IS THE COLOUR DIMORPHISM IN *DACTYLORHIZA SAMBUCINA* MAINTAINED BY DIFFERENTIAL SEED VIABILITY INSTEAD OF FREQUENCY-DEPENDENT SELECTION?

Jana Jersáková[1], **Pavel Kindlmann**[1,2] **& Susanne S. Renner**[3]

1) Department of Theoretical Ecology, Institute of Systems Biology and Ecology of the Academy of Sciences of the Czech Republic and Faculty of Biological Sciences, University of South Bohemia, Branišovská 31, CZ-370 05 České Budějovice, Czech Republic; e-mail jersa@centrum.cz
2) Agrocampus Rennes, 65 rue de St. Brieuc, F-35042 Rennes Cedex, France
3) Department of Biology, Ludwig Maximilians University, Menzinger-Str. 67, D-80638 Munich, Germany

Abstract: The European rewardless, bee-pollinated orchid *Dactylorhiza sambucina* commonly produces yellow-flowered and purple-flowered individuals in frequencies that range from balanced (per population) to very unbalanced, with parts of the species' range entirely monochromatic. We studied male and female reproductive success of the two morphs in 22 populations in the Czech Republic, relating it to morph frequency, population size and density, and presence and abundance of yellow and purple co-flowering nectar-providing species visited by the same bee species. Cumulative abundances of yellow nectar-producing co-flowering species (of which, on average, *Primula veris* made up 56%) had a negative effect on male reproductive success of the yellow morph, and spectral analyses showed that to bumblebees the colours of *P. veris* and yellow *D. sambucina* are different, permitting ready visual discrimination. The cumulative abundance of purple co-flowering species had no significant effect on morph reproductive success. Morph frequencies were unrelated to reproductive success and population size, and there was no evidence of frequency-dependent selection except in one highly unbalanced population. Density of flowering conspecifics was negatively correlated with male reproductive success of the purple morph. Seed mass, viability, and germination success depended on whether seeds resulted from outcrossed or selfed matings and on morph colour. Selfed seeds and seeds produced by the yellow morph from yellow × yellow and yellow × purple crosses had zero germination (after three months), providing the first hint that differential vegetative fitness, rather than differential reproductive fitness via pollinator selection, may explain morph frequencies in *D. sambucina*.

Keywords: *Dactylorhiza*, Flower colour polymorphism, Frequency-dependent selection, Pollinator discrimination, Rewardless flowers, Seed viability

INTRODUCTION

Plant reproductive polymorphisms provide important model systems to investigate the evolution of different sexual systems, plant-pollinator interactions, plant herbivore interactions, the maintenance of genetic diversity, and the evolution of sex and recombination (for a recent review see PANNELL et al. 2005). One such reproductive polymorphism involves flower colour, with populations comprising two or more morphs of co-flowering individuals that differ in petal colour. Colour polymorphisms occur in many species throughout the angiosperms and have been studied by evolutionary biologists since the 1970s (e.g., LEVIN & KERSTER 1970, historical review in SCHEMSKE & BIERZYCHUDEK 2001). Populations

typically comprise yellow- and purple-flowered individuals, yellow- and white-flowered individuals, or blue- and white-flowered individuals. More rarely, petal colour is polymorphic, with individuals exhibiting variable-sized spots or nectar guides on darker or lighter backgrounds (as in *Dactylorhiza maculata* (L.) Soó and the *Scrophulariaceae Bellardia trixago* (L.) ALL.) or variously coloured irregular patches as in *Ipomoea* (CLEGG & DURBIN 2000). In some cases, the genetic backgrounds of colour polymorphisms are understood, for example, in *Ipomoea* where colour patches depend on mobile genetic elements (CLEGG & DURBIN 2000) and in *Antirrhinum* and *Petunia* where particular genes determining yellow, white, and red colours have been identified (references in JONES & REITHEL 2001). A testable model for the maintenance of a colour dimorphism by frequency-dependent selection (FDS) has been proposed for *Dactylorhiza sambucina* (GIGORD et al. 2001), a terrestrial European bee-pollinated orchid that commonly contains yellow-flowered individuals and purple-flowered individuals in frequencies that range from highly balanced to very unbalanced, with parts of the species' range even monochromatic (ZIEGENSPECK 1936, NILSSON 1980, PETTERSSON & NILSSON 1983, VOGEL 1993, GIGORD et al. 2001, 2002, PELLEGRINO et al. 2005, KROPF & RENNER 2005).

Under negative FDS, rare phenotypes have higher fitness than do more common ones, with the implication that a polymorphism is protected from loss through drift and that underlying genetic variation can be maintained for long periods of evolutionary time (CLARKE & O'DONALD 1964, AYALA & CAMPBELL 1974, ROFF 1998, PANNELL et al. 2005). One mechanism through which negative FDS arises is disassortative mating, where mating is more common between different phenotypes, such that the rare phenotype(s) enjoy high fitness through their access to a greater number of compatible mating partners. Plant polymorphisms maintained by FDS through disassortative mating are sexual systems, such as heterostyly (e.g., CURTIS & CURTIS 1985, ECKERT et al. 1996, THOMPSON et al. 2003), gynodioecy (MCCAULEY & BROCK 1998), and several others (PANNELL et al. 2005). That FDS through disassortative mating also maintains the colour polymorphism in *Dactylorhiza sambucina* is supported by morph fitnesses measured in arrays of potted plants with differing frequencies of yellow- and purple-flowering individuals (GIGORD et al. 2001). In mixed populations, pollen export and import of the yellow morph was equal to that of the purple morph, and for fruit set, morph frequencies of 61% yellow and 39% purple individuals represented the equilibrium. The mechanism proposed is that pollinators, mainly bumblebee queens, that have found the flowers devoid of nectar learn to avoid the common morph, which results in over-visitation of the rare morph and hence an increase in its relative fitness. The predicted equilibrium frequency of the yellow morph of 61% was deemed to fit well with its natural mean frequency of 53% ± 2.6 [!] in 174 natural populations (GIGORD et al. 2001). The small but consistent bias in favour of the yellow morph found in the experimental arrays was explained as possibly due to an innate pollinator preference for the yellow colour.

A complicating factor in explaining the maintenance of colour dimorphisms in rewardless plants is that in one way or another all rewardless flowers [an estimated 6500–10000 *Orchidaceae* species (ACKERMAN 1986) and an estimated 3.7% of all angiosperms (RENNER 2006)], mimic rewarding species on whose presence they depend for the sustenance of their pollinators. This implies that rewardless flowers often depend on assortative mating, resulting

from pollinator constancy. Pollinator constancy refers to individual bees tending to visit that colour morph that most closely resembles the most recent rewarding flower they visited (e.g., HEINRICH 1979, WASER 1986, GUMBERT & KUNZE 2001, GIGORD et al. 2002, JOHNSON et al. 2003). Pollinators that have been rewarded by a yellow nectariferous flower will tend to visit similar yellow flowers for their next few visits, which is the basis for pollen export and import in rewardless yellow flowers before a bee encounters another rewarding yellow flower or switches its preference. Since both colour morphs of *D. sambucina* are rewardless, one morph depends on yellow co-flowering rewarding species to attract and maintain pollinator foraging interest, the other on purple co-flowering species. [That colour plays the key role for bumblebees foraging on rewardless orchids, rather than shape, size, or scent, is suggested by the experiments of GUMBERT & KUNZE (2001).] However, not only do bees learn to associate rewards with floral traits and use this acquired knowledge to discriminate between flowers, the preferences of individual bees also depend on the changing relative flower abundances during the season, which implies that bee individuals sometimes explore new flowers in spite of unfamiliar, or previously avoided, flower signals. A corollary of this context-dependent foraging behaviour is that bee-pollinated plants constantly experience frequency-dependent interactions and density-dependent selection (LITTLE 1983, ÅGREN et al. 1986, FERDY et al. 1998, CASTILLO et al. 2002, RENNER 2006).

Colour-dimorphic and nectarless species, such as *D. sambucina*, thus experience a complex interplay between negative FDS resulting from bees discriminating against the more common morph (leading to disassortative mating) and bees overvisiting whichever morph more closely resembles the most abundant rewarding species in the area (leading to assortative mating). A third factor influencing morph ratios, besides pollinator selection, may be genetic drift. Rewardless orchids typically have small effective population sizes because of low reproductive success (TREMBLAY et al. 2005), and where this is coupled with limited genetic exchange among populations, it is likely to lead to genetic drift, especially where selection is episodically intense and different among populations.

Here we present results of a field study of 22 populations of *D. sambucina* that addressed the following questions: (1) Do the two colour morphs differ in male and/or female reproductive success depending on morph frequency, implying frequency-dependent selection via disassortative mating? (2) If not, does morph reproductive success depend on the abundance of co-flowering yellow or purple nectariferous species, implying frequency-dependent interactions and assortative mating? (3) Is morph reproductive success affected negatively or positively by the density of flowering conspecifics, implying either that pollinators avoid dense patches of rewardless flowers or that high densities increase pollinator attractiveness of *D. sambucina* (KROPF & RENNER 2005). And lastly, (4) do morph ratios correlate with population size, with more unbalanced ratios in smaller populations implying likely genetic drift? We also explored the possibility raised by KROPF & RENNER (2005) that the colour polymorphism in *D. sambucina* may be maintained by differential morph fitness unrelated to mating opportunities. For example, morph fitness may differ in particular microclimates or on certain soils. As an initial test of this possibility, we assessed the viability of seeds resulting from within- and between-morph crosses. If morphs differed in vegetative fitness, this would entail an entirely new set of hypotheses to explain the maintenance of the

purple/yellow dimorphism in *D. sambucina*, instead of, or in addition to, hypotheses relying on assortative or disassortative mating mediated by pollinators.

MATERIALS AND METHODS

Study design and measured parameters

Study species

Dactylorhiza sambucina (L.) SOÓ is a gregarious spring-flowering orchid, widely distributed throughout the Mediterranean, Central Europe, and into southern Scandinavia, commonly on calcareous soils. The plants have basal leaf rosettes and 10–30 cm tall stalks bearing a dense inflorescence of 10–20 yellow or purple flowers. The two morphs are identical in plant height, number of leaves, flower number and size, and as far as known also scent (NILSSON 1980, GIGORD et al. 2001, 2002, KROPF & RENNER 2005, PELLEGRINO et al. 2005). The flowers' labellum (lip) provides a landing place for bees and bears a basal 10–15 mm long empty spur. Pollen packages (pollinia) are formed by the content of entire pollen sacs and are attached to pollinators via a viscidium and short stem. Pollen is deposited as clusters of pollen grains (massulae) that stick to the concave stigmas of the first couple of flowers visited by the pollinator. A relatively short bending time (the time it takes for the short stem to bend, such that pollen can come in contact with a stigma) in *D. sambucina* allows successful pollination shortly after a pollinium has become attached to a pollinator.

In Sweden, *D. sambucina* is pollinated mostly by *Bombus lapidarius* L. (NILSSON 1980), in France by *B. lapidarius* and *B. terrestris* L. (GIGORD et al. 2001), and in Germany by *B. lapidarius* and *B. lucorum* L. (KROPF & RENNER 2005). Honeybees have been recorded as occasional visitors, and possibly pollinators, in Sweden (NILSSON 1980), Switzerland (REINHARD et al. 1991), Austria (VÖTH 1999), and Italy (PELLEGRINO et al. 2005), and so have *Osmia* and *Halictus* bees in Sweden (NILSSON 1980), *Anthophora aestivalis* PANZER in Germany (KROPF & RENNER 2005), and *Andrena nigroaenea* (KIRBY) on an island in the Baltic, where bumblebees are extremely rare (PETTERSSON & NILSSON 1983). At our study sites, *B. lapidarius*, *B. terrestris*, *B. pascuorum* (SCOPOLI), *Psithyrus* species (cuckoo-bumblebees), and rarely honeybees, were the main pollinators (J. JERSÁKOVÁ, pers. observ.). Co-flowering species pollinated by some of the same bees include the purple *Ajuga reptans* L. and *Lamium purpureum* L., and the yellow *Primula veris* L. and *Taraxacum* sp. (NILSSON 1980, KROPF & RENNER 2005; J. JERSÁKOVÁ, pers. observ.).

Study sites and presence and density of co-flowering individuals and species

Between April and June 2002, we studied 22 populations of *D. sambucina* in the Beskydy Mountains in the eastern part of the Czech Republic, located between 49°14′ and 49°26′ N latitude and 18°04′ and 18°12′ E longitude at elevations between 510 and 760 m a.s.l. The bedrock at the study sites consisted of Magura flysch, a type of impermeable sedimentary rock consisting mainly of calcareous clay and sandstone. Population sizes ranged from 38 to more than 500 individuals (median = 110, mean = 155.5 ± 122.5 s.d., $n = 22$). For each population, we counted the total number of plants and the numbers of yellow and purple plants. Only for the two largest populations, with over 500 individuals, were morph frequencies extrapolated based on three counted squares of 10 square meters each. At each site that had yellow and/or

purple co-flowering species, we recorded the presence and abundance of nectar-offering co-flowering species visited by some of the same bee species that pollinate *D. sambucina* (see also previous section; bee foraging, however, was not the focus of our study), namely *Primula veris* (yellow) and *Ajuga reptans* (purple). We also included the nectariferous species *Taraxacum officinale* F.H. WIGG. (yellow), *Anthylis vulneraria* L. (yellow), *Lotus corniculatus* L. (yellow), *Polygala vulgaris* L. (blue), *Viola* sp. (purplish blue and yellow) *Vaccinium myrtillus* L. (pinkish white), and *Fragaria* sp. (white), because it was not clear from the outset which nectar species might be most abundant and most co-visited in the various populations. Abundances were categorized as follows: 1 = 1–5 individuals, 2 = 6–20 individuals, 3 = 20–50 individuals, 4 = 50–100 individuals, 5 = > 100 individuals. The densities of co-flowering conspecifics were measured in 3 squares of 10 square meters each and were then averaged per site.

Reproductive success

Pollinia removal (male success) and pollen (massulae) receipt (female success) were quantified usually in 25 randomly chosen individuals per colour morph and population, except in the smallest population, which had only 38 individuals, and in 5 populations with low frequency of the yellow morph. We calculated male (*mRS*) and female reproductive success (*fRS*) per plant by dividing the total number of pollinia removed by those available and the total number of pollen deposited by the number of available virgin flowers. Values were averaged per morph and population. The calculated variables were normally distributed.

Colour similarity to co-flowering nectariferous species

To assess the visual similarity between the rewardless orchids and yellow and purple nectariferous co-flowering species, we measured corolla reflectance in ten randomly chosen individuals of yellow and purple *Dactylorhiza*, and of *Primula veris* and *Ajuga reptans*, the two co-flowering species most frequently seen visited by previous *Dactylorhiza* visitors. Reflectance was measured with a Spectronic Unicam Aminco Bowman Series 2 spectrophotometer (Luminescence Spectrometer SLM Instruments), using a Xenon light source shining under a 45° angle, and with each sample placed before a black background. Spectralon was used as the white reference. Reflectance values were obtained with the AB software vs. 5.00 for the Spectronic Unicam spectrophotometer, and for each species, an average spectrum was calculated based on the ten individual measurements. To assess the position of flower colours in the bee visual spectrum, we plotted the spectra into colour hexagon diagrams (CHITTKA 1992), using the spectral sensitivity functions of *Bombus terrestris* ultraviolet, blue, and green receptors (PEITSCH et al. 1992), standard daylight irradiance spectrum D65 (WYSZECKI & STILES 1982), and the reflectance function of a typical green leaf (CHITTKA et al. 1994). The colour contrast of a flower against its background was determined by the distance of its colour locus from the centre of the colour space (CHITTKA 1992, SPAETHE et al. 2001). A flower's colour contrast and green contrast are important cues for bees to detect flower colours (SPAETHE et al. 2001).

Crossing experiments

Experimental pollinations were carried out at two sites in the Beskydy Mountains, site A at 750 m and site B at 630 m a.s.l. At each site, five randomly chosen freshly opened flowers on ten random plants of each morph were treated as follows: between-morph cross-pollinations, two flowers/plant for a total of 40 flowers overall; within-morph crosses, two flowers/plant for a total of 40 flowers; and self-pollination, one flower/plant for a total of 20 flowers. Crossings were completely reciprocal, with each morph used as a father as well as a mother. Treated plants were protected from pollinators by net cages until fruit (capsule) collection.

Between- and within-morph fruit set and seed viability

Mature capsules resulting from hand-pollinations were collected and dried. Seeds were extracted and seed biomass per capsule weighed. Seed viability was assessed by tetrazolium staining (Van Waes & Debergh 1986), with red-stained embryos recorded as viable. In addition, we carried out in vitro asymbiotic germinations to evaluate seed germination. Seeds from the two sites where hand-pollinations had been carried out were treated separately, and for each morph, seeds from each of the three crossing types (within-morph, between-morphs, and selfing) were pooled. Seeds from each group were sterilized (70% ethanol – 3 min, 7.2% calcium hypochlorite – 60 min) and sown on modified culture media in glass tubes for a total of 5–44 cultures per type of crossing (Vlašínová 1988). Seeds were judged to have germinated if they had developed protocorms after three months. The effects of the type of crossing on seed mass and viability were analyzed by a multifactorial ANOVA with the independent variables: population, morph colour and type of crossing, and the dependent variables: seed mass and viability, both normally distributed.

Data analyses

Negative FDS

In each population, the relative reproductive success of the yellow morph was calculated as $RRS_y = 2RS_y / (RS_y + RS_p)$, where RRS_y is the relative reproductive success of the yellow morph, RS_y the average reproductive success of the yellow morph, and RS_p the average reproductive success of the purple morph (Gigord et al. 2001). The relationship between relative reproductive success and morph frequency (which differed over a wide range in the 22 populations) was tested using linear regression.

Population size effects on morph frequencies

If the ideal FDS-maintained 1 : 1 morph ratio is distorted by genetic drift acting in small populations, larger distortions are expected in small populations than in large ones. To test this possibility, we calculated the absolute value of the deviation of the observed yellow morph frequencies from a .5 frequency, regressed this value against log-transformed population size, and checked whether the linear regression coefficient was significantly different from zero.

Effects of co-flowering conspecifics and nectariferous species on morph reproductive fitness

Effects of population density on morph male and female reproductive success were assessed using linear regression with Bonferroni correction. The cumulative abundances of
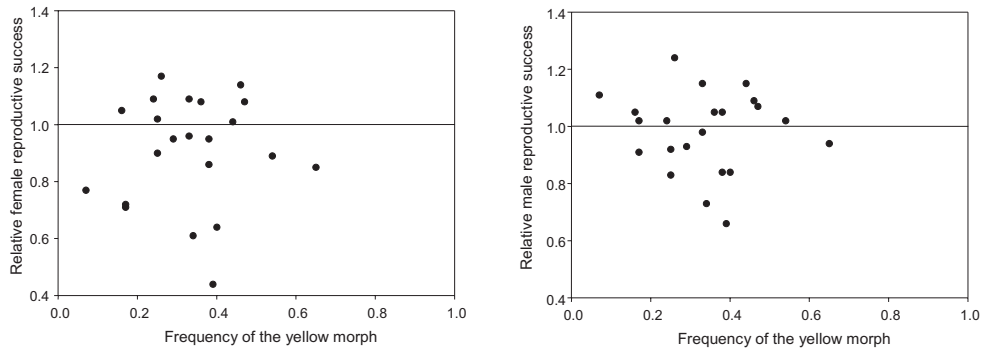
Fig. 1. Relative male and female reproductive success of the yellow morph of *Dactylorhiza sambucina* in relation to its frequency relative to the purple morph (in different populations). The yellow morph has a fitness advantage over the purple one when its relative reproductive success exceeds 1.

purple, yellow, and all bee-pollinated nectariferous co-flowering plants (i.e., the species named above) were regressed against the relative reproductive success of the yellow morph (RRS_y). Species-specific analyses were performed for *Primula veris* and *Ajuga reptans*. The relationship was tested by means of linear and logistic regressions with Bonferroni correction.

RESULTS

Morph male and female reproductive success

Both morphs typically produced 9.7 flowers/plant and did not differ in the number of flowers produced (*t*-test for dependent samples: $n = 22$ populations: $t = 1.98$; $P = 0.061$). Male and female reproductive success did not differ between morphs (*t*-test for dependent samples: $fRS_y = 0.28$ *vs. $fRS_p = 0.32$, $n = 22$, d.f. = 21, $t = 2.03$, $P = 0.055$; $mRS_y = 0.37$ *vs. $mRS_p = 0.38$, $n = 22$, d.f. = 21, $t = 0.43$, $P = 0.670$).

Morph frequency and reproductive success

Most populations were dominated by the purple morph (mean $\pm$ s.d. = 67 $\pm$ 13.5%, median = 66, $n = 22$). The frequency of the yellow morph ranged from 0.07 to 0.65. Neither relative male, nor relative female reproductive success were significantly correlated with morph frequency ($F_{(1,20)} = 0.156$, $P = 0.697$; $F_{(1,20)} = 0.051$, $P = 0.818$; respectively; Fig. 1).

Population size and reproductive success

Population size had no effect on the deviation of morph frequency from a 1 : 1 ratio (linear regression: $F_{(1,20)} = 0.007$, $P = 0.930$).

Effects of co-flowering conspecifics and nectariferous species on morph reproductive success

Densities of conspecific individuals ranged from 0.12 to 3.5 per square meter (mean $\pm$ s.d. = 0.8 $\pm$ 0.7, $n = 22$), and male and female reproductive success of both morphs tended to decrease with increasing overall densities. However, the relationship was significant only for male reproductive success of the purple morph (linear regression with Bonferroni correction:

Table 1. Reflectance characteristics of flowers of yellow and purple *Dactylorhiza sambucina* (*D. s.*), of the yellow co-flowering species *Primula veris*, and of the purple co-flowering *Ajuga reptans*. Colour contrast is measured in hexagon units, green contrast as the specific excitation of the green receptor.

Flower type measured	Colour in human visual spectrum	Colour in bumblebee visual spectrum	Colour contrast	Green contrast
Yellow morph of *D. s.*	yellow	blue-green	0.251	-0.206
Primula veris	yellow	green	0.344	-0.137
Purple morph of *D. s.*	purple	blue	0.180	0.292
Ajuga reptans	purple	blue	0.236	0.001

$F_{(1,20)} = 8.074$, $P = 0.010$). Colorimetric analyses of petal reflectance (Fig. 2A, B) revealed that in the bumblebee visual spectrum, the yellow morph of *D. sambucina* differs considerably from yellow *Primula veris*, while the purple morph of *D. sambucina* and purple *Ajuga reptans* have a similar (blue) appearance to the bumblebee. The colour contrast between flower and background decreases in the sequence: *P. veris*, yellow morph of *D. sambucina*, *A. reptans*, purple morph of *D. sambucina* (Table 1). The green contrast decreases in the sequence: purple morph of *D. sambucina*, yellow morph of *D. sambucina*, *P. veris*, *A. reptans* (Table 1). After Bonferroni correction, the relative reproductive success of the yellow morph was almost unaffected by the presence or abundance of *P. veris*, *A. reptans*, all yellow nectariferous plants (including *P. veris*), all purple nectariferous plants (including *A. reptans*), or all purple + yellow nectariferous plants (Table 2). The exception was that the cumulative abundance of yellow co-flowering plants had a negative effect on the male reproductive success of the yellow morph (Table 2, Fig. 3). On average, *Primula veris* contributed 56% of the cumulative abundance of yellow co-flowering plants.

Seed mass, viability, and germination after between- and within-morph mating

Seeds produced by the purple morph of *D. sambucina* were significantly heavier than those of the yellow morph, irrespective of whether they resulted from outcrossing or selfing (Fig. 4A), but in spite of this, out-crossed seeds of both morphs had higher viability (i.e., tetrazolium staining) and germination rates than did selfed seeds (Fig. 4B, C). Within-morph crossings of the purple morph yielded 2.64-fold heavier seeds than did within-morph yellow crossings, and between-morph crossings of the purple morph yielded 2.58-fold heavier seeds than did between-morph crossings of the yellow morph. The phenomenon of differential morph seed fitness was more pronounced at site B (significant interaction Population*Morph colour, Table 3). Morph colour also significantly influenced the proportion of viable seeds and germination rates (Table 3, Fig. 4B), with purple × purple cross pollinations on average yielding 1.19 times more viable seeds than yellow × yellow crosses, and seeds resulting from yellow × yellow and yellow × purple pollinations having zero germination success after three months (Fig. 4C).

DISCUSSION

Frequency-dependent selection is a fundamental mechanism maintaining genetic polymorphism in natural populations (CLARKE & O'DONALD 1964, AYALA & CAMPBELL
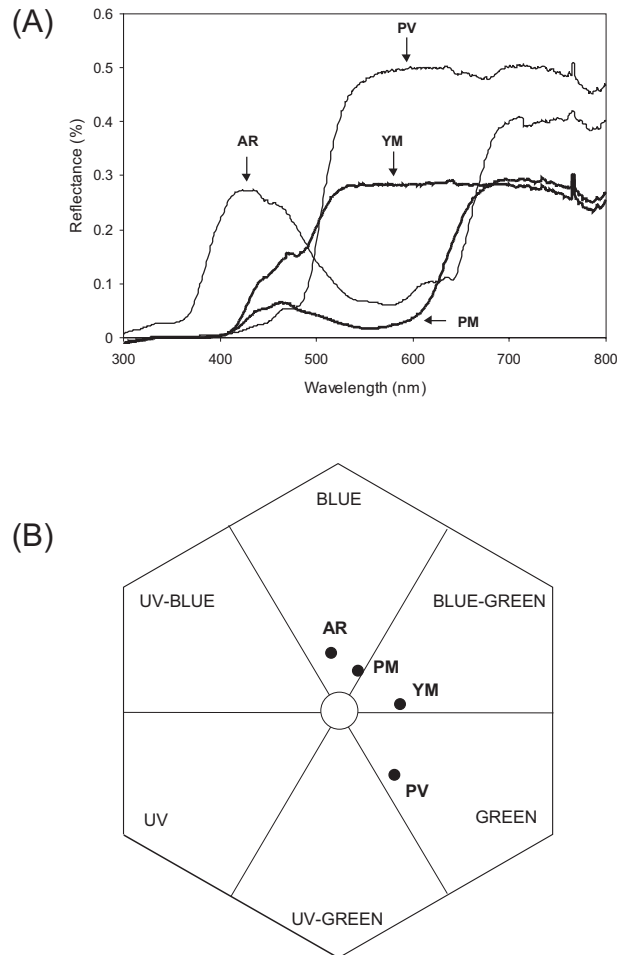
Fig. 2. Petal reflectance of the study species and the two most abundant co-flowering nectar-producing species: (A) Spectral reflection curves of the yellow morph of *Dactylorhiza sambucina* (YM), the purple morph (PM), yellow co-flowering *Primula veris* (PV), and purple co-flowering *Ajuga reptans* (AR). Bumblebee photoreceptors are maximally sensitive at about 350, 440 and 540 nm. (B) Colour loci of the four flower types in the colour hexagon. The distance between the centre and each of the hexagon's corners is unity. The colour space inside the central circle (0.1 hexagon units) appears achromatic to bumblebees.

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Table 2. Results of the logistic and linear regressions that tested the impact on the relative male and female reproductive success of yellow individuals of *Dactylorhiza sambucina* in the presence of more-or-less abundant nectar-producing *Primula veris* and *Ajuga reptans*. The abbreviations stand for $fRRS_y$ – female relative reproductive success of the yellow morph, and $mRRS_y$ – male relative reproductive success of the yellow morph; "presence" refers to the presence of co-flowering species and "abundance" to the abundance classes described in Materials and Methods. * – significant at $P < 0.05$, ** significant at $P < 0.05$ after Bonferroni corrections.

$fRRS_y$: presence	d.f.	χ^2-value	P-level
Yellow co-flowering spp.	1	2.33	0.126
Purple co-flowering spp.	1	4.96	0.026*
All co-flowering spp.	1	0.06	0.813
Primula veris	1	0.95	0.329
Ajuga reptans	1	0.16	0.733

$mRRS_y$: presence	d.f.	χ^2-value	P-level
Yellow co-flowering spp.	1	3.68	0.055
Purple co-flowering spp.	1	5.34	0.021*
All co-flowering spp.	1	0.31	0.580
Primula veris	1	2.35	0.125
Ajuga reptans	1	0.31	0.574

$fRRS_y$: abundance	d.f.	F-value	P-level
Yellow co-flowering spp.	1,11	0.15	0.707
Purple co-flowering spp.	1,17	0.47	0.502
All co-flowering spp.	1,19	0.27	0.611
Primula veris	1,10	2.85	0.121
Ajuga reptans	1,6	0.15	0.713

$mRRS_y$: abundance	d.f.	F-value	P-level
Yellow co-flowering spp.	1,11	11.77	0.006**
Purple co-flowering spp.	1,17	0.34	0.567
All co-flowering spp.	1,19	0.65	0.428
Primula veris	1,10	6.96	0.025*
Ajuga reptans	1,6	0.01	0.901

populations, such as plant density (hence density of rewardless flowers in habitat patches) and presence and abundance of co-flowering nectariferous plants, could therefore not be addressed. That these factors can impact the reproductive success of *D. sambucina* is shown by an earlier study, which found that high densities of flowering conspecifics positively affected pollen export (KROPF & RENNER 2005), while we found that high densities of conspecifics negatively impacted pollen export (at least of the purple morph), and also from our finding that the cumulative abundance of yellow co-flowering nectariferous plants negatively affected pollen export of the yellow morph. The first effect may be due to the visual attractiveness of dense patches of *D. sambucina* from a distance (KROPF & RENNER 2005), the second (contradictory) effect to pollinators learning to avoid dense patches of rewardless flowers, and the third to dense patches of nectariferous plants satiating bees' requirement, resulting in few exploratory visits to new kinds of flowers. Almost certainly much of the
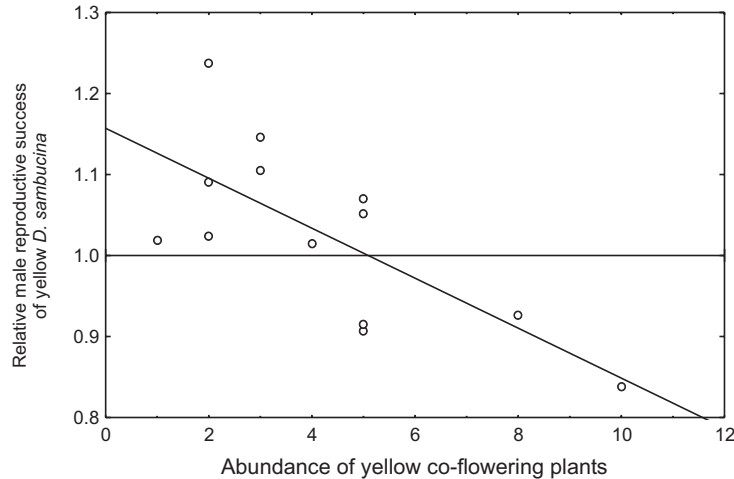
Fig. 3. Effect of the cumulative abundance of yellow co-flowering nectar-producing plants (categorical scale) on the relative male reproductive success of the yellow morph of *Dactylorhiza sambucina*.

variance in reproductive success relates to varying pollinator abundances as found in other rewardless orchids (ACKERMAN et al. 1997).

That *Primula veris*, which on average made up 56% of the yellow co-flowering plants, differs substantially in colour and green contrast from the yellow morph of *D. sambucina* (Table 1, Fig. 2B) suggests that bees can readily discriminate these flowers. There is thus no colour similarity between the nectariferous model and the rewardless mimic, which otherwise should have stimulated pollinator visits to the mimic (GUMBERT & KUNZE 2001, JOHNSON et al. 2003, GIGORD et al. 2002). Where pollinators are able to distinguish between rewarding and deceptive plants, an increase in abundance of the rewarding plant (here *P. veris*) is statistically expected to negatively affect the reproductive success of the rewardless plant (FERDY et al. 1998). Since we did not measure the spectral reflectance of other yellow species, we do not know whether some of them are rewarding models for the yellow morph of *D. sambucina*.

Regardless of *ad hoc* explanations for differential morph success through pollinator discrimination or attraction (and disassortative or assortative mating), the important point is that FDS on colour polymorphisms is likely to be extremely difficult to discern in natural populations because of other factors that influence morph reproductive success. In our 22 study populations, we detected no relationship between reproductive success and morph frequency. However, one of the 22 populations in which the yellow morph occurred at a frequency of 7% and had a higher male success fit with predictions that FDS works at rare frequencies. Similarly, a study of 17 populations in southern France (SMITHSON 2001) found weak evidence of a relationship between male reproductive success and morph frequency mainly because of four populations with purple morph frequencies below 10%. PELLEGRINO et al. (2005) found no reproductive advantage of the rare phenotype in three *D. sambucina* populations in Italy that had yellow frequencies of 50, 56, and 88%. Together, these results indicate that under natural conditions, negative FDS is best detectable when one of the morphs

Table 3. Results of a multifactorial ANOVA with interactions that tested effects of mating type on seed mass and seed viability in *Dactylorhiza sambucina*. Dependent variables: Seed mass and viability (proportion of viable seeds); independent variables: Population (sites A and B), Type of crossing (between-morphs, within-morph, self-pollination), and Morph colour (yellow or purple).

Dependent variable	d.f.	*F*-value	*P*-level
Seed mass			
Population	1	45.22	0.001
Type of crossing	2	1.02	0.364
Morph colour	1	86.81	0.001
Population × Type of crossing	2	0.91	0.405
Population × Morph colour	1	24.35	0.001
Type of crossing × Morph colour	2	0.18	0.835
Population × Type of crossing × Morph colour	2	0.12	0.885
Seed viability			
Population	1	0.09	0.753
Type of crossing	2	164.06	0.001
Morph colour	1	118.32	0.001
Population × Type of crossing	2	21.23	0.001
Population × Morph colour	1	6.65	0.014
Type of crossing × Morph colour	2	18.60	0.001
Population × Type of crossing × Morph colour	2	8.33	0.001

is extremely rare. Other studies of natural populations of rewardless orchids with flower colour polymorphisms so far have not been able to discern the role of negative FDS (*Tolumnia variegata,* ACKERMAN et al. 1997; *Psychilis monensis*, ARAGÓN & ACKERMAN 2004; *Dactylorhiza maculata*, KOIVISTO et al. 2002).

Morph frequencies in *D. sambucina* across populations in Europe vary widely. Thus, while GIGORD et al. (2001) reported ratio of 53% ± 2.6 in 174 European populations (locations not provided), 17 West German populations monitored in the Rhineland-Palatinate between 1994 and 2003 contained no purple-flowering individuals, and two contained one and two purple-flowering plants out of hundreds of plants (KROPF & RENNER 2005). A yellow bias also exists in southern France (GIGORD et al. 2001), while Swedish and Czech populations show a purple bias (NILSSON 1980; this study). These regional biases prompted us to investigate other factors that would lead one to expect unequal (rather than equal) morph frequencies. PELLEGRINO et al. (2005) hypothesized that morph frequency biases may reflect regionally different pollinator spectra. However, the main pollinators of *D. sambucina* appear to be the same in Sweden (*Bombus lapidarius*, NILSSON 1980), France (*B. lapidarius* and *B. terrestris*, GIGORD et al. 2001), Germany (*B. lapidarius* and *B. lucorum*, KROPF & RENNER 2005), and the Czech Republic (*B. lapidarius* and *B. terrestris*). For the names of all known pollinators see "Materials and Methods".

Another possible explanation for uneven morph ratios is genetic drift acting in small population, as shown for species in the rewardless, sexually deceptive, and colour polymorphic orchid genus *Lepanthes* (TREMBLAY & ACKERMAN 2001, BLANCO & BARBOZA 2005; see also TREMBLAY et al. 2005). We found no statistical correlation between population size and morph ratio; however, our smallest populations had morph ratios close to
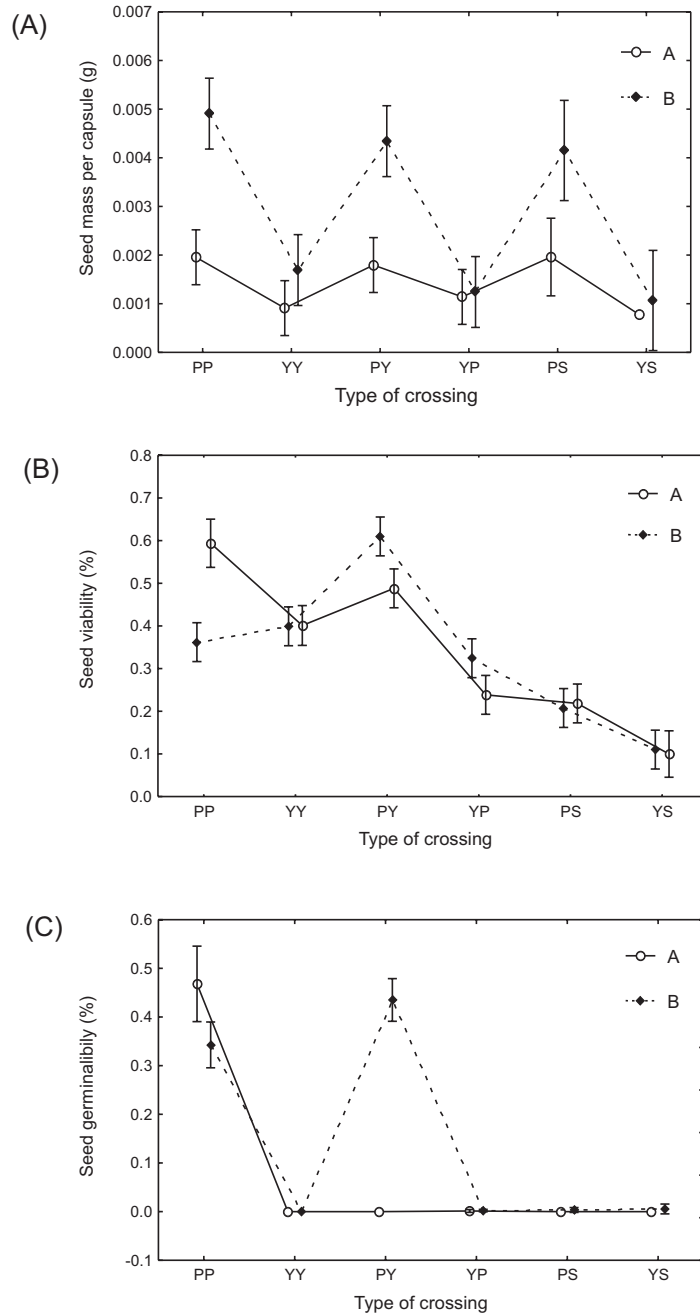
Fig. 4. Effect of type of crossing on seed mass (A), viability (B), and germination (C) in *Dactylorhiza sambucina* populations at site A (continuous line) and site B (hatched line). Abbreviations on the X-axis refer to types of crossings: within the purple and yellow morphs (PP, YY), between the morphs (PY and YP; the first letter refers to pollen receiver, the second letter to pollen donor), and self-pollination of each morph (PS and YS).

balanced, and some of the largest populations had the least balanced morph ratios. Rewardless orchids typically have low reproductive success, resulting in few reproducing individuals and small effective population sizes (TREMBLAY et al. 2005). A survey of fruit sets in natural populations of *D. sambucina* across its distribution range reported a mean fruit set of 17% ($n = 17$), with a range from 2 to 56% (KROPF & RENNER 2005, their Table 4), suggesting that small effective population sizes also characterize *D. sambucina*. Genetic drift is most intense in small populations that experience restricted gene flow, and there is preliminary evidence that the genetic connectivity between *D. sambucina* populations indeed is low (J. JERSÁKOVÁ, unpubl. data). Therefore, genetic drift is likely to play a role in the unbalanced morph ratios observed in *D. sambucina*.

The most surprising result of our study is that the yellow morph appears to have lower seed mass, seed viability (judging by tetrazolium staining), and germination success (after three months) than the purple morph. This could account for the generally low frequency of the yellow morph in our populations. Other studies also point to fitness differences among the seeds or seedlings of colour-polymorphic insect-pollinated species. For example, in *Ipomoea*, increased amounts of flavonoids in the pigmented morph, but not the white morph, alleviate adverse effects of heat stress on fertilization and early seed maturation (COBERLY & RAUSHER 2003), in *Linanthus parryae*, the blue morph outperforms the white morph in years with low spring precipitation (SCHEMSKE & BIERZYCHUDEK 2001), and in *Raphanus* and other colour-polymorphic plants, morphs consistently differ in plant secondary chemistry, which in turn can influence herbivore resistance or seed predation (IRWIN & STRAUSS 2005). It is possible that such pleiotropic effects or tight linkage with other genes also explain the maintenance of genes that influence *Dactylorhiza* flower colour. We are currently investigating this possibility.

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