 2004).

We collected data from four localities in Hiiumaa. One of the study sites is a calcareous meadow (Aruküla) and the rest three are eutrophic mires (Hagaste A, B and C). Populations of *D. incarnata* s.l. in the Hagaste area were regarded as separate populations on the basis of the distance between sites A, B and C, and especially because they were separated by a tall and thick belt of *Juniperus communis* L. Each of the four study sites as well as the study species are protected by law in Estonia.

Monitoring pollinia transfer within populations

In mid-June 2003, pollinia in the lowest 1/3 of the inflorescence of *D. incarnata* plants were stained with aqueous histochemical stains (Sigma) to monitor the pollen flow among co-occurring populations of the two different varieties (Table 1). In Hagaste A and Hagaste B populations, ripe pollinia from 22 to 53% of individual plants in each variety were treated with the stain to get enough marked pollinia for the monitoring of pollen transfer. In the Aruküla population, there were only a few flowering var. *ochroleuca* plants, and, therefore, all flowering var. *ochroleuca* individuals in this site were treated with the stain. Stains were prepared according to Peakall (1989). Because using syringe to inject the liquid to the pockets protecting pollinia proved to be extremely difficult in field conditions, we used a fine art brush to "paint" the pollinia. Brush with stain was gently pushed against the opening in the middle of the "pocket" protecting each pollinium, and the stain was spread to the surface of a pollinium with a few up and down movements within the pocket. During this process the region of the column containing the pollinia was stained, but other parts of the flower usually remained clean of stain.

Table 1 Number of flowering plants of different varieties of *D. incarnata* s.l., number of plants with stained pollinia, and the staining chemical used for a pollinia transfer experiment conducted in three Estonian populations

Population	Variety	*N* of plants	*N* of stained	Stain used (1%)
Hagaste A	*incarnata*	45	23	Neutral red
	ochroleuca	55	23	Brilliant green
Hagaste B	*incarnata*	60	13	Neutral red
	ochroleuca	17	9	Brilliant green
Aruküla	*incarnata*	20	12	Neutral red
	ochroleuca	7	7	Brilliant green

In all three populations, the *D. incarnata* flowers were checked for pollen deposition during four consecutive days after the staining treatment. Stigmas of all open flowers were checked from the non-treated plants and from flowers of the upper 2/3 of the inflorescence of the stained plants. Stigmas of stained flowers were also checked for interspecific pollen deposition with differently coloured stain. A magnifying class was used to count the number of flowers with stained pollen on the stigma from each plant. For the analysis, the pollinia movements were categorised at the plant level regarding a plant as a receiver if it had at least one flower with stained pollen on its stigma. The number of intra- and inter-variety movements of pollinia was counted for each population.

Hand-pollination experiment

In June 2000, a total of 30 flowering plants of *D. incarnata* var. *incarnata* ($n = 17$) and var. *ochroleuca* ($n = 13$) were chosen for a controlled hand-pollination experiment conducted in the Hagaste C area. The study plants were marked with numbered sticks and covered with light mesh bags to exclude the pollinators. Pollinia were removed from the experimental plants using wooden sticks to prevent autogamous pollination. Two to four freshly opened flowers of each study plant were pollinated with one pollinium from (a) the same inflorescence (self-pollination), (b) another plant of the same variety (within-variety pollination) and (c) a plant from the other variety (between-varieties pollination) by placing one pollinium to stigmas with tweezers. The hand-pollinated flowers were individually marked with light plastic rings of different colour indicating the pollination treatment. The mesh bags were removed after a few days when stigmas of the hand-pollinated flowers had lost their receptivity. Upper flowers of the experimental plants opened normally after the removal of the mesh bags, and some of them developed to mature capsules. These capsules were treated as a reference for the results from the hand-pollination treatments and are later referred to as "open-pollination".

In the end of July, mature capsules were collected, sorted by pollination treatment and stored in a freezer. Capsules were then oven-dried in 80°C for 24 h and weighed using an analytical scale. The proportion of embryonic seeds was counted using a microscope from samples of >100 seeds. A seed was counted as embryonic when (1) it had a normally shaped dark brown embryo and (2) when the width of the embryo was at least 2/3 of the width of the seed.

In situ germination experiment

In June 2003, ten *D. incarnata* var. *incarnata* plants were chosen from the Hagaste A area for a hand-pollination experiment. These plants grew far away from the main population and were not included in the pollinia staining study described above. Plants were marked with number sticks and covered with mesh bags to exclude pollinators. Two to three flowers of each selected plant were pollinated with one pollinium from (a) the same inflorescence (self-pollination), (b) another var. *incarnata* plant (within-variety pollination), (c) var. *ochroleuca* plant (between-varieties pollination) and (d) *Orchis militaris* L. (between-species pollination).

In August 2003, mature capsules were collected. Seeds from capsules from the same plant and treatment were mixed and divided into two batches (if there were enough seeds). All seeds from each batch (or alternatively a few hundred seeds, if there were too many seeds for a comparable batch) were placed to each in situ germination unit prepared from a glassless slide mount and piece of plankton netting (Sefar Nitex 03-36/28) (see Rasmussen and Whigham 1993 for detailed methodology). The slide mounts were marked with plant and treatment codes. These germination units were then tied to a nylon line leaving approximately 10 cm between the units. They were taken to the Hagaste A site and placed in the ground in two separate sets. Ends of the lines were anchored to the ground with bamboo sticks and the evenly spread germination units were lightly covered with detritus.

At the end of May 2004, the germination units were removed from the ground and gently rinsed under running water. After opening the mount, the inner side of the netting was placed against the surface of 1.2% water agar (see Rasmussen and Whigham 1993) on a Petri dish. Agar with transferred seeds was placed under stereomicroscope and the seeds were counted. Because there were no protocorms in the samples, the proportion of seeds with ingrown fungal hyphae was examined. Growth of internal hyphae is not a straightforward estimate of seed germination, but fungal growth is an obligate factor in the natural seed germination process (see e.g. Zelmer et al. 1996).

Statistical tests

Differences in the capsule dry weight and the proportion of embryonic seeds from all seeds between different varieties and different pollination treatments were tested using two-way ANOVA.

Differences in frequencies of plants producing or failing to produce capsules after different pollination treatments were tested using Cochran's Q test, and the differences in proportion of seeds infected with mycorrhizal fungus was tested using Friedman's test, in which every plant acted as a block including all four pollination treatments.

To test the differences in pollen movements within and between var. *incarnata* and var. *ochroleuca*, the relative number of movements of stained pollinia from one plant to other plants was counted taking the proportion of plants with stained pollinia from all flowering plants of the variety in the population into account. Because the registered movements between plants had the same pattern (direction and relative number of movements within and between varieties) in all three populations, a combined contingency table was created from the relative number of within- and between-varieties movement of stained pollinia. Because the sum of numbers in each row was not fixed, the general test for fourfold contingency tables was used by calculating P values to all tables more extreme than the observed table and using their sum as the P-value (Ranta et al. 2004).

Results

Pollen transfer within and between varieties

A total of 70 pollinator movements that can be considered as transfer of pollinia from a flower with stained pollinia to another flower either within or between inflorescences were counted in the three populations during the 4-day study period. In most plants, none or only one flower had received stained pollen on stigma, but some plants had up to six flowers with stained pollen. Frequencies of movements of the stained pollinia within and between varieties differed significantly from random (General two-way test for fourfold contingency tables: $P < 0.001$, Fig. 1). Transport of stained pollinia was far more frequent between than within varieties, and var. *ochroleuca* pollen was found on stigmas of var. *incarnata* more often than var. *incarnata* pollen on stigmas of var. *ochroleuca.*

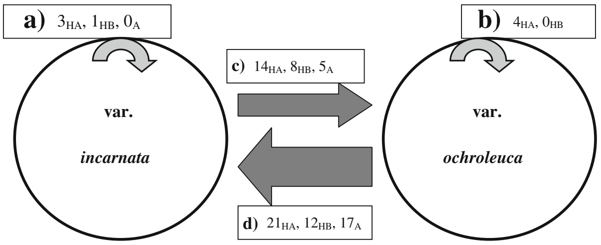

Fig. 1 Number of pollinia transferred within (**a**, **b**) and among (**c**, **d**) two varieties in mixed *D. incarnata* s.l. populations (*HA* Hagaste A, *HB* Hagaste B, *A* Aruküla)

Seed production

The four pollination treatments (self-pollination, cross-pollination with pollen from the same variety, cross-pollen with pollen from different variety and open-pollination) differed in influence on the dry weight of capsules (two-way ANOVA MS = 426.77, $F = 5.73$, $df = 3$, $P = 0.001$, Fig. 2). Post-hoc tests revealed that dry weight of capsules was lower after natural pollination compared to self-pollination ($P = 0.002$), within-variety pollination ($P = 0.006$) and between-variety pollination ($P = 0.001$). There was no difference in dry weight of capsules produced between self-pollination, within-variety pollination or between-variety pollination ($P > 0.960$ for all tested pairs). The mean dry weight of capsules was greater in var. *ochroleuca* than in var. *incarnata* (two-way ANOVA: MS = 864.57, $F = 5.73$, $df = 1$, $P = 0.001$, Fig. 1), but there was no significant interaction between variety and pollination treatment (MS = 35.70, $F = 0.479$, $df = 3$, $P = 0.697$).

There were no significant differences between the varieties (MS = 0.004, $F = 0.053$, $df = 1$, $P = 0.818$) or pollination treatments (MS = 0.024, $F = 0.295$, $df = 2$, $P = 0.745$) concerning the proportion of embryonic seeds from all seeds produced, and there was no significant interaction between the treatments and varieties (MS = 0.014, $F = 0.168$, $df = 3$, $P = 0.845$).

Seed germination

Some of the var. *incarnata* plants failed to produce capsules after self-pollination and after cross-pollination with pollinium from another var. *incarnata* plant, but the difference in capsule production between pollination treatments was not statistically significant (Cochran's test: $Q = 3.273$, $df = 3$, $P = 0.351$). Germination of seeds after incubation of 8 months in situ was very low in all treatments. No protocorm formation was found, but some of the seeds were already infected by fungi. There no differences in proportion of infected seeds from all seeds amog the four pollination treatments (Friedman: $\chi^2 = 3.875$, $df = 3$, $P = 0.275$, Fig. 3)

Discussion

Pre-pollination barrier

We did not find any pre-pollination barrier reducing pollinia transfer between *D. incarnata* var. *incarnata* and var. *ochroleuca* plants. In the light of this result, co-occurrence of different varieties cannot be explained with a restricted pollen flow between the varieties, as suggested by Hedrén (2002). On the contrary, in all study populations, pollinia moved more often between than within them. This difference between intra- and inter-variety pollinator movements may, in fact, be even more striking, because we could not separate geitonogamous pollination of stained plants from the real intra-variety pollen transfer. Effect of geitonogamous pollination may be important, however, as Kropf and Renner (2007) have shown the geitonogamy levels as high as 40% in the flower-dimorphic orchid *Dactylorhiza sambucina*. The observed higher frequency of between-variety pollen transfer is probably a result of an avoidance behaviour of the pollinating insects. After visiting a series of empty flowers, pollinators tend to move to differently coloured flowers to increase the probability of finding a reward (Neiland and Wilcock 2000; Smithson and Gigord 2003).

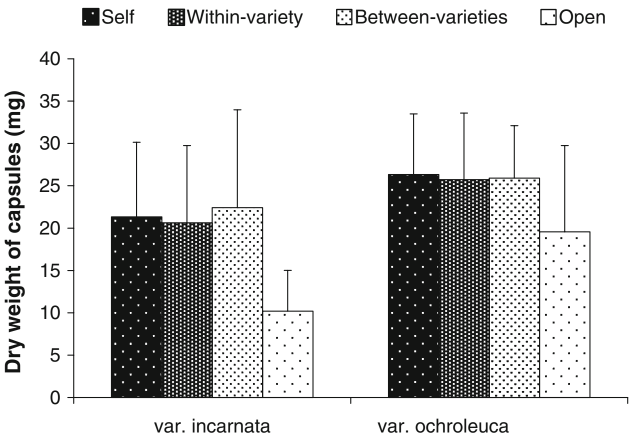

Fig. 2 Dry weight of capsules produced after pollination with self-pollen, within-variety cross-pollen, between-varieties cross-pollen and natural pollination (*open*) in two varieties of *D. incarnata* s.l.

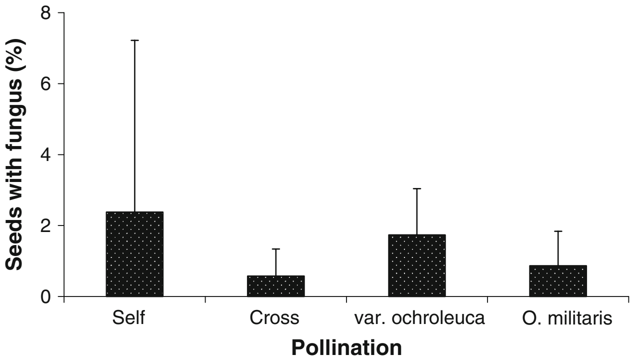

Fig. 3 The proportion of seeds (%) with ingrown fungus hyphae in a 9-month in situ germination experiment with *D. incarnata* var. *incarnata*. Seeds used in the experiment originated from controlled hand-pollinations using pollen from the same inflorescence (self), another var. *incarnata* plant (cross), another *D. incarnata* variety (var. *ochroleuca*) and a related orchid species (*Orchis militaris*)

Among inter-variety transfer, pollinia were moved more frequently from var. *ochroleuca* than from var. *incarnata*. This may be a result of pollinators moving to *D. incarnata* from other rewarding species in the area. Pollinators' choices of inflorescence are affected by a generalised searching image (Gumbert 2000). In addition to two pink-flowered species (*Primula farinosa* L. and *Gymnadenia conopsea* (L.) R. Br.), pollinators mainly had food-plants with white or yellow flowers (e.g. *Galium boreale* L., *G. palustre* L., *Potentilla erecta* (L.) Räuschel, *Ranunculus acris* L.) in the study sites. The latter group of species is probably frequently visited by bees also capable of pollinating *Dactylorhiza* orchids. With a closer resemblance with food-flowers the pale-flowered var. *ochroleuca* plants received more first-visits than the purple-flowered var. *incarnata* plants. Furthermore, after visiting deceptive flowers pollinators tend to move to an inflorescence with a different coloration, thus transferring pollen from var. *ochroleuca* flowers preferentially to var. *incarnata* flowers. This may also explain why negative frequency dependence was not found in our previous study (Vallius et al. 2004). As in most populations the number of flowering var. *ochroleuca* plants was lower than that of var. *incarnata*, and because flowers of the rewarding neighbour species were mainly white and yellow in colour, pollen flow was mainly from the rare to the common variety. The variety with lower frequency (*ochroleuca*) thus acted as a pollen donor, while the more common variety (*incarnata*) had higher female reproductive success. Smithson et al. (2007) got similar results with *D. sambucina* in which pollinia removal in particular was higher in populations with a high proportion of the yellow-flowered plants compared to the red-flowered ones. This could be a result of an innate preference of bees for yellow (Gigord et al. 2001) or the yellow flowers being more conspicuous to bees against the background (Spaethe et al. 2001). The former hypothesis was not supported by the results of Gumbert (2000), who found a strong innate preference of bumblebees for violet (wavelengths 400–420). The latter hypothesis, however, seems more plausible for the *D. incarnata* populations. Bees can also see UV wavelengths (e.g. Spaethe et al. 2001), and therefore the main UV-absorbing white flowers of var. *ochroleuca* (Vallius et al. 2004) form a striking contrast with the UV-reflecting green background vegetation, thus enhancing their detectability.

Post-pollination barrier

There was no functional post-pollination reproductive barrier between the var. *incarnata* and var. *ochroleuca* plants as measured by seed production. This is not surprising, since a high genetic resemblance has been found between these varieties (Hedrén 1996b). In their study with *Serapias* L. (Orchidaceae), Pellegrino et al. (2005c) found that despite the absence of floral isolation, the co-occurring species were easily identified with the help of microsatellite data. Consequently they argued that reproductive barriers acting post-zygotically could explain the observed reproductive isolation. A post-zygotic reproductive barrier could act, for example, via low seed quality (seed mass, viability and germination success) as was found in the dimorphic *Dactylorhiza sambucina* (Jersáková et al. 2006). Pellegrino et al. (2005a) also found that the pink-flowered *D. sambucina* plants had much lower fertility than the more common morphs, which fact could be explained by an association between the anthocyanin pigment synthesis and plant fertility. These differences in female reproductive success between intra- and inter-morph crossings cannot be seen in fruit production, because normal capsule formation can be found also after intra-morph crosses in *D. sambucina* (Pellegrino et al. 2005b).

In our study, the var. *incarnata* plants even managed to produce seeds following pollination with inter-specific *O. militaris* pollen. Capsules produced following inter-specific pollination were small, but contained at least some normal seeds, which did not differ from seeds produced after intra-specific pollination in frequency of fungal infection (Fig. 3). Hybridisation experiments (Malmgren 1992) have shown that in most cases orchids are able to produce seeds also after inter-specific pollination, but there were only a few viable ones in a sample of 1,000 seeds. This kind of seed production may be possible because also genetically incompatible and inviable pollen may trigger the maturation of ovules and the production of agamospermous seeds (E. Vallius and J. Stout, unpublished).

There was no difference in proportion of seeds with a fungus infection between the seeds from different pollination treatments. After 9 months in the ground, all seeds were more or less intact with only a few of them having a fungus infection. Despite the presence of fungi, the seeds were not swollen or showed any other sign of germination. At least in Estonia, the germination of *D. incarnata* seeds seems not to take place before May. This is probably due to a snow cover during winter and low temperature of soil water in spring. It is also possible that the placing of the germination units was not most favourable for the presence of mycorrhizal fungi essential for the germination, even though there were several *D. incarnata* plants growing in the area. Germination of *Dactylorhiza* seeds has been shown to be related to soil nutrient levels (Dijk and Eck 1995) and light availability (Rasmussen et al. 1990). In their in situ germination experiment, Rasmussen and Whigham (1993) also found zero germination rates in some areas and some orchid species used. Among Mediterranean deceptive orchids, different species have been shown to share pollinators (Cozzolino et al. 2005), and post-mating reproductive isolation acts as late as in the F_1 hybrid generation, which usually suffers from low fertility (Cozzolino and Widmer 2005b; Cozzolino et al. 2006).

Concluding remarks

Our results show that pollinia movements are frequent between *D. incarnata* var. *incarnata* and var. *ochroleuca*, and no functional post-pollination reproductive barrier could be found in seed production. The presence of these varieties in mixed populations thus cannot be explained by pollinator behaviour or incompatibility, which leaves us with three additional hypotheses to be tested in future. First, there is a possibility for a post-pollination barrier acting later in the F_1-generation, as it was found in the hybrids of some Mediterranean orchids (Cozzolino and Widmer 2005b; Cozzolino et al. 2006). Secondly, differences in pigmentation may be a result of extremely high phenotypic plasticity. This explanation does not seem probable, because co-occurring varieties often grow closely together and differences in e.g. light availability or soil properties thus cannot be significant. Kropf and Renner (2005) argued that in *D. sambucina*, the fitness of different colour morphs may be related to microclimates and soil type and the existing colour dimorphism was not maintained by pollinators. This third explanation seems to be at least partially true also for *D. incarnata*, because var. *ochroleuca* and var. *cruenta* are very rare in more acid soils of eastern Estonia and Scandinavia compared to the calcareous soils of western Estonia. Because the colour differences among the varieties are mainly based on the amount of pigmentation (especially anthocyanin) e.g. purple or pale flower coloration could be inherited in a single or few genes (Hedrén 1996b). If this is true, then in calcareous soils, the dominance relations of these alleles, and probably the number of each in a plant would determine if it will perform as var. *incarnata* or var. *ochroleuca*. The roles of phenotypic plasticity and genetic determination in pigmentation of *Dactylorhiza* should thus be studied in future.

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References

Cozzolino S, Widmer A (2005a) Orchid diversity: an evolutionary consequence of deception? Trends Ecol Evol 20:487–494

Cozzolino S, Widmer A (2005b) The evolutionary basis of reproductive isolation in Mediterranean orchids. Taxon 54:977–985

Cozzolino S, Schiestl FP, Müller A, De Castro O, Marco Nardella A, Widmer A (2005) Evidence for pollinator sharing in Mediterranean nectar-mimic orchids: absence of premating barriers? Proc Roy Soc B 272:1271–1278

Cozzolino S, Nardella AM, Impagliazzo S, Widmer A, Lexer C (2006) Hybridization and conservation of Mediterranean orchids: should we protect the orchid hybrids or the orchid hybrid zones? Biol Conservation 129:14–23

Dafni A (1987) Pollination of *Orchis* and related genera: evolution from reward to deception. In: Arditti J (ed) Orchid biology: reviews and perspectives, vol. IV. Cornell University Press, New York, pp 80–104

Dijk E, Eck N (1995) Axenic in vitro nitrogen and phosphorus responses of some Dutch marsh orchids. New Phytol 131:353–359

Gigord LDB, Macnair MR, Smithson A (2001) Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina*. Proc Natl Acad Sci USA 98:6253–6255

Goulson D (1999) Foraging strategies of insects for gathering nectar and pollen, and implications for plant ecology and evolution. Perspect Pl Ecol Evol Syst 2:185–209

Gumbert A (2000) Color choices by bumble bees (*Bombus terrestris*): innate preferences and generalization after learning. Behav Ecol Sociobiol 48:36–43

Hedrén M (1996a) Genetic differentiation, polyploidization and hybridization in northern European *Dactylorhiza* (Orchidaceae): evidence from allozyme markers. Pl Syst Evol 201:31–55

Hedrén M (1996b) Notes on the esterase variation in Swedish *Dactylorhiza incarnata* s.l. (Orchidaceae). Nord J Bot 16:253–256

Hedrén M (2002) Speciation patterns in the *Dactylorhiza incarnata/maculata* polyploid complex (Orchidaceae): evidence from molecular markers. Jour Eur Orch 34:707–731

Jersáková J, Kindlman P, Renner SS (2006) Is the colour dimorphism in *Dactylorhiza sambucina* maintained by differential seed viability instead of frequency-dependent selection? Folia Geobot 41:61–76

Johnson SD, Peter GI, Ågren J (2004) The effects of nectar addition on pollen removal and geitonogamy in the non-rewarding orchid *Anacamptis morio*. Proc Roy Soc Lond B 271:803–809

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

Kropf M, Renner S (2005) Pollination success in monochromic yellow populations of the rewardless orchid *Dactylorhiza sambucina*. Pl Syst Evol 254:185–197

Kropf M, Renner S (2007) Pollinator-mediated selfing in two deceptive orchids and a review of pollinium tracking studies addressing geitonogamy. Oecologia 155(3):497–508

Kuusk V (1984) Orchidaceae. In: Eichwald K, Kukk E, Lellep E, Mäemets A, Rebassoo H, Sander R, Talts S, Trei T, Viljasoo L (eds) Eesti NSV Flora IX. Valgus, Tallinn, pp 320–408

Kuusk V, Cepur te B, Ryla M (2003) *Dactylorhiza*. In: Kuusk V, Tabaka L, Tankevičiene R (eds) Flora of baltic countries, compendium of vascular plants III. Estonian Agricultural University, Tartu, pp 378–384

Malmgren S (1992) Hybridisering bland svenska orkideer—korsnings- och odlingsförsök. Svensk Bot Tidskr 86:337–346; (In Swedish)

Neiland MRM, Wilcock CC (1999) The presence of heterospecific pollen on stigmas of nectariferous and nectarless orchids and its consequences for their reproductive success. Protoplasma 208:65–75

Neiland MRM, Wilcock CC (2000) Effects of pollinator behaviour on pollination of nectarless orchids: floral mimicry and interspecific hybridisation. In: Wilson KL, Morrison DA (eds) Monocots: systematics and evolution. CSIRO, Melbourne, pp 318–325

Nilsson LA (1980) The pollination ecology of *Dactylorhiza sambucina* (Orchidaceae). Bot Notiser 133:367–385

Nilsson LA, Rabakonandrianina E, Pettersson B (1992) Exact tracking of pollen transfer and mating in plants. Nature 360:666–668

Peakall R (1989) A new technique for monitoring pollen flow in orchids. Oecologia 79:361–365

Pedersen HÆ (1998) Allozyme variation and genetic integrity of *Dactylorhiza incarnata* (Orchidaceae). Nord J Bot 18:15–21

Pellegrino G, Bellusci F, Musacchio A (2005a) Evidence of post-pollination barriers among three colour morphs of the deceptive orchid *Dactylorhiza sambucina* (L.) Soó. Sex Pl Reprod 18:179–185

Pellegrino G, Caimi D, Noce ME, Musacchio A (2005b) Effects of local density and flower colour dimorphism on pollination and reproduction in the rewardless orchid *Dactylorhiza sambucina* (L.) Soó. Pl Syst Evol 251:119–129

Pellegrino G, Musacchio A, Noce ME, Palermo AM, Widmer A (2005c) Reproductive versus floral isolation among morphologically similar *Serapias* L. species (Orchidaceae). J Hered 96:15–23

Pillon Y, Fay MF, Shipunov AB, Chase MW (2006) Species diversity versus phylogenetic diversity: a practical study in the taxonomically 3difficult genus *Dactylorhiza* (Orchidaceae). Biol Conservation 129:4–13

Ranta E, Rita H, Kouki J (2004) Biometria. Yliopistopaino, Helsinki; (in Finnish)

Rasmussen HN, Whigham DF (1993) Seed ecology of dust seeds in situ: a new study technique and its application in terrestrial orchids. Amer J Bot 80:1374–1378

Rasmussen HN, Andersen TF, Johansen B (1990) Light stimulation and darkness requirement for the symbiotic germination of *Dactylorhiza majalis* (Orchidaceae) in vitro. Physiol Pl 79:226–230

Smithson A, Gigord LDB (2003) The evolution of empty flowers revisited. Amer Naturalist 161:537–552

Smithson A, Macnair MR (1997) Negative frequency-dependent selection by pollinators on artificial flowers without rewards. Evolution 51:715–723

Smithson A, Juillet N, Macnair MR, Gigord LDB (2007) Do rewardless orchids show a positive relationship between phenotypic diversity and reproductive success? Ecology 88:434–442

Spaethe J, Tautz J, Chittka L (2001) Visual constraints in foraging bumblebees: flower size and color affect search time and flight behaviour. Proc Natl Acad Sci 98:3898–3903

Tremblay RL (1992) Trends in the pollination ecology of the Orchidaceae: evolution and systematics. Canad J Bot 70:642–650

Vallius E, Salonen V, Kull T (2004) Factors of divergence in co-occurring varieties of *Dactylorhiza incarnata* (Orchidaceae). Pl Syst Evol 248:177–189

Waddington KD (1983) Foraging behaviour of pollinators. In: Real L (ed) Pollination biology. Academic Press, London, pp 213–239

Zelmer CD, Cuthbertson L, Currah RS (1996) Fungi associated with terrestrial orchid mycorrhizas, seeds and protocorms. Mycoscience 37:439–448