

Asymmetrical mating patterns and the evolution of biased style-morph ratios in a tristylous daffodil

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Summary

Non-random mating in plant populations can be influenced by numerous reproductive and demographic factors, including floral morphology and inter-plant distance. Here, we investigate patterns of outcrossed mating through male function in *Narcissus triandrus*, a tristylous, bee-pollinated wild daffodil from the Iberian Peninsula, to test pollen transfer models which predict that floral morphology promotes asymmetrical mating and biased morph ratios. Unlike other tristylous species, *N. triandrus* has an incompatibility system that permits intra-morph mating and long-level rather than mid-level stamens in the L-morph. Incomplete sex-organ reciprocity should result in significant intra-morph mating in the L-morph. We measured mating patterns in two L-biased populations – dimorphic (two style morphs) and trimorphic (three style morphs) – using multilocus genotyping and maximum-likelihood-based paternity analysis. We also examined the spatial distribution of style morphs and neutral markers to investigate the potential consequence of spatially restricted mating on morph ratios. As predicted, we detected significant amounts of intra-morph mating in the L-morph in both populations. Pollen transfer coefficients generally supported predictions based on the Darwinian hypothesis that anthers and stigmas of equivalent level promote pollinator-mediated cross-pollination in heterostylous populations. There was evidence of significant spatial aggregation of both style morphs and neutral markers in populations of *N. triandrus*, probably as a result of restricted pollen and seed dispersal. Our results provide empirical support for theoretical models of pollen transfer, which indicate that the commonly observed L-biased morph ratios in *Narcissus* species result from significant intra-morph mating in the L-morph because of its atypical floral morphology.

1. Introduction

Mating is typically non-random within plant populations as a result of diverse reproductive and demographic factors that influence pollen dispersal (e.g. Barrett *et al.*, 1987; Jones & Reithel, 2001; Oddou-Muratorio *et al.*, 2005; Weis, 2005). Consequently, understanding the determinants of mating patterns within plant populations represents an important challenge for plant evolutionary biologists. Until recently, the measurement of mating patterns has largely been concerned with estimating the proportion of self and outcross progeny produced by maternal parents using allozyme markers (reviewed in Barrett & Eckert, 1990; Vogler & Kalisz, 2001). However, the

development of hypervariable molecular markers such as microsatellites has enabled parentage assignment to offspring and more refined analysis of mating patterns, including estimates of outcrossing through male function (reviewed in Jones & Ardren, 2003; Garant & Kruuk, 2005). This advance should allow the causes and consequences of non-random mating to be directly investigated in plant populations.

Mating patterns in typical heterostylous populations are non-random because of shared incompatibility type among plants of the same mating group. Heterostylous species have two (distyly) or three (tristyly) floral morphs that differ reciprocally in the placement of their anthers and stigmas (Darwin, 1877; Barrett, 1992, Fig. 1*a*). These polymorphisms function primarily as mechanisms promoting cross-pollination and generally result in inter-morph

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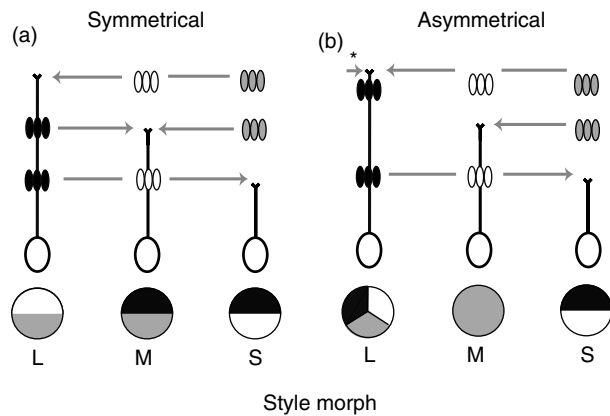


Fig. 1. Symmetrical and asymmetrical patterns of mating in heterostylous plant populations. (a) Typical tristily, in which heteromorphic incompatibility only allows mating between stigmas and anthers of equivalent height, resulting in symmetrical disassortative mating, as indicated by the arrows. (b) Tristily with imperfect reciprocity. The diagram illustrates the predicted asymmetrical mating patterns for *Narcissus triandrus* because reciprocity between sexual organs is not complete and this species possesses a self-incompatibility system that allows intra-morph (assortative) mating. Circles illustrate the predicted share of mating obtained by each morph. L, M and S refer to the L-, M- and S-morphs. The arrow below the asterisk represents pollen transfer from the long-level anthers of the L-morph to the long-level stigma, rather than self-fertilization in the L-morph. Black, L-morph; white, M-morph; grey, S-morph. (After Barrett & Hodgins, 2006).

(disassortative) mating among the morphs (Darwin, 1877; Barrett *et al.*, 1987; Lloyd & Webb, 1992*a, b*). Most heterostylous species possess a heteromorphic incompatibility system in which intra-morph (assortative) mating is prevented physiologically. This ensures symmetrical mating among morphs and typically results in equal morph ratios (isoplethy) at equilibrium due to the action of negative frequency-dependent selection (Fisher, 1941; Charlesworth & Charlesworth, 1979; Heuch, 1979*a*; Barrett & Hodgins, 2006). However, some heterostylous species lack heteromorphic incompatibility (reviewed in Barrett & Cruzan, 1994) and these species provide opportunities to study directly the role of floral morphology in determining patterns of mating, without the confounding effect of heteromorphic incompatibility.

Populations of many heterostylous species deviate from the predicted isoplethic equilibrium. In most cases, biased morph ratios (anisoplethy) result from interactions between stochastic forces (genetic drift and founder effects) and clonal propagation in non-equilibrium populations (reviewed in Barrett, 1993). However, anisoplethy can also be caused by changes in the form of negative frequency-dependent selection because of asymmetrical mating among morphs. Asymmetrical mating can potentially result from a number of mechanisms, including morph-specific

changes in compatibility relations or differences in rates of self-fertilization among the morphs (Weller, 1992; Barrett & Hodgins, 2006). In species that lack heteromorphic incompatibility, changes in the position of sexual organs affecting pollen dispersal could also result in asymmetrical mating and biased equilibrium morph ratios.

Narcissus triandrus is a heterostylous wild daffodil from the Iberian Peninsula that possesses population style-morph ratios that commonly deviate from isoplethy (Fernandes, 1965; Barrett *et al.*, 1997, 2004). The species is tristylous with three morphs (long-, mid- and short-styled morphs, hereafter referred to as L-, M- and S-morphs) and a self-incompatibility system that permits both inter- and intra-morph mating. Allozyme markers generally indicate that populations of *N. triandrus* maintain high outcrossing rates with low rates of self-fertilization that do not differ among the style morphs (Barrett *et al.*, 1997; Hodgins & Barrett, 2006*a*). In common with other *Narcissus* species with stylar polymorphisms (reviewed in Barrett *et al.*, 1996; Barrett & Harder, 2005), *N. triandrus* has incomplete sex-organ reciprocity, with the L-morph possessing elongated upper-level stamens (Fig. 1*b*). Consequently, unlike any known tristylous species, all three morphs of *N. triandrus* have long-level anthers, only the S-morph possesses mid-level anthers, and in common with other tristylous species the L- and S-morphs have short-level anthers. If floral morphology influences mating patterns and Darwin's (1877) proposal that most cross-pollination in heterostylous populations results from pollen transfer between anthers and stigmas of equivalent height is true (see Lloyd & Webb, 1992*b*), then the imperfect pattern of sex-organ reciprocity displayed by *N. triandrus* should result in significant levels of assortative mating in the L-morph, at the expense of pollen transfer from the L-morph to the M-morph.

Theoretical models of heterostylous populations have explored the influence of pollen transfer on equilibrium morph ratios (Charlesworth, 1979; Lloyd & Webb, 1992*b*). This general approach has enabled investigations of the consequences of incomplete sex-organ reciprocity on pollen transfer, mating and morph-ratio evolution (Barrett *et al.*, 1996, 2004; Baker *et al.*, 2000; Hodgins & Barrett, 2006*b*). These models indicate that in populations with the stigma-anther placement that is characteristic of *N. triandrus*, assortative mating in the L-morph should result in L-biased morph ratios (Barrett *et al.*, 2004; Hodgins & Barrett, 2006*b*). Large-scale surveys of style-morph ratios in populations of *N. triandrus* are consistent with this prediction and have revealed that over 90% of populations are L-biased, implicating asymmetrical mating as the cause of anisoplethy (Barrett *et al.*, 2004). However, direct measurements of the patterns

of outcrossed mating among morphs in *N. triandrus* have not been conducted. The main goal of this study was to test the hypothesis that, unlike other heterostylous species, asymmetrical mating is a feature of the reproductive biology of *N. triandrus* populations.

In addition to floral morphology, demographic factors can also influence mating patterns in plant populations. In particular, because of the sessile nature of plants the distance between individuals and the size and composition of mating neighbourhoods should also influence patterns of mating (Cresswell, 2006). Spatial genetic structure (SGS), or the spatial distribution of genotypes, reflects the extent of local gene flow through pollen and seed dispersal and is a common feature of plant populations (Heywood, 1991; Vekemans & Hardy, 2004). The development of SGS depends on a variety of factors including plant density, the number of compatible mates, and pollinator foraging behaviour (Van Rossum *et al.*, 2004; Vekemans & Hardy, 2004). Restricted pollen and seed dispersal can also influence the distribution of mating types within populations (Olson *et al.*, 2006; Stehlik *et al.*, 2006), although the degree of spatial structure will also be affected by sexual recruitment and the intensity of negative frequency-dependent selection (Muirhead, 2001; Schueler *et al.*, 2006). In plant species with polymorphic sexual systems and local pollen dispersal, mating patterns will depend on the number, frequency and spatial distribution of mating types within populations. Therefore, restricted pollen and seed dispersal within heterostylous populations should have consequences for mating patterns and hence morph-frequency evolution. Because *N. triandrus* is potentially capable of both assortative and disassortative mating, populations may be especially sensitive to the influence of the morph structure of local neighbourhoods on mating patterns.

Here, we employ paternity analysis to measure patterns of outcrossed mating through male function in natural populations of *N. triandrus* to test the prediction that populations engage in asymmetrical mating. We also examine the spatial structure of morphs and of neutral genetic markers to investigate the extent to which the spatial patterning of morphs may influence non-random mating. Our study specifically addressed two main questions: (1) What are the patterns of outcrossed mating among the style morphs? We predicted significant levels of assortative mating in the L-morph, whereas mating in the M- and S-morphs should be largely disassortative. We also predicted that because only the S-morph possesses true mid-level anthers most mating in the M-morph should result from pollen dispersal from the S-morph. (2) Are the style morphs and neutral genetic markers spatially structured within populations? The spatial structuring of morphs within populations should influence mating patterns, particularly in populations

that are genetically subdivided and where pollen dispersal is restricted. For example, the hyperdispersion of morphs could promote disassortative mating (Wolfe, 2001), whereas spatial aggregation should promote assortative mating (Stehlik *et al.*, 2006).

2. Materials and methods

(i) Study organism

Narcissus triandrus is a non-clonal, bee-pollinated geophyte, common in the central and northern parts of the Iberian Peninsula. Flowering begins in early March and continues until late April and early May at higher elevations. Flowering plants produce a single stem with pale yellow to white flowers, ranging in number from 1–9 (mean = 1.6), which last up to 14 days. Flowers are pendulous with reflexed tepals and have a narrow floral tube with a prominent corona. Solitary bees (primarily *Anthophora* spp.) are the main visitors in the southern portion of the range but are largely replaced by *Bombus* spp. in the cooler Atlantic zone of northern Spain and Portugal. Pollinator visitation rates are generally low in populations of *N. triandrus*, although pollen limitation is not a common feature of populations (Hodgins & Barrett, 2006b), probably because of the extended longevity of flowers.

(ii) Spatial distribution of style morphs

In 2003 and 2004 we sampled 33 populations (13 dimorphic and 20 trimorphic) in central Portugal and north-west Spain, recording latitude and longitude at each site. Localities and morph ratios for all populations are available from the first author upon request. In each population, we also estimated the style-morph ratios (see Barrett *et al.*, 2004 for details). Populations were identifiable as discrete colonies of plants separated from other populations usually by several kilometres. In 26 of these populations (12 dimorphic and 14 trimorphic), we selected focal individuals (mean = 37.4, range = 13–46) and recorded the morph of the nearest neighbour to these plants. Near-neighbour pairs were randomly selected from individuals in the population that were not previously sampled and hence sampling was without replacement. In 10 of these populations we also mapped the location of style morphs in areas ranging in size from 32.5 to 553.4 m² ($N = 124$ – 517 individuals), depending on the density of individuals.

(iii) Data analysis of style-morph distribution

Clustering of morphs will influence patterns of mating within populations if pollen dispersal is local. To determine whether the style morphs were

spatially segregated we compared the local style-morph ratios for each of the morphs to the population morph ratio using Pielou's (1961) coefficient of segregation: $S' = 1 - (O/E)$, where O is the observed number of focal and near-neighbour pairs comprised of different morphs and E is the expected number. The expected number of each pair type was calculated assuming random pair formation with respect to style morph. Positive values of the coefficient of segregation (S') indicate spatial clumping of the morphs, whereas negative values indicate an affinity between opposite morphs (Pielou, 1961). This method has been used previously to test for spatial structure of morphs in several heterostylous species (e.g. Levin, 1974; Ornduff & Weller, 1975; Wolfe, 2001). To evaluate whether populations possessed significant spatial structuring of morphs, we used a t -test to determine whether the average coefficient of segregation (S') from the 26 populations was significantly different from zero. We also used goodness of fit G -tests to determine whether nearest neighbour pairs were more often formed from opposite morph pairs than expectations based on population morph frequencies for each population. We used the false discovery rate procedure (MULTTEST procedure, SAS) to correct for multiple tests (Benjamini & Hochberg, 1995).

To test for spatial aggregation of morphs in the 10 populations that we mapped, we calculated the average difference between the neighbourhood and population morph frequencies (d_i) for morph i as:

$$d_i = \frac{\sum_1^{10} \sum_1^{n_i} (f_i - F_i)}{\sum_1^{10} n_i}$$

where f_i and F_i represent the neighbourhood and the population morph frequencies of the i th morph, respectively, and n_i represents the number of individuals of the i th morph in each population (see Stehlik *et al.*, 2006 for details). We calculated the neighbourhood morph frequency, f_i , as the number of plants of the L-, M- or S-morph divided by the total number of plants within a certain radius of each focal plant. We computed the mean differences (d_i) for a range of neighbourhood radii (1–9 m). We performed significance testing using 1000 permutations of the data within each population. The morphs exhibited significant spatial structure at each distance if the observed d_i value was greater than the 97.5% percentile or less than the 2.5% percentile of the d_i values based on permutations at each distance.

(iv) Population sampling and genotyping for paternity analysis

During 2003 and 2004 we located one dimorphic (139 individuals) and two trimorphic populations of *N. triandrus* (113 and 154 individuals). The sites at

which these populations occurred were separated from conspecifics by at least 200–300 m to reduce the likelihood of pollen flow from unsampled individuals. In each population we tagged every individual and collected a leaf sample. We also mapped the location of all individuals and recorded their style morph. Although most plants were flowering, we identified individuals that were in bud and removed them from populations. For those individuals that were past anthesis, we identified the style morph, where possible, and location, and collected leaf tissue in case these individuals had already sired seeds. Four to five weeks later, we collected capsules from all individuals that produced seed.

We extracted total genomic DNA from leaf tissue using the Puregene DNA isolation kit (Gentra Systems). For two of these populations (populations 204 and 254) we extracted total genomic DNA from the progeny (population 204 mean = 5.4 seeds from 41 maternal families; population 254 mean = 6.1 seeds from 50 maternal families) using the Qiagen DNeasy kit according to the manufacturer's instructions for low quantities of DNA. We dried the capsules and then stored the seeds in the dark at 4 °C. Prior to extraction, we soaked the seeds in distilled water to loosen the seed coat and to induce germination. We then extracted DNA from the germinated cotyledons, or, as germination rates were very low, from embryos, which we removed using a dissecting scope and fine forceps. We measured the quality and quantity of all DNA using a mass spectrometer and diluted the DNA to a final concentration of 50 ng/ μ l for parental samples and 25 ng/ μ l for offspring samples. We discarded samples of low quality and re-extracted parental samples while we replaced low-quality offspring samples with other individuals from the same maternal family.

To assess mating patterns among morphs and SGS we used five microsatellite primer pairs (NT26, NT63, NT113, NT154 and NT155) to genotype adults and progeny (Hodgins *et al.*, 2007). We performed DNA amplification using the following conditions: 50 ng of genomic DNA in a 25 μ l PCR volume, along with 0.1 μ M of primer, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 1.25 U *Taq* Polymerase (Fermentas) and 1 \times PCR buffer with (NH₄)₂SO₄. DMSO was added to NT26 reactions to a final concentration of 5%. The cycling conditions were 4 min initial denaturing followed by 40–50 cycles of: denaturing for 30 s at 94 °C, annealing at 59–63 °C (depending on the primer pair) for 30 s, and extension for 30 s at 72 °C with a 72 °C 10 min final extension. We sent the PCR reactions to the Genetic Analysis Facility of the Centre for Applied Genomics (The Hospital for Sick Children, Toronto, ON) for fragment analysis and we conducted size alignments using GeneMapper v.3.5 software.

(v) *Spatial structure of neutral alleles*

We used autocorrelation analysis (Epperson, 1990; Heywood, 1991; Smouse & Peakall, 1999) to investigate spatial genetic structure (SGS) in three populations (populations 204, 207 and 254). We assessed SGS using the kinship coefficient F_{ij} (Loiselle *et al.*, 1995), which has been shown to perform well under a wide range of conditions, including in populations with rare alleles and significant levels of inbreeding (Vekemans & Hardy, 2004). Significantly positive values of F_{ij} are expected for short distance intervals when localized dispersal results in spatial aggregation of individuals with common ancestry. Therefore, the slope of the regression (b_F) between F_{ij} and geographic distance is predicted to be negative when there is SGS. For each population, we plotted the multilocus kinship estimators against the logarithm of distance and tested the regression slopes (b_F) for significance by Mantel tests with 1000 permutations. We assigned each pair of individuals to a distance class using the Euclidean distance separating the pair and selected classes so the number of pairs in each class was equal (~ 1000 pairs). This resulted in nine distance classes in population 204, ten classes in population 207 and six classes in population 254. To facilitate comparisons among populations we used the 'Sp' statistic, where $Sp = -b_F / (1 - F_1)$, and F_1 is the kinship estimator F_{ij} between adjacent individuals. Therefore, higher values of Sp reflect stronger spatial structuring. The Sp statistic allows for comparison among species and populations because the kinship estimator, F_{ij} , depends on the sampling scheme used, whereas Sp does not (Vekemans & Hardy, 2004). We conducted the analysis using SPAGeDi v.1.2 (Hardy & Vekemans, 2002).

(vi) *Paternity analysis and measurements of mating patterns*

We performed a maximum likelihood (ML)-based paternity analysis using Cervus 3.0 (Marshall *et al.*, 1998; Kalinowski *et al.*, 2007) in populations 204 and 254. Cervus calculates the probability of paternity for each potential father based on Mendelian segregation probabilities given the genotypes of offspring, their known maternal parents and potential fathers. Paternity is assigned to the male with the highest log-likelihood ratio (LOD score; Meagher, 1986). The difference in the LOD scores between the most likely and second most likely male (Δ) is calculated for each offspring. Using Cervus, we conducted simulations of paternity to determine whether the difference in the LOD scores (Δ) between the first and the second most likely father were statistically significant. We permitted self-fertilization in the analysis. We determined critical Δ values using

the simulated distributions of Δ scores for cases where the most likely father was the true father and for cases where the most likely father was not the true father. We calculated the critical Δ scores such that 95% (strict criterion) or 80% (relaxed criterion) of the Δ scores exceeding this value resulted from a true father. Higher confidence criteria were simply not possible with our data; however, 95% and 80% are the standard range of criteria considered generally acceptable in paternity studies (e.g. Marshall, 1998; Vassiliadis *et al.*, 2002; Nishizawa *et al.*, 2005). Although we genotyped all potential fathers in the population, distant individuals may have contributed to the pollen pool. Therefore, in the simulations we estimated that 90% of the candidate parents were sampled and included a 0.01 genotyping error rate. Prior to analysis, we removed all the genotypes of offspring where the known mother could not have been a parent based on the Mendelian segregation (approximately 5% in both populations), as this is likely to have resulted from errors in genotyping or contamination.

We assessed mating patterns among morphs in both populations by identifying progeny for which a single father was assigned by the ML-based categorical analysis using 80% and 95% confidence criteria. We then identified the morph of the most likely father for each offspring. To examine the significance of mating patterns among the morphs, we used goodness-of-fit tests (G -tests; Sokal & Rohlf, 1995) and compared the observed patterns of mating with those that would be expected given random mating among the morphs. We derived the expected frequencies from the frequencies of morphs in the population and the number of offspring that were successfully assigned a father from each maternal morph. This allowed us to determine: (1) whether the mating patterns among morphs were non-random, (2) the level of assortative and disassortative mating for each morph, (3) whether the S-morph sired the majority of seeds produced by the M-morph.

(vii) *Assessment of Darwin's pollen transfer hypothesis*

For heterostylous species, Lloyd & Webb (1992b) developed a method for comparing pollen transfer among morphs from the viewpoints of both pollen donation and pollen receipt. This method can be used to assess the mating consequences of Darwin's pollen transfer hypothesis. Using previously published data on stigmatic pollen loads, Lloyd & Webb (1992b) determined the probability of transfer of a single pollen grain of morph i to stigmas of morph j . We estimated pollen transfer coefficients, q_{ij} , using a similar method. Specifically, for populations 204 and 254 we indirectly calculated pollen transfer

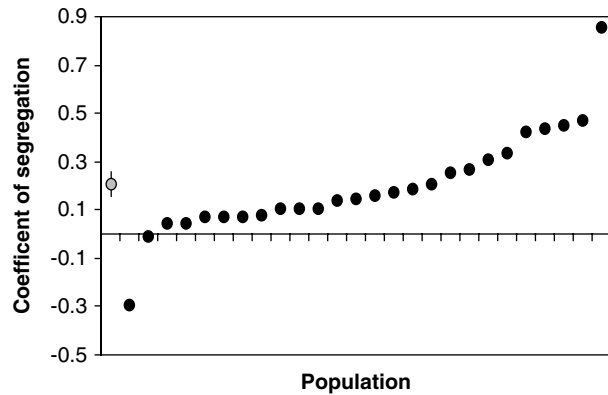


Fig. 2. Intra-population morph structure for 26 populations of *Narcissus triandrus* sampled from the Iberian Peninsula. The grey circle represents the population mean \pm SE for the coefficient of segregation (S'), while the black circles represent the coefficient of segregation for each population. Values greater than zero represent spatial aggregation of the morphs. The populations are ranked in ascending order for values of the coefficient of segregation.

coefficients using the proportion of seeds sired in each population by morph i on morph j and dividing by the frequency of morph i in the population. This value represents the average siring success of an individual of morph i on morph j and is analogous to Lloyd & Webb's (1992b) pollen transfer coefficients, except that it measures the mating consequences of particular pollen transfers. This approach cannot be applied to typical heterostylous species because heteromorphic incompatibility only permits disassortative mating whereas in *N. triandrus* compatible cross-pollination is independent of style morph.

3. Results

(i) Style-morph distribution

Goodness of fit G -tests revealed that 7 of the 26 populations had significantly aggregated style morphs ($\alpha=0.05$). On average, populations of *N. triandrus* had coefficients of segregation that were significantly greater than zero, indicating spatial aggregation ($t_{25}=4.65$, mean=0.20, SE=0.042, $P<0.001$; Fig. 2). Only two out of 26 populations had coefficients of segregation equal to or less than zero. This pattern reveals that morphs are generally spatially aggregated within populations of *N. triandrus*. The coefficient of segregation for dimorphic (mean=0.22, SE=0.063, $N=12$) and trimorphic populations (mean=0.17, SE=0.058, $N=14$) was not significantly different ($F_{1,24}=0.38$, $P=0.54$). Our analysis of the spatial distribution of morphs using mapped populations revealed that, over short distances, all three morphs were significantly clumped despite the non-clonal habit of the species (Fig. 3). The S-morph exhibited

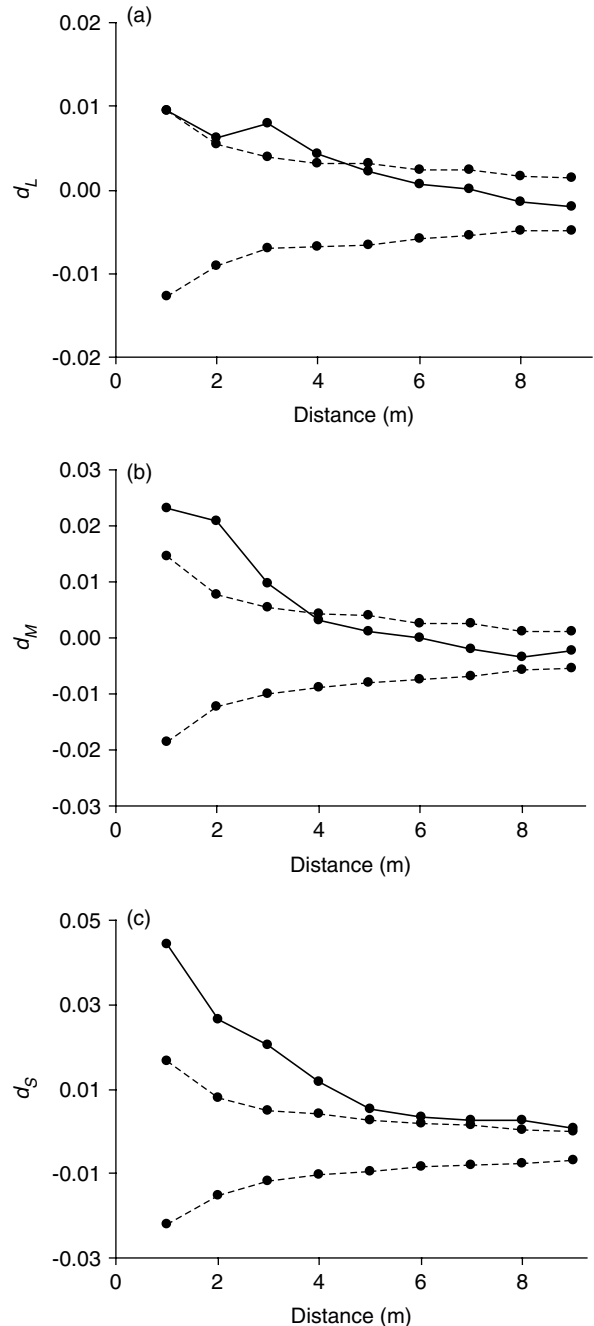


Fig. 3. The morph structure of ten mapped populations of *Narcissus triandrus* from the Iberian Peninsula. The deviation of the local morph frequency from the population morph frequency at each distance is shown, for (a) the L-morph, d_L ; (b) the M-morph, d_M ; and (c) the S-morph, d_S . The dashed lines represent the permutation envelopes calculated based on 1000 permutations of the morphs among locations within each population (see text for details). Values above or below this envelope are significant at $P<0.05$.

the strongest clumping (significant aggregation from 1 to 9 m), followed by the M-morph (significant aggregation from 1 to 3 m). The L-morph exhibited weak aggregation at short distances (significant aggregation from 1 to 4 m).

Table 1. The spatial genetic structure within three populations of *Narcissus triandrus*

Population	b_F	F_1	Sp
204	-0.018***	0.028	0.022
207	-0.020***	0.033	0.021
254	-0.0047**	0.0082	0.0047
Mean	-0.014 ± -0.008	0.023 ± -0.013	0.015 ± -0.009

b_F is the regression slope of pairwise kinship coefficients, F_{ij} , and the logarithm of distance; F_1 is the average F_{ij} among individuals in the first distance class; and the Sp statistic follows Vekemans & Hardy (2004). Means ± SD are shown for each statistic. The significance of the regression slope was tested using a one-sided Mantel test with 1000 permutations.

** $P < 0.01$, *** $P < 0.001$.

(ii) Spatial population substructure

Mantel tests identified a significant decline of pairwise kinship coefficients with increasing distance indicating significant SGS in all three populations (Table 1, Fig. 4). In population 204, significant positive kinship coefficients were found for distance classes 1–3 (<3.8 m) and significant negative kinship coefficients were found for the last three distance classes (>7.2 m, $b_F = -0.018$, $P < 0.001$). Significant SGS was also found in population 207, where significantly positive kinship coefficients were identified for the first three distance classes (<4.3 m) and significantly negative coefficients were found for the last four distance classes (>7.9 m, $b_F = -0.020$, $P < 0.001$). There was significant but weak SGS in population 254 ($b_F = -0.0047$, $P < 0.01$) compared with the other two populations. Significant positive kinship coefficients were evident for the first two distance classes (<3.5 m) and a significant negative kinship coefficient was identified for only the fourth distance class (10.5–12.5 m). The greatest spatial structure was found in population 207 ($Sp = 0.021$), while population 254 had the weakest spatial structure ($Sp = 0.0047$). The average Sp for all three populations was 0.015 ± 0.009 .

(iii) Categorical paternity analysis and patterns of outcrossed mating

In the dimorphic population (204), morph frequencies were 0.89 and 0.11 for the L- and S-morph, respectively. Using the categorical paternity analysis, we could assign fathers to 8% and 25% of the offspring ($N = 224$) using the 95% and 80% criteria, respectively (Fig. 5a). Therefore, for 75% of the offspring sampled paternity was unresolved. Because of the small number of progeny whose paternity was determined with the 95% criterion, we analysed mating patterns in the dimorphic population using the 80%

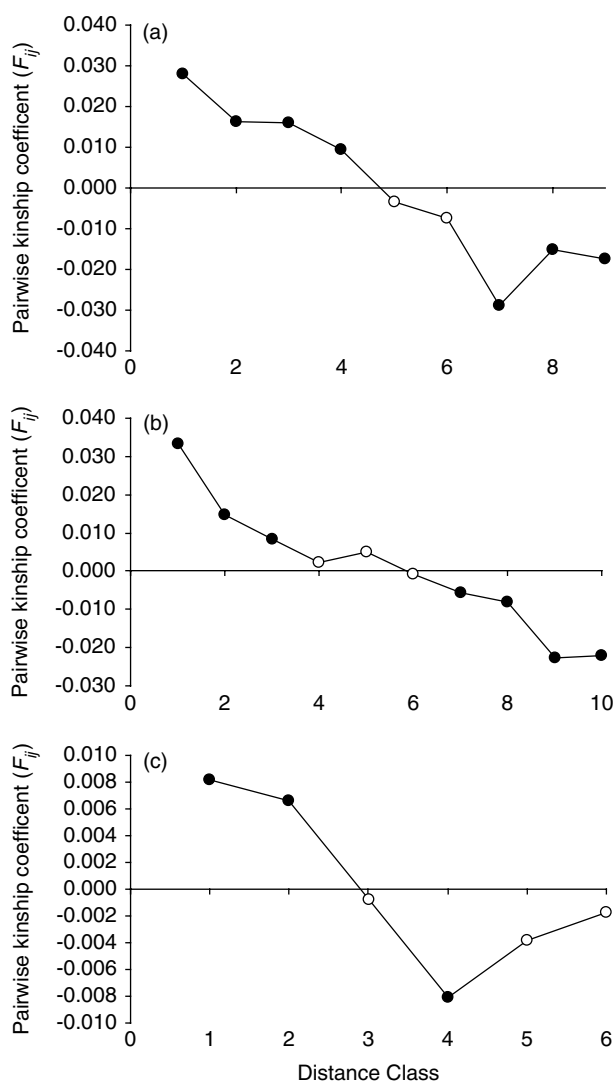


Fig. 4. Spatial correlograms of mean pairwise kinship coefficients F_{ij} of distance classes for three populations of *Narcissus triandrus* from the Iberian Peninsula. Filled symbols indicate a significant deviation of F_{ij} from the average of 1000 permutations. (a) Population 204, (b) population 207 and (c) population 254.

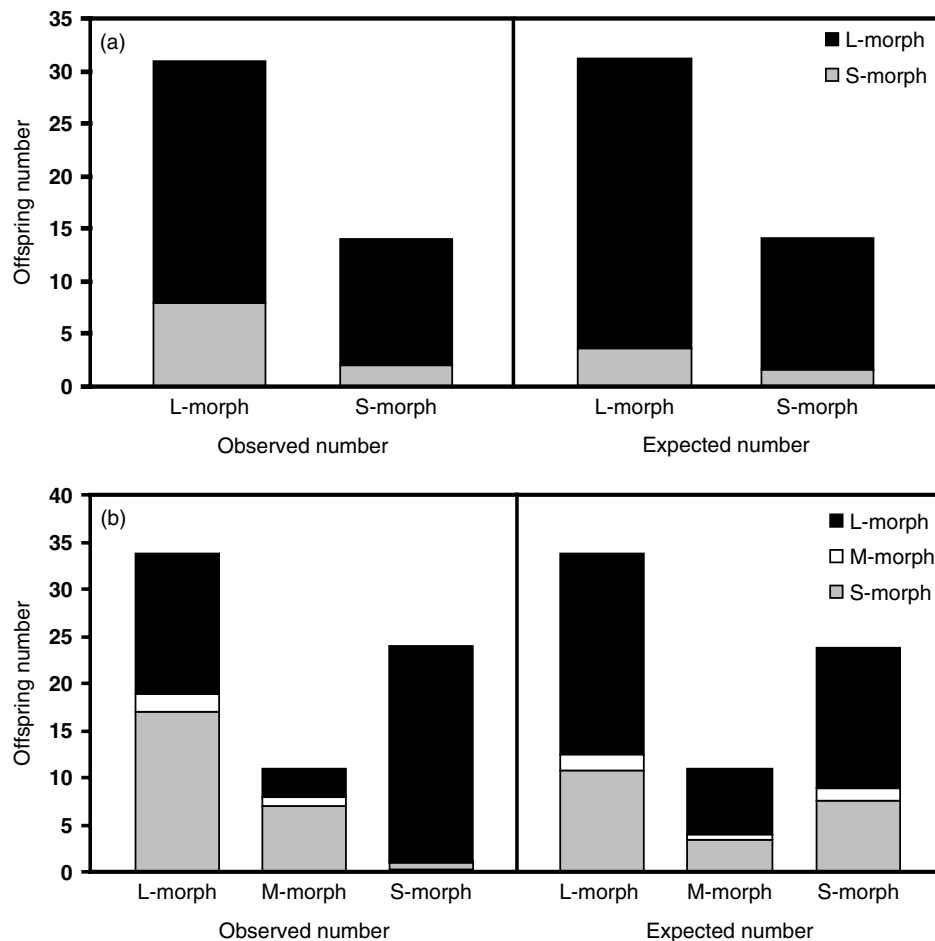


Fig. 5. Mating patterns among the morphs in two populations of *Narcissus triandrus* from the Iberian Peninsula. The results are from a paternity analysis conducted in Cervus 3.0 (80% confidence criteria); see Section 2 for details. (a) A dimorphic population (L-morph = 0.89 and S-morph = 0.11) and (b) a trimorphic population (L-morph = 0.63, M-morph = 0.05, S-morph = 0.32). The observed number of progeny sired by each morph for each maternal morph is shown, as is the expected number of progeny sired by each morph based on morph frequencies in the population. Black, L-morph; white, M-morph; grey, S-morph.

criterion. Overall, there were significant deviations from random mating among the morphs ($G_1 = 7.16$, $P < 0.01$). The percentage of offspring produced by the L-morph sired by pollen from the L- and S-morphs was 74% and 26%, respectively. This indicates that there were significantly more offspring sired by the S-morph than expected from random mating ($G_1 = 5.89$, $P < 0.01$). Two L-morph offspring were products of self-fertilization and were included in estimates of assortative mating, although their inclusion did not qualitatively influence mating patterns. The percentage of offspring produced by the S-morph sired by the L- and S-morphs was 95% and 5%, respectively, and mating in the S-morph was not significantly different from random ($G_1 = 1.28$, $P = 0.26$).

In the trimorphic population (254), morph frequencies were 0.63, 0.05 and 0.32 for the L-, M- and S-morphs, respectively. The results of the categorical paternity analysis indicated that of the 311 offspring

genotyped, 5% and 24% of the offspring could be assigned a father using the 95% and 80% criteria, respectively (Fig. 5b). Using the 80% criterion, there were significant deviations from random mating among the morphs ($G_4 = 27.44$, $P < 0.001$). The percentage of seeds produced by the L-morph sired by the L-, M- and S-morphs were 44%, 6% and 50%, respectively, demonstrating that mating was non-random in the population ($G_2 = 5.73$, $P = 0.05$). Specifically, there were more offspring sired by the M- and S-morphs than would be expected if mating was random. Although assortative mating gave rise to nearly one-half of matings in the L-morph, it was significantly less than would be expected with random mating ($G_1 = 5.07$, $P < 0.05$). The percentage of seeds produced by the M-morph sired by the L-, M- and S-morphs was 27%, 9% and 64%, respectively, indicating that significantly more offspring were sired by the S-morph than random expectations ($G_2 = 5.95$, $P < 0.05$). However, levels of assortative mating were

Table 2. Estimated pollen transfer coefficients for a dimorphic and a trimorphic population of *Narcissus triandrus*

Male parent	Female parent				
	Population 204		Population 254		
	L	S	L	M	S
L	0.84	1.07	0.63	0.44	1.53
M	–	–	1.12	1.72	0
S	2.36	0.45	1.58	2.00	0.13

The pollen transfer matrix was calculated using offspring whose paternity was assigned with Cervus 3.0 at 80% confidence and was calculated as the average number of progeny sired by an individual of each morph within each maternal morph.

not significantly different from random ($G_1=0.26$, $P=0.6$). Finally, the percentage of offspring produced by the S-morph that were sired by the L-, M- and S-morphs was 95%, 0 and 5%, respectively ($G_2=15.77$, $P<0.001$). Significantly more offspring were sired by the L-morph and the level of assortative mating was lower than random expectations ($G_1=11.44$, $P<0.001$). No progeny were identified as the products of self-fertilization in this population.

We did not explicitly test for correlated paternity in our analyses. However, we observed that several maternal plants produced offspring sired by single paternal parents. This is not surprising given the limited pollen dispersal and high variance in male mating success that we have found in these populations (K. A. Hodgins & S. C. H. Barrett, unpublished manuscript). We note that the G -tests we have used above assume independent observations and hence a significant amount of correlated paternity violates this assumption. However, non-independence is unlikely to affect our overall conclusion that mating patterns deviate strongly from those occurring in typical tristylous species and that the L-morph engages in a significant amount of assortative mating.

(iv) Estimates of pollen transfer based on mating patterns

Estimates of pollen transfer based on measurements of outcrossed siring success generally reflected expectations based on the atypical floral morphology of *N. triandrus* and largely supported Darwinian predictions (Table 2). In the dimorphic population, as predicted, pollen transfer among individuals of the S-morph was much less frequent than pollen transfer from the S-morph to the L-morph ($q_{SL}/q_{SS}=5.20$). In contrast, pollen transfer from the L-morph to the S-morph was similar to pollen transfer among plants

of the L-morph ($q_{LS}/q_{LL}=1.29$). In the trimorphic population, pollen transfer was less frequent from the L-morph to the M-morph compared with pollen transfer to the L- and S-morphs ($q_{LL}/q_{LM}=1.61$, $q_{LS}/q_{LM}=3.5$). Pollen transfer among individuals of the S-morph occurred less often than pollen transfer from the S-morph to the other two morphs ($q_{SL}/q_{SS}=12$, $q_{SM}/q_{SS}=15$). However, contrary to predictions, pollen transfer among individuals of the M-morph was greater than pollen transfer from the M-morph to the other two morphs ($q_{ML}/q_{MM}=0.65$, $q_{MS}/q_{MM}=0$).

4. Discussion

The distribution of style morphs within heterostylous populations provides the spatial template for mating when pollen is locally dispersed. We detected significant structuring of style morphs and neutral markers in populations, probably because of restricted pollen and seed dispersal. Spatially aggregated morphs and restricted pollen dispersal have implications for mating patterns and morph-ratio evolution. As predicted, we identified asymmetrical mating among the style morphs, including significant levels of assortative mating in the L-morph. In addition, we found that the S-morph sired most seeds produced by the M-morph, a result expected because only the S-morph possesses mid-level anthers. Below we discuss the mechanisms and implications for morph-ratio evolution of the spatial genetic structure and patterns of asymmetrical mating which our study has revealed.

(i) The causes and consequences of spatially structured mating

Our analysis of neutral genetic markers revealed that populations of *N. triandrus* are spatially structured. This phenomenon is a common feature of plant populations and has been reported in species with diverse life histories, pollen and seed dispersal strategies, and sexual systems (reviewed in Heywood, 1991; Vekemans & Hardy, 2004), including species with multi-allelic incompatibility systems (e.g. *Prunus avium*: Schueler *et al.*, 2006) and heterostyly (e.g. *Primula elatior*: Van Rossum & Triest, 2006; and *P. sieboldii*: Ishihama *et al.*, 2005). Both limited pollen and seed dispersal, as well as restricted seed dispersal alone, can create the spatial structure we observed (Hamrick & Nason, 1996; Kalisz *et al.*, 2001). Because *N. triandrus* has no obvious means of seed dispersal and is diminutive in stature, seeds fall in the vicinity of maternal parents. Estimates of near-neighbour mating also indicate that pollen is locally dispersed, with greater than 64% of matings occurring among individuals 5 m apart (K. A. Hodgins & S. C. H. Barrett, unpublished manuscript). In addition, a study of mating-system variation in *N. triandrus*

(Hodgins & Barrett, 2006a) detected significant biparental inbreeding, as would be expected in populations with limited seed dispersal and local pollinator foraging. These features of the reproductive biology of *N. triandrus* enhance opportunities for mating among relatives and contribute to the pattern of kinship structuring that we found.

The magnitude of spatial structuring in all three populations of *N. triandrus* was similar to expectations based on previous studies of outcrossing plants (Vekemans & Hardy, 2004). Animal pollination, gravity-dispersed seeds and low plant density result in greater kinship structuring, while self-incompatibility should reduce spatial structure. A comparison of our findings with a recent survey of 47 plant species revealed that the average *Sp* statistic for *N. triandrus* (0.014 ± 0.009) was very similar to that of other self-incompatible species (0.013 ± 0.008), but was 10-fold lower than the average for self-compatible species (Vekemans & Hardy, 2004). Self-fertilization is known to increase spatial structure, by enhancing genetic drift and reducing gene flow because of diminished pollen dispersal. Although *N. triandrus* possesses a self-incompatibility system, limited selfing can occur (Barrett *et al.*, 1997; Hodgins & Barrett, 2006a) and two selfed progeny were detected in one population of this study. However, marker-based estimates of inbreeding depression indicate that few selfed progeny reach reproductive maturity (Hodgins & Barrett, 2006a), and, consequently, self-incompatibility and strong inbreeding depression are likely to contribute to moderate levels of SGS in *N. triandrus*.

In species with polymorphic sexual systems, disassortative mating among morphs results in negative frequency-dependent selection (Fisher, 1941; Heuch, 1979b). With restricted pollen and seed dispersal, reduced spatial structuring of the mating types relative to neutral alleles is expected because locally rare mating types have a fitness advantage (Schueler *et al.*, 2006). Therefore, in heterostylous species in which sexual reproduction predominates and heteromorphic incompatibility governs mating patterns, the style morphs should exhibit weak or no spatial structure (but see Levin, 1974; Ornduff, 1980). However, in *N. triandrus*, assortative mating is permitted and therefore the strength of frequency-dependent selection will be reduced, providing greater opportunities for the spatial aggregation of morphs.

Spatial structuring of the style morphs has recently been detected in *Narcissus assoanus*, a species with stigma-height dimorphism (Stehlik *et al.*, 2006). These authors interpreted this pattern as resulting from assortative mating in the L-morph and restricted seed dispersal. We also found significant spatial structuring of the morphs within populations of *N. triandrus* and this probably results from similar causes. The

spatial structure of morphs in *Narcissus* populations should have consequences for mating patterns reinforcing local aggregation through assortative mating, particularly given the evidence for restricted pollen dispersal. Experiments investigating the influence of the spatial structure of morphs on patterns of assortative and disassortative mating in *Narcissus* species with stylar polymorphisms are clearly warranted.

(ii) *Mating patterns among morphs and the evolution of L-biased morph ratios*

In both populations of *N. triandrus* in which we investigated patterns of mating there was clear evidence of deviations from random expectations. Non-random mating is a requirement for the maintenance of sexual polymorphism through negative frequency-dependent selection. However, non-random mating is a necessary but not sufficient condition because levels of disassortative mating must exceed assortative mating within each morph for stylar polymorphism to be maintained (Baker *et al.*, 2000). In the dimorphic population, the L-morph sired 74% of seeds produced by the L-morph. Correcting for morph frequencies, assortative mating therefore represented 26% of L-morph matings. A similar pattern occurred in the trimorphic population, where the L-morph sired 44% of L-morph seeds, and after adjusting for morph frequency, assortative mating represented 20% of matings. Therefore, although the L-morph of *N. triandrus* engaged in a significant amount of assortative mating the levels recorded did not exceed that of disassortative mating. With the exception of the M-morph, disassortative mating far exceeded assortative in the remaining morphs and hence conditions for the maintenance of polymorphism in both populations were generally met.

Observed mating patterns in the L-morph support a key prediction of the pollen-transfer model for *N. triandrus* populations (Barrett *et al.*, 2004; Hodgins & Barrett, 2006b). The model indicates that elongated upper levels stamens in the L-morph should result in significant amounts of assortative mating and the evolution of L-biased morph ratios. Using our estimates of assortative mating for the L-morph in the model (i.e. dimorphic $Q=0.26$, trimorphic $Q=0.20$) results in equilibrium morph frequencies of 0.57 and 0.43 for the L- and S-morphs, and 0.38, 0.28 and 0.33 for the L-, M- and S-morphs in dimorphic and trimorphic populations, respectively. Consequently, the levels of assortative mating in the L-morph help to explain the L-bias that occurs in dimorphic and trimorphic populations of this species. However, predictions of morph-frequency variation and evolution in *N. triandrus* also depend on accurate estimates of patterns of mating in the M- and S-morphs.

Our measurements of mating patterns in the M- and S-morphs of *N. triandrus* were limited because each morph was at low frequency (M-morph, 0.05 in the trimorphic population; S-morph, 0.11 in the dimorphic population) resulting in a lack of statistical power. Consequently, given the exclusion probability of 0.93, paternity was determined for only 11 M-morph progeny in the trimorphic population and 22 S-morph progeny in the dimorphic population. However, despite this limited sampling of progeny, patterns of mating in the M- and S-morphs generally supported our predictions. First, levels of assortative mating in these morphs should be low in comparison with the L-morph as neither morph has anthers positioned at a similar height as their stigmas. In the trimorphic population, 95% of seeds produced by the S-morph were sired by the L-morph and levels of assortative mating were significantly less than would be expected with random mating. After adjusting for morph frequency, levels of assortative mating in the S-morph were estimated as 7%. Seeds produced by the M-morph should largely result from mating with the S-morph because this is the only morph with mid-level anthers. This was indeed the case with 64% of offspring from the M-morph sired by the S-morph.

In heterostylous populations, Darwin (1877) proposed that the reciprocal positioning of female and male sex organs in different morphs promotes cross-pollen transfer through segregation of pollen on the pollinator's body. Several lines of evidence support this hypothesis (reviewed in Lloyd & Webb, 1992*a, b*). For example, studies examining the distribution of pollen on bees have revealed that pollen from different anther levels is spatially segregated on contrasting insect body parts (e.g. Wolfe & Barrett, 1989). Experimental studies of pollen loads in natural populations of heterostylous plants indicate preferential pollen transfer among sex organs of equivalent height (reviewed in Lloyd & Webb, 1992*b*). Finally, studies using style-morph (Barrett *et al.*, 1987) or allozyme (Kohn & Barrett, 1992) markers have measured mating patterns in self-compatible species and demonstrated significant levels of inter-morph mating. Collectively, these studies provide strong support for the Darwinian prediction that the reciprocal positioning of sex organs in heterostylous flowers promotes cross-pollination between anthers and stigmas of equivalent height resulting in disassortative mating.

Pollen transfer coefficients estimated from patterns of outcrossed mating in *N. triandrus* can be used to evaluate the Darwinian hypothesis on the function of heterostyly. Our results provide support for the role of disassortative mating in maintaining tristylity. For example, in the trimorphic population individuals of the L-morph should sire more seeds produced by the

L- and S-morphs than the M-morph, because only the L-morph possesses long- and short-level anthers (and not mid-level anthers). Accordingly, pollen transfer estimates from the L-morph to the L- and S-morphs were 1.6 and 3.5 times, respectively, higher than transfer from the L- to the M-morph. Similarly, individuals of the S-morph should sire more seeds produced by the L- and M-morphs compared with the S-morph because the S-morph possesses only long- and mid-level anthers. As predicted, pollen transfer among individuals of the S-morph was much less likely than pollen transfer from the S-morph to the other two morphs ($q_{SL}/q_{SS}=12$, $q_{SM}/q_{SS}=15$). However, contrary to predictions pollen transfer among individuals of the M-morph was greater than pollen transfer from the M-morph to the other two morphs. This result may be associated with aggregation of morphs and stochastic sampling variance because of the low frequency of the M-morph in this population. In the dimorphic population, patterns of siring success also reflected stigma-anther locations as pollen transfer from the S-morph to the L-morph was over 5 times greater than pollen transfer among individuals of the S-morph. In addition, pollen transfer from the L-morph to the S-morph was similar to pollen transfer from the L-morph to the L-morph ($q_{LS}/q_{LL}=1.29$). These results based on patterns of outcrossed mating generally support predictions based on the hypothesis that the positioning of anthers and stigmas in heterostylous populations promotes insect-mediated cross pollination. However, it is important to recognize that this analysis assumes that post-pollination mechanisms play no significant role in influencing siring success, as they would in a species with heteromorphic incompatibility. Studies of pollen–pistil interactions in *N. triandrus* provide no evidence that different outcross pollinations vary in siring success based on pollen tube growth (Sage *et al.*, 1999) or seed set (Barrett *et al.*, 1997).

Narcissus species with stylar polymorphisms provide a unique opportunity to demonstrate the role of morphology in influencing patterns of outcrossed mating. This is because, unlike the majority of heterostylous species, both intra- and inter-morph mating are permitted. Our finding of significant intra-morph mating in the L-morph in two natural populations of *N. triandrus* provides the first direct evidence for the occurrence of an asymmetrical mating system in a heterostylous species. This finding also supports predictions based on floral morphology and pollen-transfer models that the predominance of L-biased populations in this species is a result of elevated levels of intra-morph mating in the L-morph in comparison with the M- and S-morphs (Barrett *et al.*, 1996, 1997, 2004). Our results also illustrate that small differences in the location of sexual organs among the morphs can have important functional

consequences for mating patterns, the nature of frequency-dependent selection and the evolution of morph ratios.

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