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Research article

The potential for selection on pollen colour dimorphisms in *Nigella degenii*: morph-specific differences in pollinator visitation, fertilisation success and siring ability

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Abstract. Two subspecies of *Nigella degenii* (Ranunculaceae) possess a dimorphism in pollen colour and vary extensively in frequency of the two morphs in natural populations. Here we investigate the role of selection on pollen colour during the pollination phase in the two subspecies and its potential contribution to the maintenance of this colour variation. In a combination of common garden experiments and field observations, we obtained data on pollinator visitation rates and explored the effect of pollen colour on fertilisation success and siring ability under conditions of low vs. high pollen competition. In experimental gardens, naïve pollinators responded differently to plants with different pollen colour, but the favoured morph varied between dates and locations, and colour morphs were not visited in a frequency-dependent manner. Donor plants with dark pollen had a reproductive advantage (higher seed set) in single-donor pollinations, but the realised siring ability (measured by progeny morph ratio) was highly variable between different two-donor crosses with no general bias towards the light or dark morph. Therefore, although the dark pollen type appears to have a general selective advantage in terms of fertilisation success, our data are also consistent with a scenario involving the maintenance of both colour morphs, particularly under conditions of high pollen competition, a variable genetic background and/or spatial or temporal variation in the pollinator fauna.

Key words: Cyclades, gametophyte fitness, pollen colour, pollen competition, Ranunculaceae

Introduction

Flower colour polymorphisms are conspicuous examples of intraspecific variation in plants, especially in species whose flowers contain blue, red or purple anthocyanin pigments. Studies of such polymorphisms have contributed significantly to our understanding of the evolutionary mechanisms contributing to the maintenance of the great diversity in floral morphology exhibited by natural populations of plants (Clegg and Durbin, 2000; Schemske and

Bierzychudek, 2001). Variation in petal colour is the most common form of floral colour polymorphism (Warren and Mackenzie, 2001), but naturally-occurring colour polymorphisms involving pistils, stamens and pollen have also been reported. Examples include stigma colour dimorphisms in *Crocus scepuensis* (Rafinski, 1979) and pollen colour dimorphisms in *Campanula* (Lau and Galloway, 2004), *Erythronium* (Thomson, 1986), *Linum* (Wolfe, 2001), *Lythrum* (Darwin, 1877) and *Nigella* (Strid, 1970).

There is a wide variety of ecological factors that could exert selection on flower colour and thereby maintain colour polymorphisms. Numerous authors have documented associations between flower colour and pollinator visitation, fecundity or outcrossing rate (e.g. Mogford, 1974; Waser and Price, 1981, 1983; Stanton, 1987; Stanton *et al*., 1989; Rausher and Fry, 1993; Jones, 1996; Jones and Reithel, 2001; for exceptions, see e.g. Stone, 2000). Some of these associations have been found to be frequency-dependent in a way that contributes to the maintenance of the colour polymorphisms (Brown and Clegg, 1984; Gigord *et al*., 2001). In some cases, the under-visited morph shows higher selfing rates, thus enhancing the transmission advantage of those alleles that express this phenotype (Fisher, 1941; Brown and Clegg, 1984).

Selection on flower colour polymorphisms may also involve morph-specific differences in pollen performance (Mo *et al*., 1992; Snow, 1994) or correlated responses to selection during vegetative stages, mediated through pleiotropic relationships between floral anthocyanins and related compounds that affect, for example, plant-microbe interactions, herbivore defence, stress tolerance, and UV protection (Koes *et al*., 1994; Shirley, 1996; Graham, 1998; Steyn *et al*., 2002). Evidence for such selection pressures is provided by Levin and Brack (1995), who documented differences in survivorship and flower production between plants of *Phlox drummondii* with white and pigmented flowers, and Simms and Bucher (1996) and Coberly and Rausher (2003) who found colour morphs of *Ipomoea purpurea* to differ in vulnerability to herbivores and in fertilisation success at high temperatures, respectively (see also Schemske and Bierzychudek, 2001).

Most studies on floral colour polymorphisms have been based on species showing intraspecific variation in petal colour. Only one study has, to our knowledge, examined the role of pollinators in exerting selection on pollen colour: halictid bees responded differently to the amount of pollen exposed by plants of different pollen colour morphs in *Campanula americana*, but showed no preference for particular colour morphs (Lau and Galloway, 2004). Clearly, more data are required to draw general conclusions about the importance of pollinators and other ecological forces in the evolution and maintenance of flower colour polymorphisms.

In the present study, we evaluate the potential for selection on pollen colour in *Nigella degenii* during the pollination phase. The dimorphisms in pollen colour occur in two subspecies of *N. degenii* (Strid, 1970) and involve plants with either yellow (henceforth 'light') or violet ('dark') pollen. The dark morph varies in frequency, between 0 and 0.93, in different populations (Jorgensen and Andersson, 2005). We tested for morph-specific differences in pollination success using both naïve pollinators in experimental gardens and indirect measures of pollination success in 10 natural populations. Using controlled crosses, we also explored the effect of pollen colour on fertilisation success and siring ability under conditions of low vs. high pollen competition.

Materials and methods

Study system

Nigella degenii Vierh. (Ranunculaceae) is a self-compatible, diploid annual with four local allopatric races (referred to as subspecies) on different islands in the Cyclades (Greece). It occupies a variety of more or less disturbed habitats such as roadsides, stone walls, phrygana vegetation and sea shores. The 15–25 mm wide, protandrous flowers have a double perianth with five petal-like sepals, eight stalked, nectariferous petals and a variable number of stamens, which shed their pollen as the filaments curve outwards during the staminate phase. The central gynoecium consists of 5–10 partly united follicles, each having a 15–20 mm long style, which is receptive on the adaxial surface. The styles are erect in young flowers, but become excurved and finally curled during the receptive stage. Styles on fertilised follicles straighten within 1 day of pollination, whereas unpollinated styles remain curled (Strid, 1970). Previous work indicates that the partly fused follicles allow some pollen tubes to cross between adjacent carpels (TH Jorgensen, pers. obs.). Protandry coupled with spatial separation of anthers and stigmas enhance the potential for outcrossing. Fertilised flowers develop into capsules, each consisting of 20–40 seeds (occasionally up to 100 seeds) with no special mechanisms for long-distance dispersal.

Plant material, garden sites and experimental conditions

This study involves populations of two morphologically distinct subspecies: *N. degenii* subsp. *barbro* Strid, endemic to the island of Mykonos and a few adjacent islands, and subsp. *jenny* Strid, endemic to the island of Syros. Plants of subsp. *barbro* produce dark yellow or violet pollen in dark red anthers, whereas plants of subsp. *jenny* produce pale yellow or dark violet pollen in

anthers with the same colour as the pollen grains (Strid, 1970). Differences in pollen colour are not associated with differences in the expression of anthocyanin pigments in leaves, stems and perianths (TH Jorgensen, pers. obs.) and there are no between-morph differences in pollen size, external pollen morphology (surface structure), flower size, plant height and flower production (TH Jorgensen and S Andersson, unpubl. data). Experimental populations were based on plant material from one locality of *N. degenii* subsp. *barbro* (Mykonos, ca. 2.5 km NNW of the town) and one locality of *N. degenii* subsp. *jenny* (Syros, ca. 300 m S of the village Kini), both sampled in 1993 and maintained for several generations by random outcrossing within populations in a greenhouse at the University of Lund, Sweden. The two study populations were polymorphic for pollen colour, the initial frequency of the dark morph being ca. 14% in both cases.

Differences in pollen colour are controlled by a single major gene with dominance towards the dark morph (Andersson and Jorgensen, 2005). Based on this information, we carried out a series of within-population crosses or self-pollinations to produce (i) seeds homozygous for the dominant allele (dark pollen), (ii) seeds homozygous for the recessive allele (light pollen), and (iii) heterozygotes (dark pollen). Plants from the different seed categories will be referred to as 'light homozygotes', 'dark homozygotes' and 'dark heterozygotes', respectively. Comparisons of morphs in experimental studies either involved light homozygotes and dark heterozygotes from segregating full-sib families (each derived from a separate cross between two unrelated individuals differing in pollen colour), or light and dark homozygotes (derived from a number of inbred lines that had reached fixation for the intended morph after four or five generations of self-pollination).

Pollinator studies were performed in the sunny part of two experimental gardens at the University of Lund, one in the Botanical Garden (site B) and another at the Genetics department (site G). The two garden sites are separated by approximately one kilometre. Flower visitors are sometimes seen in natural populations, but the insects are too infrequent to allow assessments of visitation rates to individual plants (TH Jorgensen and S Andersson, pers. obs.). Experimental pollinations were, unless otherwise stated, carried out in a greenhouse chamber with regulated watering and 12 h day lighting (60% humidity, ≥24°C). All statistical analyses were performed using SPSS version 10.

Pollinator preferences in experimental populations

To examine whether naïve pollinators preferentially visit plants of the dark or light pollen morph, we established artificial populations in the two garden sites using plants of *N. degenii* subsp. *jenny*, the subspecies with the most conspicuous colour dimorphism. A bulked sample of seeds from about 100 segregating

full-sib families (representing light homozygotes and dark heterozygotes) were germinated in the greenhouse (in early spring). When the first flower buds became visible (June), we transferred the plants to the two garden sites. At each site, the plants were set up in three adjacent subplots, each with 70 plants in the following morph ratios (dark:light): 10:60, 35:35 and 60:10. Plants within each subplot were assigned to random positions in a 3×4.5 m grid with 0.5 m spacing between individuals. The subpopulations were covered by fine-mesh nets to prevent insect visitation before the first observation period. During a period of 9 days in early August, the three subplots at each site were alternately exposed to insects for 1 or 2 h (between 10.00 and 16.30) by removing the net on one subplot while keeping the other two still covered. Before exposing a subplot, we cut off flowers that had passed the staminate phase (i.e. flowers lacking visible pollen and anthers) and counted the remaining flowers on each plant to determine the morph frequency at the time of insect visitation. The visitation rate to each morph was determined by following insects that entered the exposed subplot and recording the pollen colour of each flower visited. Only insects that were sufficiently large to contact the dehisced anthers (which are positioned at the same level as the receptive stigmas) were considered (honey bees, bumble bees and large hover flies). After the exposure of a particular subplot, all subplots were covered for 2 or 3 days to ensure that flower visitors would loose the 'search image' of the two colour morphs between observation periods. Each subplot was exposed to pollinators once or twice.

Log-likelihood G-tests for goodness-of-fit (Sokal and Rohif, 1995) were employed to compare the observed visitation rate of pollinators to each colour morph with the visitation rate predicted under the null hypothesis of random visitation (estimated from the relative frequency of the two colour morphs in the subplot).

Pollen viability and fertilisation success

Morph-specific differences in pollen viability were estimated by comparing one light homozygote and one dark heterozygote within each of 10 segregating full-sib families per population. Pollen from 3 to 5 newly dehisced anthers on each individual was stained with aniline blue lactophenol (cotton blue) to distinguish between viable (stained) and nonviable (unstained) pollen grains (Stanley and Linskens, 1974). A total of 200 pollen grains per anther were scored for viability under a light microscope.

To provide a more direct estimate of pollen viability, we quantified the fertilisation success of each pollen type after single-donor pollinations. To this end, we took advantage of plants in a separate experiment investigating the existence of morph-by-environment interactions in survival and flower production (Jorgensen and Andersson, 2005). These plants represented light

homozygotes or dark heterozygotes in each of 10 segregating fullsib families per population, grown in a mix of nutrient-poor peat soil (80%) and sand (20%), and watered regularly with a 0.5% nutrient solution (NPK 6-1-5, Superba®S).

Fertilisation success was assessed in a pollination experiment involving 83 sets of flowering plants. Each set consisted of two 'pollen donors', representing the same full-sib family but differing in pollen colour, and one 'pollen recipient', representing a randomly selected individual with light or dark pollen in one of the remaining families. Single-donor crosses were made by applying excess pollen from each donor on separate stigmas (different follicles) in the same (emasculated) flower on the recipient. The two pollinated follicles were separated by at least one unpollinated follicle to minimise confounding effects of pollen tubes traversing the border between adjacent follicles. Morph-specific differences in fertilisation success were determined by comparing (i) the number of well-developed seeds in each of the two pollinated follicles in each flower (male fertilisation success), and (ii) the total number of seeds in the two pollinated follicles on recipients with light vs. dark pollen (female fertilisation success).

The proportion of stainable pollen was averaged for each individual, transformed to natural logarithms and subjected to a two-way ANOVA without replication (Sokal and Rohlf, 1995) using colour morph as a fixed factor and full-sib family as a random factor. Interaction effects were assumed to be zero. The paired data on male fertilisation success did not follow a normal distribution and were therefore analysed with Wilcoxon's matched-pairs signed-ranks tests (Sokal and Rohlf, 1995). Likewise, differences in female fertilisation success were analysed with the non-parametric Mann–Whitney U-tests (Sokal and Rohlf, 1995). Experimental flowers that failed to set seed in both follicles were excluded from the analyses.

Siring ability

The relationship between pollen colour and siring ability, i.e. the proportion of plants sired by pollen of each colour in the progeny generation, was examined in a pollination experiment involving pairs of competing pollen donors. To this end, we established 15–19 sets of simultaneously flowering plants from each population. Each set consisted of two competing pollen donors, one light homozygote and one dark homozygote, and one light homozygote as a pollen recipient. The plants in each triplet represented three unrelated homozygous lines. Two flowers of each pollen recipient were emasculated and assigned to one of two pollination treatments: (i) application of excess pollen from both donors at the tip of the receptive stigmas (tip pollination), and (ii) application of excess pollen from both donors at the base of the stigmas (base pollination).

In both treatments, pollen from the two donors was positioned just next to each other and the pollen morph closest to the ovules was alternated randomly between stigmas. All pollinations in a flower were performed within 1 or 2 min. The siring ability of each pollen donor was estimated as the proportion of offspring with light and dark pollen (assumed to represent light homozygotes and dark heterozygotes, respectively). All offspring seeds were planted in separate pots and scored for pollen colour at the onset of flowering. Given the 1–1.5 cm longer distance to the ovules after tip pollination compared to base pollination, we expected the morph-specific differences in pollen performance (if any) to be accentuated, i.e. to cause more deviant morph ratios, in this treatment group. Any effect of inbreeding (resulting from the use of inbred parent lines) was assumed to be unrelated to pollen colour.

The frequency data were subjected to log-likelihood analyses based upon a model with plant triplet, treatment (tip vs. base pollination) and progeny pollen colour as categorical factors, to test whether one of the morphs or one of the pollination treatments was consistently more successful than the other. All two- and three-way interactions were entered in the original model and tested for significance using a backward elimination procedure with a P-to-remove value of 0.05 (Sokal and Rohlf, 1995).

Fruit set in natural populations

The relationship between pollen colour and pollination success under natural conditions was assessed in five populations of *N. degenii* subsp. *jenny* on Syros and five populations of *N. degenii* subsp. *barbro* on Mykonos, chosen to represent the range of morph ratios in each subspecies. Between 12 and 29 plants per population and morph were marked during the flowering stage and collected at the fruiting stage to determine their fruit set, quantified as the proportion of successfully pollinated follicles on each individual (as indicated by the presence of 'straightened' styles). In another population of each subspecies, we recorded the fruit set of plants that were bagged just before the first flower reached the pistillate stage. The bags prevented visitation by large to medium-sized bees and flies, but probably allowed some pollen transfer by wind or small insects (e.g. thrips). A total of 13–15 individuals per colour morph were bagged in each population.

Differences between populations or colour morphs were tested by one- or two-way ANOVAs with both factors considered as fixed and tested over the error term. Residuals had bell-shaped distributions, but showed significant non-normality in analyses of natural pollination success (Kolmogorov–Smirnov test for normality, $p < 0.001$ in both subspecies), whereas data from bagged plants resulted in normally distributed residuals ($p > 0.05$ in both subspecies).

As no transformation enhanced normality, all ANOVAs were performed on untransformed data.

The use of fruit set as a measure of pollination success relies on the assumption that female fertility is at least partly determined by the amount of pollination achieved. If female fertility is limited by resources rather than by pollen, there is no reason to expect a positive relationship between pollinator visitation and fruit set.

Results

Pollinator preferences

Honey bees were the most frequent visitors (54% of all pollinator visits) compared to bumble bees (29%) and hover flies (17%). A minimum of 2 and a maximum of 16 different visitors (mean = 7.4) were recorded at each site on different days, resulting in 15–315 visits at one site on 1 day (mean = 101). All three groups of insects were found at both sites, but bumble bees dominated at site G (73%) and honey bees dominated at site B (77%). Overall, the pollinators did not visit the two morphs in proportion to their relative frequency in the subplot (pooled $G = 26.5$, $p < 0.001$, df $= 8$, Fig. 1). On August 16, site G showed a significant excess of visits to the light morph (all involving bumble bees), while on August 15 and 16 there was a significant excess of visits to the dark morph on site B (all involving honey bees). Morph-specific differences in

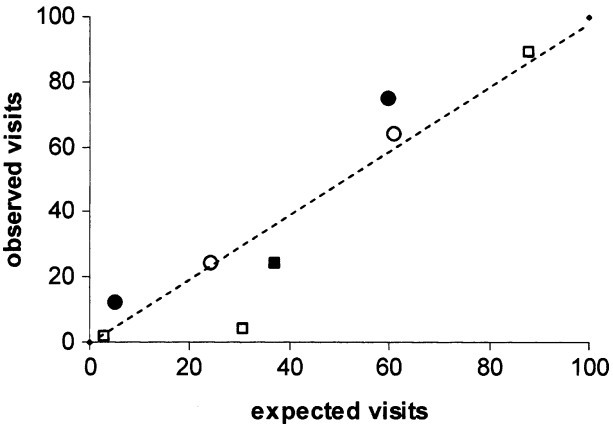

Figure 1. The observed and expected number of pollinator visits to *N. degenii* flowers with dark pollen in experimental populations at two garden sites (circles, site B; squares, site G). Black symbols refer to occasions where observed visitation rates were significantly different from expectation ($p < 0.05$, G-test). Significant G-values remained significant after Bonferroni correction. Overall, the pollinators did not visit the two morphs in proportion to their relative frequency (pooled $G = 26.5$, $p < 0.001$, df $= 8$).

visitation rate were not present on other days (results from each insect group analysed separately not shown). Flower visitors did not seem to change their visitation behaviour according to the frequency of light and dark flowers in the populations (Fig. 1).

Pollen viability and fertilisation success in single-donor pollinations

Analysis of data pooled over colour morphs and families revealed a higher percentage of stainable pollen for plants in the Mykonos population (0.91 ± 0.55 SD) than for plants from the Syros population (0.77 ± 0.14 SD; $F_{1,38} = 22.12$, $p < 0.001$). However, there was no significant difference in pollen stainability between the two colour morphs in our limited sample of 10 full-sib pairs ($F_{1,9} < 0.13$, $p > 0.05$ for both populations) and no added variance component between individuals from different full-sib families ($F_{9,9} < 2.50$, $p > 0.05$ for both populations).

Flowers from the Mykonos population produced significantly more seeds per follicle after pollination with dark pollen than with light pollen ($Z = -2.82$, $p = 0.005$, $n = 33$; Wilcoxon's matched-pairs signed-ranks test, Table 1). Plants from the Syros population showed an excess of flowers in which dark pollen had a higher siring ability than light pollen (Table 1), but the difference in seed set was too small to be declared significant ($Z = -1.39$, $p = 0.16$, $n = 35$). Female seed set after single-donor pollinations varied greatly between pollen recipients (range 1–17 for Mykonos plants; range 1–28 for Syros plants), but there was no significant difference in seed set between maternal plants with dark vs. light pollen ($U = 73.50$, $p = 0.93$, $n = 25$ for Mykonos plants; $U = 108.00$, $p = 0.31$, $n = 33$ for Syros plants; Mann–Whitney U-test).

Siring ability after two-donor pollinations

Between 11 and 49 progenies from each flower and pollination treatment survived to flowering (mean = 27). There was extensive among-family variation

Table 1. Comparison of seed set in one follicle after pollination with either dark or light pollen for plants of *N. degenii*

Population	Total	Fruits	D seeds	L seeds	D < L	D > L	Z
Mykonos	44	33	4.9 ± 4.4	2.1 ± 3.2	8	22	-2.82^{**}
Syros	39	35	5.1 ± 3.9	3.9 ± 4.0	11	21	1.39 ns

$^{**}p < 0.01$, ns = not significant ($p > 0.05$).

Entries are the total number of flowers pollinated (Total), the number of flowers setting fruit (Fruits), the mean seed set (± SD) of follicles pollinated by dark (D seeds) and light pollen (L seeds), and the number of flowers in which dark pollen produced fewer (D < L) or more (D > L) seeds per follicle than light pollen. The significance of the difference in seed set was evaluated using the Wilcoxon's matched-pairs signed-ranks test.

in the proportion of progeny that produced dark pollen (range 0–1.0 for tip pollinations; range 0–0.88 for base pollinations, Fig. 2). The position of the pollen grains on the stigma (tip vs. base) and the identity of parent plants interacted in their effect on the progeny morph ratio, as shown by a significant interaction between pollination treatment, plant triplet and progeny morph colour (Likelihood-ratio $\chi^2 = 74.2$, $df = 14$, $p < 0.001$ for Syros plants; $\chi^2 = 131.3$, $df = 18$, $p < 0.001$ for Mykonos plants; G-test of independence). As

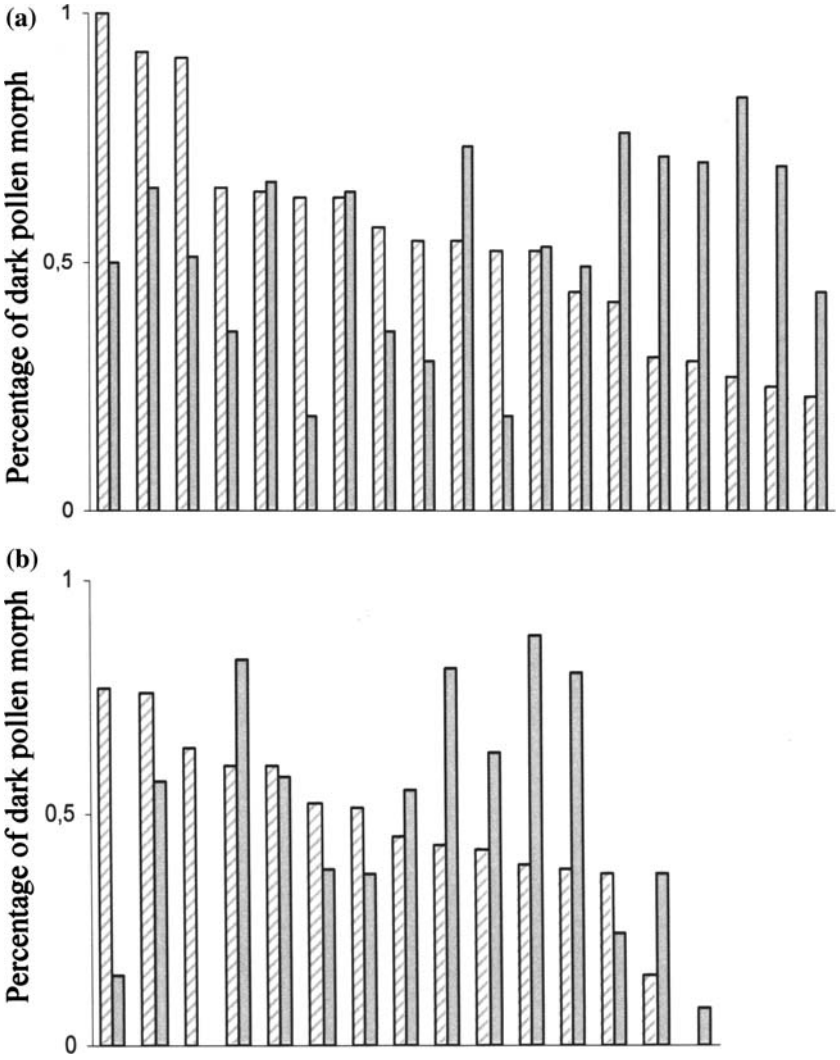

Figure 2. The frequency of the dark pollen morph in offspring produced from pollination with dark and light pollen at the tip (hatched bars) or base (grey bars) of the stigma on the pollen recipient. Each cluster of two bars represents one pollen recipient. A. Mykonos = *N. degenii* subsp. *barbro*, B. Syros = *N. degenii* subsp. *jenny*.

shown in Figure 2, tip pollination favoured the dark morph in some cases and the light morph in other cases; hence, no morph was consistently more successful than the other.

Fruit set in natural populations

Our field data revealed no consistent difference in fruit set between the two pollen morphs ($F_{(1,227)} = 0.55$, $p > 0.05$ on Mykonos; $F_{(1,213)} = 0.11$, $p > 0.05$ on Syros). The mean fruit set showed significant between-population variation on Mykonos (range 0.72–0.88, Table 2; $F_{(4,227)} = 5.87$, $p < 0.001$), but not on Syros (range 0.69–0.88, Table 2; $F_{(4,213)} = 2.35$, $p > 0.05$). There was no significant interaction between pollen morph and population ($F_{(4,227)} = 0.38$, $p > 0.05$ on Mykonos; $F_{(4,213)} = 1.90$, $p > 0.05$ on Syros). The fruit set of bagged plants averaged 0.50 in the Mykonos population and 0.58 in the Syros population, with no significant difference between colour morphs ($F_{(1,27)} = 0.07$, $p > 0.05$ for the Mykonos population; $F_{(1,26)} = 0.05$, $p > 0.05$ for the Syros population).

Discussion

The pollen colour dimorphisms in *N. degenii* contrasts sharply with the uniformly light pollen of related species (Strid, 1970), indicating that the dimorphism(s), or more strictly the dark pollen type(s), evolved in situ rather than being inherited from some remote ancestor. Populations of *N. degenii* on Syros and Mykonos vary greatly in morph frequency, although few have reached

Table 2. Mean percent fruit set (with SD) for open-pollinated plants with dark or light pollen

	$N_{D/L}$	Pop. freq.	Dark		Light	
			Mean	SD	Mean	SD
A. Mykonos	28/18	0.04	0.72	0.17	0.71	0.23
	25/27	0.06	0.74	0.20	0.75	0.18
	25/22	0.13	0.88	0.14	0.82	0.25
	23/23	0.46	0.86	0.15	0.81	0.24
	25/21	0.72	0.87	0.23	0.89	0.22
B. Siros	26/13	0.02	0.83	0.20	0.86	0.20
	17/12	0.05	0.61	0.33	0.80	0.27
	29/24	0.05	0.86	0.22	0.78	0.30
	26/25	0.16	0.87	0.17	0.89	0.17
	24/27	0.44	0.86	0.22	0.76	0.35

A. Populations from Mykonos (*N. degenii* subsp. *barbro*). B. Populations from Syros (*N. degenii* subsp. *jenny*). Populations are arranged according to increasing frequency of the dark morph in the population (Pop. freq.). $N_{D/L}$ = the number of dark/light plants examined.

fixation for the dark morph (Jorgensen and Andersson, 2005). These patterns indicate either that the different populations are at different stages on the way to the fixation of the dark morph, or that selection is operating in a way that facilitates the co-occurrence of both pollen morphs at most sites, albeit at widely different frequencies. Alternatively, non-adaptive processes like genetic drift and gene flow may contribute to the persistence of the colour dimorphisms. Comparison of the variation at the pollen colour locus with the variation in neutral AFLP markers clearly suggests that the observed dimorphism is under diversifying rather than uniform selection in both subspecies Jorgensen and Andersson, 2006. Although results from the present study agree with a general advantage of the dark pollen type in single-donor pollinations, our data also provide support for a 'balancing selection scenario' involving the maintenance of both colour morphs. First, the interactive effect of pollen placement (tip vs. base) and parent combination (plant triplet) on progeny morph ratio after two-donor pollinations should obscure the overall advantage of the dark morph under conditions of high pollen competition and a variable genetic background. Second, based on the widely different visitation patterns in the garden experiment, there should be potential for changes in the pollinator fauna to cause temporally or spatially variable selection on the colour dimorphism.

Observations of flower visitors in two garden populations of *N. degenii* subsp. *jenny* indicated that pollinators responded differently to plants with different pollen (and anther) colour, but the magnitude of this effect depended on the date and the type of pollinator: bumble bees preferentially visited the light pollen morph on one date at site G, whereas honey bees preferentially visited the dark morph on two dates at site B. On each of these occasions, the number of pollinator visits was low; hence, it is possible that individual visitors were expressing constancy rather than manifesting a general preference for a particular colour morph. The relative visitation rate to the morphs did not vary in a frequency-dependent manner, as demonstrated for some petal colour polymorphisms (Brown and Clegg, 1984; Gigord *et al*., 2001). Given that these results can be generalized to natural populations, one would expect temporal or spatial variation in the pollinator fauna to influence the relative fitnesses of the dark and light pollen morph, a factor that could stabilise pollen colour dimorphism in *N. degenii*.

There was no detectable effect of pollen colour on pollination success (fruit set) in natural populations of *N. degenii*. The (apparent) lack of morph effects in the field study has at least three possible explanations: (i) that the natural pollinators of this species play a minor role in exerting selection on pollen colour; (ii) that fruit set was limited by resources rather than by pollen; or (iii) that our measure of fruit set—the proportion of straightened styles—also responded to wind- or self-pollination, a factor that would obscure existing

differences in the rate of pollinator visitation. Consistent with the occurrence of some wind- or self-pollination, plants of both colour morphs were found to have a relatively high proportion of straightened styles after exclusion of (large) pollinators. More data on pollinator visitation and male reproductive success in natural populations are required to distinguish between these possibilities.

Plants of *N. degenii* subsp. *barbro* with dark pollen had a reproductive advantage in single-donor pollinations; pollinations with dark pollen resulted in more seeds per follicle than pollinations with light pollen. A similar difference was observed in *N. degenii* subsp. *jenny*, although this result failed to reach significance. These patterns were not caused by differences in pollen viability, nor can they be attributed to differences in pollen grain number or competition between pollen donors: each comparison involved two follicles in the same flower, pollinated with excess pollen from a single male parent. The relationship between pollen colour and male seed set in the single-donor experiment is therefore likely to reflect differences in the proportion of pollen grains that failed to germinate on the stigma, the proportion of pollen tubes that were arrested in the style, or the proportion of fertilised ovules that aborted or failed to initiate embryo development. We have no direct evidence as to the mechanism(s) underlying the difference in fertilisation success, but note that the production of floral anthocyanins also results in compounds that influence the growth of pollen tubes (Mo *et al.*, 1992).

The overall reproductive advantage of the dark pollen morph, as observed in the single-donor pollinations, disappeared when pollen from both morphs were applied on the same stigma and when the reproductive success of each morph was quantified as progeny morph ratio (realised siring ability). One interpretation of this finding is that dark pollen has a relatively low competitive ability during pollen germination, pollen tube growth or ovule development, thereby counteracting the morph-specific difference seen in the single-donor experiment. However, we found no tendency for differences in siring ability to become more accentuated when pollen tubes had to grow a longer distance to reach the ovules (tip pollination), as would be expected if differences in competitive ability were important. Alternatively, there is potential for confounding factors, for example genetic background effects and subtle differences in the timing and placement of pollen deposition etc., to affect the relative siring ability of the competing pollen donors. Consistent with the existence of genetic background effects and other confounding factors, we found different combinations of male and female parents (plant triplets) to produce widely different progeny morph ratios after the two pollination treatments (tip vs. base pollination). In this context, it is worth noting that the two-donor experiment contrasted unrelated pollen donors (plants from different homozygous lines), while the single-donor pollinations contrasted related pollen donors

(plants from the same full-sib family). Consequently, one would expect the single-donor experiment to provide greater statistical control for differences in the genetic background, thus enhancing the statistical power of this experiment to detect a general morph effect.

Although the role of selection has become a major topic in studies of flower colour polymorphisms, our study is among the first to examine the importance of pollinators and other ecological forces in exerting selection on pollen colour (Lau and Galloway, 2004). We found significant relationships between pollen colour and three fitness components directly associated with pollination (visitation rate, fertilisation success and siring ability) that may contribute to the stabilisation of pollen colour dimorphisms in *N. degenii*. A similar role for selection in maintaining colour polymorphisms through the pollination phase has previously been documented for petal colour polymorphisms (Brown and Clegg, 1984; Gigord *et al.*, 2001).

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