

Female reproductive success and the evolution of mating-type frequencies in tristylous populations

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Summary

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- In tristylous populations, mating-type frequencies are governed by negative frequency-dependent selection typically resulting in equal morph ratios at equilibrium. However, *Narcissus triandrus* generally exhibits long-styled (L)-biased populations with a deficiency of the mid-styled (M)-morph.
- Here we used a pollen-transfer model and measurements of female fertility in natural populations to investigate whether these uneven morph ratios were associated with variation in female reproductive success.
- Our theoretical analysis demonstrated that morph ratio bias can result from maternal fitness differences among the morphs, and that these effects were magnified by asymmetrical mating. In nine out of 15 populations of *N. triandrus*, seed set differed significantly among the morphs, but pollen limitation occurred in only two of 11 populations investigated. Average seed set of the M-morph was positively associated with its frequency in populations. Flower size was negatively correlated with the seed set of the M-morph.
- Our results suggest that interactions between mating patterns and female fertility are responsible for variation in morph frequencies and loss of the M-morph from tristylous populations of *N. triandrus*.

Key words: female fertility, frequency-dependent selection, *Narcissus triandrus*, plant sexual polymorphisms, pollen limitation, pollen-transfer model, tristily.

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Introduction

Sexual polymorphisms influence mating patterns, gene transmission and the evolutionary dynamics of populations. They are maintained in populations by negative frequency-dependent selection, whereby the fitness of a sexual morph depends on its frequency relative to the frequency of partners with which it can mate. This class of polymorphisms has provided valuable insights into the genetics, ecology and evolution of plant populations (Ford, 1971; Charlesworth & Charlesworth, 1979a; Ganders, 1979; Lewis, 1979; Barrett, 1992; Geber *et al.*, 1999). As the sexual morphs in populations can be readily identified in the field and their inheritance is often relatively simple, predictions can be made about expected morph frequencies at equilibrium.

Heterostylous species illustrate the role of mating phenotype in controlling equilibrium morph ratios. This sexual

polymorphism includes two or three floral morphs that typically differ in morphological and physiological traits (Darwin, 1877; Barrett, 1992). In tristylous populations there are three floral morphs that differ reciprocally in the placement of anthers and stigmas within flowers (long-, mid- and short-styled, hereafter referred to as L-, M- and S-morphs). Most tristylous species also possess a heteromorphic incompatibility system that prevents self- and intramorph mating. The resulting pattern of intermorph mating that occurs within populations (disassortative mating) generally gives rise to an isoplethnic (1 : 1 : 1) equilibrium of morph ratios (Fisher, 1941, 1944; Charlesworth, 1979; Heuch, 1979; Barrett, 1993). However, some tristylous species are self-compatible or have alternative incompatibility systems (Barrett & Cruzan, 1994), and this can provide opportunities for self- and intramorph mating, resulting in biased morph ratios within populations (Barrett

et al., 1989; Eckert & Barrett, 1992, 1995; Barrett *et al.*, 2004).

Narcissus triandrus is a tristylous species that lacks heteromorphic incompatibility, and population morph ratios usually deviate strongly from isoplethy (Fernandes, 1965; Barrett *et al.*, 1997). This species possesses a late-acting ovarian self-incompatibility system in which self pollen-tube growth results in the abortion of developing ovules, preventing significant self-fertilization (Sage *et al.*, 1999). This process results in the loss of ovules that could otherwise have been available for outcrossing, a phenomenon known as ovule discounting (Barrett *et al.*, 1996). Based on large-scale surveys conducted in the Iberian Peninsula, three distinctive patterns characterize population morph ratios of *N. triandrus* (Barrett *et al.*, 2004): (1) most populations are L-morph biased; (2) there is a negative association between the frequencies of the L- and M-morphs; and (3) some populations are dimorphic and lack the M-morph. These unusual morph ratios vary geographically with decreasing frequency of the M-morph from the south-east to the north-west of the Iberian Peninsula. This variation in morph ratios is accompanied by an increase in flower size and allometric changes in the size and positional relations of sex organs (Barrett *et al.*, 2004). Geographical variation in floral morphology could affect morph ratios through its influence on pollen transfer, mating and female fertility within local populations.

Here we investigate whether variation in female reproductive success among the morphs of *N. triandrus* could explain the unusual morph ratios that characterize populations of this species. We addressed this question using two different approaches. First, we use a pollen-transfer model to explore how variation in female fertility and mating among the morphs could affect morph frequencies. Because the incompatibility system in *N. triandrus* permits intramorph mating, we were particularly interested in examining how fertility variation might interact with assortative mating to influence morph ratios. Having established the potential significance of female fertility in contributing to morph-ratio variation in theoretical populations, we next investigated whether there was evidence from natural populations of *N. triandrus* for the requisite variation in female fertility. We examined seed set of the morphs in 15 populations of *N. triandrus* and determined whether variation in female fertility was associated with the flower size and morph ratios of populations. Finally, we investigated if differences in pollen limitation among morphs could be a mechanism responsible for variation in female reproductive success among morphs.

Description

Pollen-transfer model

We modified the pollen-transfer model of Barrett *et al.* (2004) to investigate the consequences of morph-specific differences in female fertility for the evolution of morph ratios. We used

two separate sets of equations. The first incorporates ovule discounting; the second examines the effects of pollen limitation on morph-ratio evolution. For both sets of equations, pollen from a given morph competes for access to ovules in proportion to its relative abundance on the stigmas of the receiving morph. The total pollen receipt (x_i) of the i th morph is given by:

$$x_i = q_{ii}f_i + q_{ji}f_j + q_{ki}f_k \quad \text{Eqn 1}$$

We assume that there is no pollen carryover, and that individuals of all morphs export equivalent amounts of pollen, where q_{ij} represents the proportion of pollen collected from an individual of morph i that is deposited on the next stigma, given that the next plant visited is of morph j . The frequency of morph i is represented by f_i . We also assume that there are no differences in the selfing rates of morphs. This assumption has been validated for *N. triandrus* (Hodgins & Barrett, 2006).

Our first pollen-transfer model examines the effect of female fertility differences and assortative mating on morph ratios. We assume seed production is limited by resource availability, rather than by pollen receipt. The proportion of ovules of the i th morph that are not matured into seed is represented by δ_i . This would reflect a situation where morph-specific differences in ovule discounting occur (Barrett *et al.*, 1996). Hence the relative fitness of the i th morph is given by:

$$w_i = 1/2(1 - \delta_i) + 1/2[(1 - \delta_i) \times (q_{ii}f_i/x_i) + (1 - \delta_j) \times (q_{ij}f_j/x_j) + (1 - \delta_k) \times (q_{ik}f_k/x_k)] \quad \text{Eqn 2}$$

The first term, $1/2(1 - \delta_i)$, represents a plant's contribution of genes as a maternal parent. The remaining terms on the right represent a morph's paternal contributions realized through pollen competition in the pistils of the L-, M- and S-morphs, respectively.

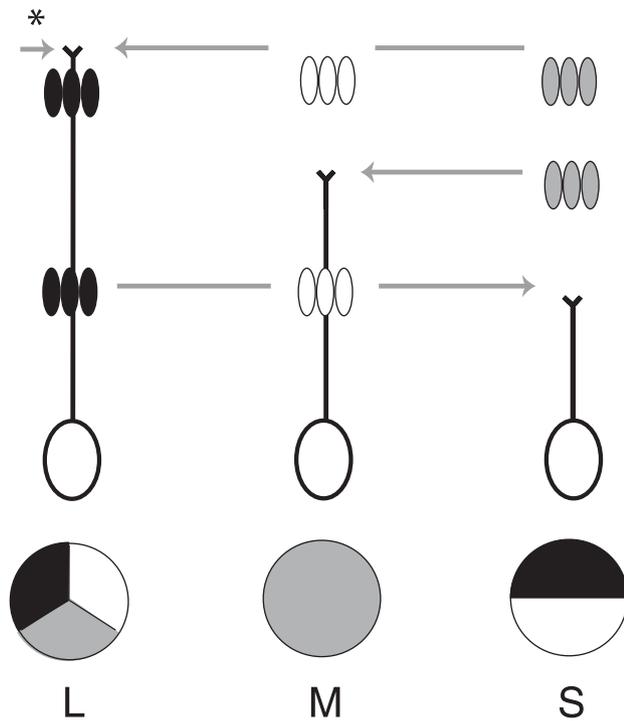
The second pollen-transfer model examines the effect of pollen limitation on female fertility. To do this, we assumed that the female fertility of a morph was a saturating function of the amount of pollen deposited on the stigma (Kohn & Waser, 1985; Waser & Price, 1991; Mitchell, 1997):

$$F(x_i) = 1 - e^{-\alpha x_i} \quad \text{Eqn 3}$$

where x_i represents the pollen receipt of morph i (equation 1) and α is a constant. We substituted this into the above pollen-transfer model (equation 2). For this model we assume no difference in ovule discounting among the morphs:

$$w_i = 1/2F(x_i) + 1/2[(F(x_i) \times (q_{ii}f_i/x_i) + F(x_j) \times (q_{ij}f_j/x_j) + F(x_k) \times (q_{ik}f_k/x_k))] \quad \text{Eqn 4}$$

Again the first term, $1/2F(x_i)$, represents a plant's contribution of genes as a maternal parent. Thus, fitness gained through female function is the result of total pollen receipt of that morph. Therefore, if pollen receipt differs among morphs, the fertility of the morphs could also differ depending on the value of α , or the level of pollen limitation. When $\alpha = \infty$, $F(x_i) = 1$ for any level of pollen receipt (x_i). Thus fertility is



Style morph

Fig. 1 Arrangement of sex organs in the three floral morphs of tristylous *Narcissus triandrus*. Note the imperfect reciprocity between anther and stigma positions that characterizes this species. Grey arrows, expected pollen transfers between anthers and stigmas of each morph. Circles below, proportion of seeds sired by each morph if $Q = 0.5$. Black, L-morph; white, M-morph; grey, S-morph. *, Pollen transfer is among plants of the L-morph and does not represent self-pollination.

determined solely by resources and is equal for all three morphs. When $\alpha = 0$, $F(x_i) = 0$ for any level of pollen receipt. However, as fertility for all three morphs must be >0 for a population to be maintained, α was constrained to values >0 . The remaining terms on the right represent a morph's paternal contribution realized through pollen competition in the pistils of the L-, M- and S-morphs, respectively.

The matrix of pollen-transfer proficiencies are those detailed by Barrett *et al.* (2004), and reflect Darwin's (1877) original proposal that in heterostylous plants pollen disperses primarily between anthers and stigmas of equivalent height, because of segregated placement of pollen on the pollinator's body. Several empirical studies of pollen dispersal in heterostylous populations provide evidence supporting this hypothesis (reviewed by Lloyd & Webb, 1992). The anomalous elevated position of the upper-level stamens of the L-morph in *N. triandrus* (Fig. 1) should promote pollen transfer from the L-morph to other plants of the L-morph (q_{LL}) at the expense of pollen transfer to the M-morph (q_{LM}). We represent this by $q_{LL} = Q$ and $q_{LM} = 0.5 - Q$, with $0 \leq Q \leq 0.5$. This pattern of pollen transfer should result in a significant level of assortative

mating in the L-morph. Pollen transfer coefficients for mid- and short-level anthers and their corresponding stigma heights are those expected from the complete reciprocity that characterizes these sex-organ positions in *N. triandrus* (Fig. 1). These morphs are therefore unlikely to engage in significant assortative mating. The pollen-transfer patterns that result from the elevated position of upper-level stamens of the L-morph give rise to equilibrium morph ratios with a predominance of the L-morph and a relative deficiency of the M-morph, a pattern that occurs in populations of *N. triandrus* (Barrett *et al.*, 2004). Therefore in our study we consider only the impact of assortative mating in the L-morph, in conjunction with variation in female reproductive success caused by ovule discounting or pollen limitation, on equilibrium morph ratios.

With frequency-dependent selection, the morph frequencies in tristylous populations evolve until they reach a stable equilibrium at which $w_L = w_M = w_S$. Both models are analytically intractable, so that the equilibrium morph ratio must be identified by a numerical set of initial frequencies. Simulations of genetically explicit models lead to the same equilibria as the phenotypic models (K.A.H., unpublished) when there is a single unique equilibrium, so here we consider only the simpler phenotypic models.

Materials and Methods

Study organism

Narcissus triandrus L. (section Ganymedes) is a bee-pollinated geophyte, common in the central and northern parts of the Iberian Peninsula. Several intraspecific taxa have been described in *N. triandrus* (Blanchard, 1990); here we focus only on the widespread *N. triandrus* var. *triandrus* (hereafter *N. triandrus*) as this taxon displays the greatest morph-frequency variation, including all dimorphic populations reported for the species (Barrett *et al.*, 2004). Flowering commences in early March and populations at higher elevations flower until late April and early May. Flowering plants produce a single stem with a mean of 1.6 pale yellow to white flowers (range 1–9) that last up to 14 d. Flowers are pendulous with reflexed tepals and have a narrow floral tube with a prominent corona. Long-tongued solitary bees are the primary pollinators of *N. triandrus*. *Anthophora* spp. are the main visitors in the southern portion of the range in central Portugal, but are largely replaced by *Bombus* spp. in the cooler Atlantic zone of northern Spain and Portugal. Pollinator visitation rates are commonly very low in populations of *N. triandrus* (K.A.H., unpublished).

Morph-specific female reproductive success in natural populations

We estimated morph-specific seed set and morph ratios in *N. triandrus* based on random samples of plants in six dimorphic and nine trimorphic populations distributed throughout the

range, during the spring of either 2003 or 2004. Localities for all populations are available from the first author on request. As *N. triandrus* does not propagate clonally, the sampling of genets is unambiguous. We collected between 30 and 50 individuals of each morph in each population, except where the rarity of a morph prevented equivalent sample sizes. On average, we collected 33.7 (range = 13–54) individuals per morph in each population. We preserved all capsules from each individual in 70% ethanol. Using a dissecting microscope, the contents of capsules were classified into three categories (seeds, unfertilized ovules and aborted seeds) and then counted. We considered brown, shrivelled seeds to be aborted, while mature seeds were plump and black. In addition, in eight of the nine trimorphic populations (except population 195) we measured with digital calipers the length of the distal-most flower on the inflorescence. An average of 105 randomly sampled individuals (range = 22–224 individuals) per population, representing all three morphs, were measured for flower size.

Pollen limitation

We investigated morph-specific differences in pollen limitation in five trimorphic and six dimorphic populations during spring 2004. We conducted supplementary hand pollinations using fine forceps on flowers 1–2 d after opening, using two pollen donors located 5–10 m from the recipient. We pollinated a single flower on 20 individuals per morph in each population. The rarity of the M- and S-morph in several of the populations prevented larger morph-specific sample sizes. In order to control for density effects, pollination treatments of open pollination or hand pollination were randomly assigned either to the nearest neighbours of the same morph for single-flowered individuals, or to the first or second flowers of multi-flowered inflorescences. When control flowers and flowers receiving supplemental pollen are on the same plant, the effect of hand pollination has been shown in other species to be inflated by reallocation of resources from control flowers to flowers that receive a surplus of pollen (Zimmerman & Pyke, 1988). However, a preliminary experiment in *N. triandrus* did not show an inflated effect of supplementation (K.A.H., unpublished). The morph of the pollen donor was not considered in the experiment, as in *N. triandrus* all crosses among morphs are fully compatible (Barrett *et al.*, 1997; Sage *et al.*, 1999). To facilitate pollination of the S-morph, the floral tube was slit with forceps and both hand- and open-pollinated flowers received this treatment. Because of herbivory, we collected capsules from an average of 16 (range = 9–20) open- and hand-pollinated pairs per morph in each population. Capsules were sampled at maturity and seeds were counted as described in the preceding section.

Data analysis

We conducted all statistical analyses on fertility variation using SAS ver. 9.1 (SAS Institute Inc., Cary, NC, USA) and analysed

differences in proportional seed set among the morphs using the generalized linear model for categorical data (PROC GENMOD SAS; McCullagh & Nelder, 1989). We define proportional seed set (sometimes referred to here as seed set) as the total number of seeds divided by the sum of the total number of ovules, aborted seeds and seeds for each individual. We analysed the proportional data for seed set using binomially distributed errors and corrected for overdispersion of variance using the PSCALE option. We analysed data on seed set in two ways. First, we compared dimorphic and trimorphic populations, analysing data for the L- and S-morphs only. We treated morph, sexual system (dimorphic vs trimorphic) and population nested within sexual system as fixed effects. We included in this model interactions between morph and sexual system, and morph and population nested within sexual system. Second, to compare differences among the three morphs within trimorphic populations, we removed dimorphic populations from the analysis. In this analysis we treated morph and population as fixed effects in the model, as well as the interaction between morph and population. We tested for significant interactions with independent contrasts, and used the false discovery rate procedure to correct the level of significance for multiple tests (Benjamini & Hochberg, 1995). Back-transformed least-squared means and standard errors are used for presentation in figures.

We calculated the relative seed set of morphs in each population by determining the average seed set of a morph and dividing it by the average seed set of the other two morphs weighted by their relative abundance in the population. Relative seed set was calculated as our model shows that the relative reduction in the fertility of one of the morphs will influence its frequency. We used the logit transformation of morph frequency and tested the association of morph frequency and its proportional seed set or relative seed set using generalized linear models with binomially distributed errors (PROC GENMOD, SAS). Similarly, we investigated the correlation between average flower length for each population and the proportional seed set, as well as the relative seed set of each morph. Spearman's rank correlations are presented as the data were not normally distributed (PROC CORR, SAS).

For the pollen limitation experiment, we analysed proportional seed set using generalized linear models with binomially distributed errors (PROC GENMOD, SAS). As above, we analysed our data in two ways because of the absence of the M-morph in dimorphic populations. We considered pollination treatment, morph, sexual system and population nested within sexual system as fixed effects, and included all interactions in the model. We investigated pollen limitation in trimorphic populations by comparing proportional seed set of the L-, M- and S-morphs. We treated pollination treatment, morph and population as fixed effects in the model, along with all interactions. As we designed the experiment by pairing supplementary and open-pollinated flowers based on location, in both analyses we included paired flowers nested within

population and morph as a repeated measure (PROC GENMOD, REPEATED option, SAS).

Results

Influence of female fertility and assortative mating on morph ratios

The results of our pollen-transfer model, incorporating ovule discounting, demonstrate that differences in female fertility among morphs can influence morph frequencies in populations with disassortative mating (Fig. 2). Lower fertility of a given morph relative to the other morphs in a population leads to a lower equilibrium frequency of that morph. In our model, with complete disassortative mating, a reduction in fertility of 100% of one of the morphs results in the evolution of dimorphic populations through morph loss in populations (Fig. 2a–c). In this situation, each morph is mathematically and functionally interchangeable. However, when we introduce assortative mating (Q) in the L-morph, striking mating asymmetries, not normally found in tristylous species, are created. At the equilibrium, the intensity of competition should be balanced by the availability of mating opportunities. When assortative mating in the L-morph (Q) increases, the intensity of competition for mating with the L-morph increases while that of the M-morph declines. Consequently, as a result of these changes in competition for mating, the L-morph increases in frequency while the frequency of the M-morph is reduced.

These mating asymmetries also have the effect of magnifying the influence of maternal fitness differences on morph ratios.

Important differences are evident in the responses of the morphs to the interaction between fertility and assortative mating. The reduction in equilibrium frequency of the L- or M-morph with respect to either δ_L or δ_M occurs more rapidly with increased Q (Fig. 2d,e,g,h). In addition, the loss of the L- or M-morph from populations occurs with equal levels of ovule discounting. For example, the L- and M-morphs can be lost from populations when they have maternal fitness disadvantage of $\delta_L \approx \delta_M \approx 0.40$ when $Q = 0.30$ (Fig. 2g,h). Finally, loss of the L-morph results in equal equilibrium frequencies of the S- and M-morphs (Fig. 2d,g), whereas the loss of the M-morph results in L-biased morph ratios (Fig. 2e,h). However, any reduction in frequency of the S-morph caused by ovule discounting is independent of the level of assortative mating in the L-morph (Q) (cf. Fig. 2c with f,i).

The pollen-limitation model provides results similar to the ovule-discounting model. In both models, the levels of assortative mating in the L-morph are important for determining the influence of fertility on equilibrium morph ratios. When α is large (e.g. $\alpha = 100$), there is no pollen limitation as $F(x_i) = 1$ for each morph (Fig. 3a). In this situation, the results are identical to the resource-limited model (Barrett *et al.*, 2004). As α decreases, the fertility differences caused by pollen limitation begin to influence morph ratios. In all cases the equilibrium frequency of the L-morph increases and that of the M- and S-morphs declines when the level of assortative mating in the

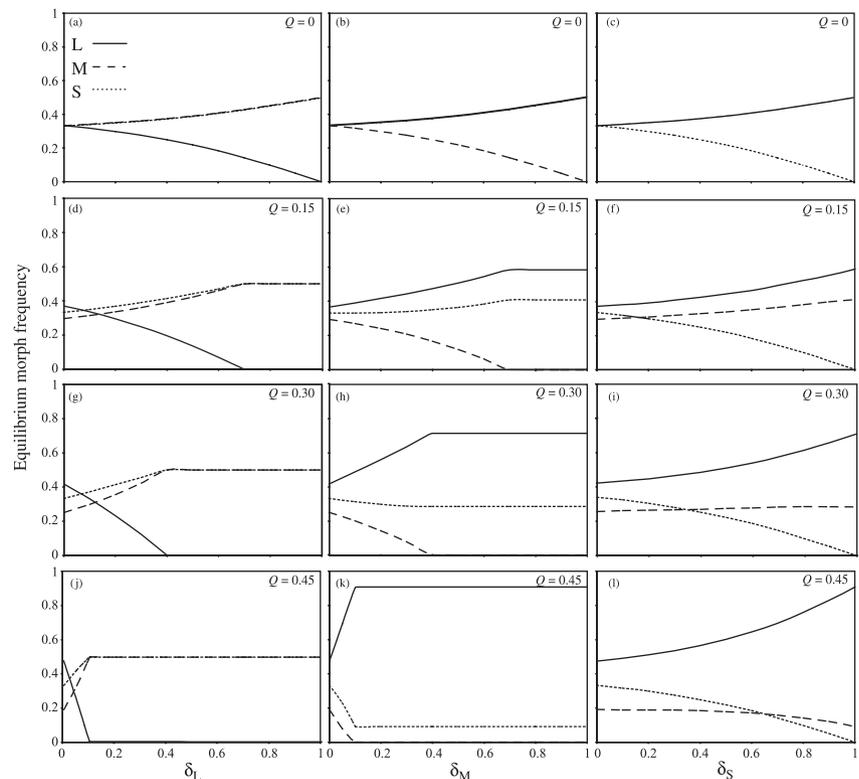


Fig. 2 Effects of interaction between variation in female fertility in the L-, M-, and S-morphs and of levels of assortative mating in the L-morph on equilibrium morph ratios in tristylous populations. The proportion of ovules in the L-, M- and S-morphs that do not reach reproductive maturity is represented by δ_L , δ_M and δ_S , respectively. (a–c) Complete disassortative mating among the morphs. (d–i) Consequence of elevation of upper-level stamens of the L-morph, where Q represents assortative mating in the L-morph at the expense of pollen transfer to the M-morph from the L-morph. Solid line, L-morph; dashed line, M-morph; dotted line, S-morph.

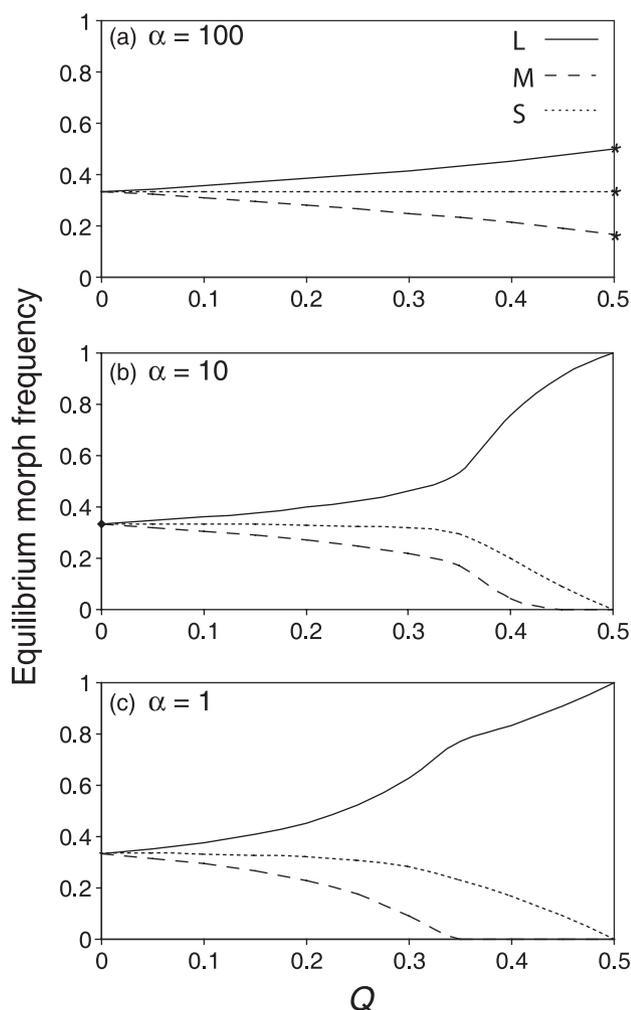


Fig. 3 Effects of pollen limitation and levels of assortative mating in the L-morph on equilibrium morph ratios in tristylous populations. Increasing levels of assortative mating in the L-morph (Q) occur at the expense of pollen transfer to the M-morph from the L-morph. Increased pollen limitation is represented by lower values of α , where α is a constant in the function $F(x_i) = 1 - e^{-\alpha x_i}$. Solid line, L-morph; dashed line, M-morph; dotted line, S-morph. Asterisks indicate that the equilibrium illustrated is one of many possibilities (Barrett *et al.*, 2004).

L-morph increases. This is because of differences in the levels of pollen receipt among morphs as a result of increased assortative mating in the L-morph (Q). However, with decreased values of α , lower levels of assortative mating in the L-morph (Q) are required for the M-morph to be lost (Fig. 3b,c). Once the M-morph is lost from populations, the L-morph will reach fixation when the level of assortative mating in the L-morph reaches 1/2.

Morph-specific differences in female reproductive success

Dimorphic populations of *N. triandrus* had higher proportional seed set than trimorphic populations (dimorphic = 0.652,

Table 1 Comparison of proportional seed set in the L- and S-morphs from dimorphic and trimorphic populations of *Narcissus triandrus* var. *triandrus*

Source	df	χ^2	P
Morph	1	1.3	0.2535
Population (sexual system)	13	120.4	< 0.0001
Population (sexual system) \times morph	13	51.7	< 0.0001
Sexual system	1	45.97	< 0.0001
Sexual system \times morph	1	0.7	0.4026

A generalized linear model was used where morph, sexual system, population (sexual system) and all interactions are fixed effects. Proportional seed set has binomially distributed errors.

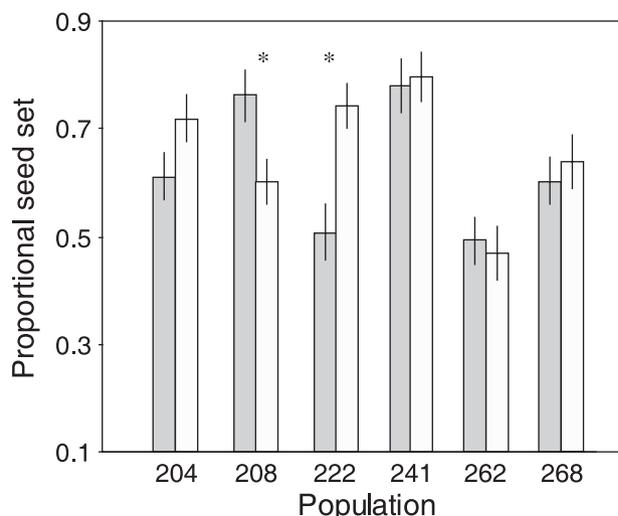


Fig. 4 Mean proportional seed set of L- and S-morphs in six dimorphic populations of *Narcissus triandrus* var. *triandrus*. Closed bars, L-morph; open bars, S-morph. Means \pm 1 SE. *, Significant differences between morphs in each population.

$SE \pm 0.014$; trimorphic = 0.528, $SE \pm 0.011$; $P < 0.0001$; Table 1). There was no significant morph effect ($P = 0.254$; Table 1), although there was a significant population (sexual system) effect ($P < 0.0001$; Table 1). However, there was also a significant interaction between morph and population (sexual system) ($P < 0.0001$; Table 1). Independent contrasts of morphs within dimorphic populations identified two out of six populations with significant differences in seed set between the L- and S-morphs. In populations 208 and 222, the L- and S-morphs had 10.8 and 28.0% higher proportional seed set than the S- and L-morphs, respectively (Fig. 4). Among trimorphic populations, both morph and population were significant ($P < 0.0001$ for both effects; Table 2). However, there was also a significant population \times morph interaction ($P < 0.0001$; Table 2). Independent contrasts revealed that seven of the nine populations had significant differences in female fertility among morphs (Fig. 5). In the three populations with the lowest M-morph frequency, the seed set of the M-morph

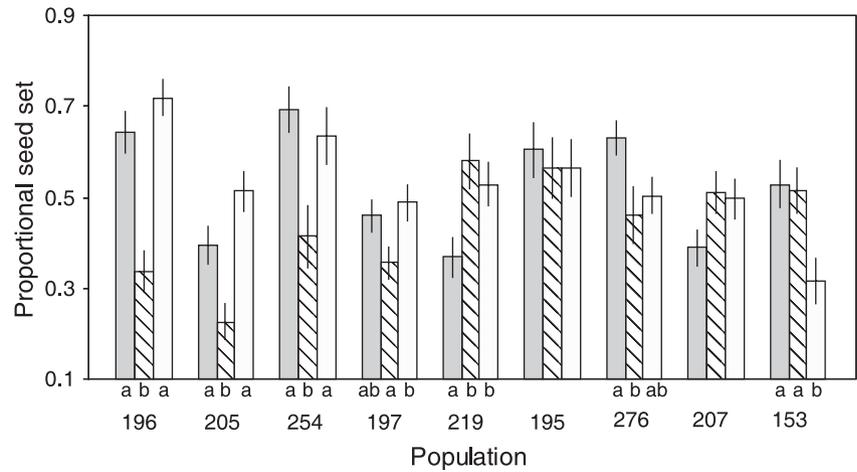


Fig. 5 Mean (± 1 SE) proportional seed set of the L-, M- and S-morphs in nine populations of *Narcissus triandrus* var. *triandrus*. Closed bars, L-morph; hatched bars, M-morph; open bars, S-morph. Different letters represent significant differences among morphs within each population. Populations are ordered from lowest to highest M-morph frequency.

Table 2 Comparison of proportional seed set in the L-, M- and S-morphs of trimorphic populations of *Narcissus triandrus* var. *triandrus*

Source	df	χ^2	P
Morph	2	24.64	< 0.0001
Population	8	49.57	< 0.0001
Population \times morph	16	89.79	< 0.0001

A generalized linear model was used where morph, population and their interaction are fixed effects. Proportional seed set has binomially distributed errors.

was significantly lower than the L- and S-morphs (populations 196, 205 and 254, respectively). In one population (197), seed set of the M-morph was significantly lower than the S-morph, but only marginally lower than the L-morph. In these four populations, M-morph frequency ranged from 0.16 to 0.23; in the remaining populations, M-morph frequency was higher, ranging from 0.28 to 0.68. In population 219, the L-morph had significantly lower seed set compared with the M- and S-morphs, while in population 276 the L-morph set more seed than the M-morph, with the S-morph intermediate. In population 153 the L- and M-morphs set significantly more seed than the S-morph.

There was a marginally positive association between the proportional seed set of the M-morph and its frequency in trimorphic populations ($\chi^2_1 = 3.96$, $P = 0.054$; Fig. 6a). However, there was no association between seed set of the L- and S-morphs and their respective frequencies within trimorphic populations (L-morph, $\chi^2_1 = 0.39$, $P = 0.532$; S-morph, $\chi^2_1 = 1.59$, $P = 0.207$). The same pattern was true for relative seed set. There was a positive association between the relative seed set of the M-morph and its frequency in trimorphic populations of *N. triandrus* ($\chi^2_1 = 19.25$, $P < 0.0001$). In contrast, there was no association between the relative seed set of the L- and S-morphs and their respective frequencies within trimorphic

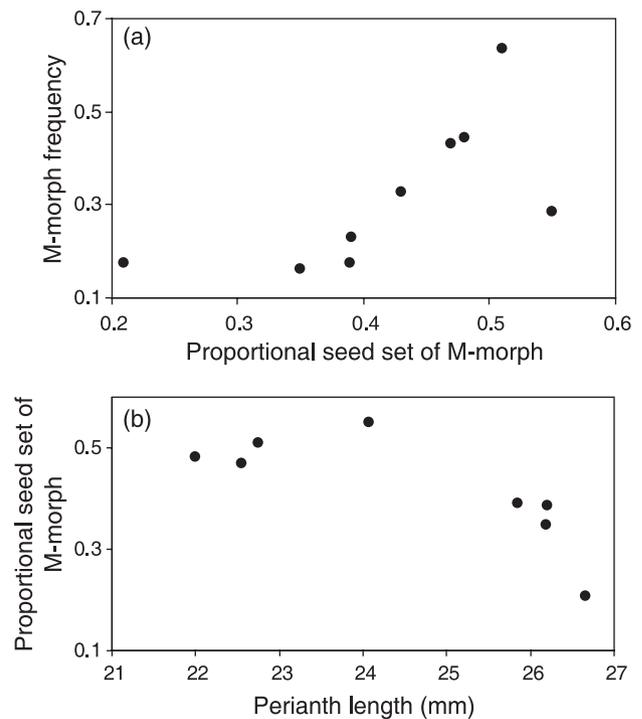


Fig. 6 Correlates of proportional seed set of the M-morph in tristylous populations of *Narcissus triandrus* var. *triandrus*. (a) Relationship between M-morph frequency and proportional seed set; (b) relationship between proportional seed set in the M-morph and flower length.

populations (L-morph, $\chi^2_1 = 2.78$, $P = 0.096$; S-morph, $\chi^2_1 = 0.22$, $P = 0.637$).

Flower length was negatively correlated with proportional seed set of the M-morph in trimorphic populations ($n = 8$, Spearman's $\rho = -0.761$, $P = 0.028$; Fig. 6b), but there was no relationship between these variables for the L- and S-morphs in trimorphic populations (L-morph, $n = 8$, $\rho = -0.0714$, $P = 0.867$; S-morph, $n = 8$, $\rho = 0.548$, $P = 0.160$). Similarly, flower length was negatively correlated with the relative seed set of

Source	df	χ^2	<i>P</i>
Morph	1	2.12	0.1451
Sexual system	1	20.38	< 0.0001
Treatment	1	6.58	0.0103
Population (sexual system)	9	20.61	0.0145
Morph \times sexual system	1	0.00	0.9766
Morph \times treatment	1	0.05	0.8169
Morph \times population (sexual system)	9	31.35	0.0003
Sexual system \times treatment	1	0.19	0.6670
Treatment \times population (sexual system)	9	28.85	0.0219
Morph \times sexual system \times treatment	1	0.77	0.3787
Morph \times treatment \times population (sexual system)	9	7.96	0.5383

A generalized linear model was used where treatment, morph, sexual system and population (sexual system), as well as all interactions, are fixed effects. Proportional seed set has binomially distributed errors.

the M-morph in trimorphic populations ($n = 8$, $\rho = -0.738$, $P = 0.037$) but there was no relation between flower length and relative seed set of the L- or S-morphs in trimorphic populations (L-morph, $n = 8$, $\rho = 0.336$, $P = 0.385$; S-morph, $n = 8$, $\rho = 0.619$, $P = 0.102$).

Pollen limitation

Despite infrequent pollinator service, pollen limitation was not a general feature of *N. triandrus* populations. In only two of the 11 populations that we investigated were there differences in the proportional seed set of open- vs hand-pollinated flowers. There was no significant interaction between sexual system and pollination treatment ($P = 0.667$), indicating that the treatment effects on proportional seed set did not depend on sexual system (Table 3). Similarly, there was no significant morph \times treatment interaction ($P = 0.817$), or morph \times population (sexual system) \times treatment interaction ($P = 0.538$). This reveals that the treatment effects on proportional seed set did not depend on morph across all populations or within each population. There was a significant treatment effect ($P < 0.010$; Table 3); however, there was also a significant interaction between treatment and population (sexual system) ($P = 0.022$). Similarly, the analysis of trimorphic populations also identified a significant treatment effect ($P = 0.005$; Table 4) as well as a population \times treatment interaction ($P = 0.005$). There was no significant morph \times treatment interaction ($P = 0.823$) or morph \times population \times treatment interaction ($P = 0.303$). This also indicates that the treatment effects on proportional seed set did not depend on morph. Independent contrasts revealed no significant differences between the open- and hand-pollinated treatments in dimorphic populations. In two of the five trimorphic populations, open-pollinated flowers set significantly less seed than hand-pollinated flowers (populations 205 and 254) (Fig. 7). These two populations exhibited the lowest M-morph frequencies among the populations sampled (0.17 in both populations).

Table 3 Comparison of the proportional seed set of open-pollinated and hand-pollinated flowers of the L- and S-morphs in six dimorphic and five trimorphic populations of *Narcissus triandrus* var. *triandrus*

Table 4 Comparison of proportional seed set of open-pollinated and hand-pollinated flowers of the L-, M- and S-morphs in five trimorphic populations of *Narcissus triandrus* var. *triandrus*

Source	df	χ^2	<i>P</i>
Morph	2	4.90	0.2982
Treatment	1	7.93	0.0049
Population	4	4.90	0.2982
Population \times treatment	4	11.79	0.0049
Morph \times treatment	2	0.39	0.8232
Morph \times population	8	8.91	0.3502
Morph \times treatment \times population	8	9.48	0.3033

A generalized linear model was used where treatment, morph and population as well as all interactions are fixed effects. Proportional seed set has binomially distributed errors.

Discussion

Narcissus triandrus exhibits unusual floral morphology and style-morph frequencies for a tristylous species. Because of the absence of heteromorphic incompatibility, floral morphology plays an important role in governing pollen dispersal, female fertility and patterns of outcrossed mating. Our theoretical results indicate that variation in female fertility has the potential to play a significant role in morph-ratio evolution, particularly when the strength of frequency-dependent selection varies among the morphs because of asymmetrical mating patterns. Our empirical studies of natural populations of *N. triandrus* revealed that this component of female reproductive success was associated with sexual system, flower size and morph frequencies within populations. We discuss the potential mechanisms responsible for these associations and their consequence for the evolution of sexual systems.

The majority of studies that have investigated seed production in tristylous species have not detected morph-specific differences in female fertility (Dulberger, 1970; Barrett, 1977;

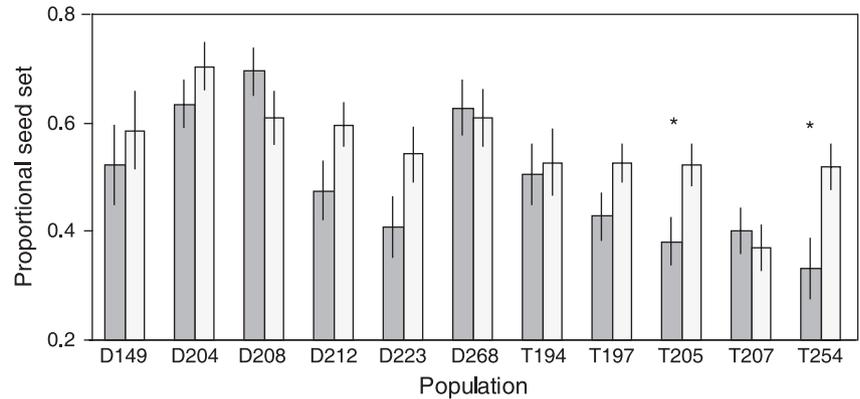


Fig. 7 Mean (± 1 SE) proportional seed set for open- and hand-pollinated flowers in six dimorphic and five trimorphic populations of *Narcissus triandrus* var. *triandrus*. Closed and open bars represent open- and hand-pollinated flowers in each population, respectively.

Price & Barrett, 1982; Barrett *et al.*, 1989). For example, *Oxalis alpina* resembles *N. triandrus* in several respects, with low frequencies of the M-morph characteristic of tristylous populations and dimorphic populations missing the M-morph. However, reduced female fertility of the M-morph was not evident based on extensive surveys of natural populations (Weller, 1981, 1986). A few cases are reported of morph-specific differences in female fertility in tristylous species. For example, in *Decodon verticillatus* (Eckert & Barrett, 1995) and *Lythrum salicaria* (Ågren, 1996; Ågren & Ericson, 1996), lower seed set of the L-morph is reported in some L-biased populations, perhaps reflecting frequency-dependent effects. In *N. triandrus* we found significant differences in proportional seed set in seven of the nine trimorphic populations (Fig. 5) and two of the six dimorphic populations (Fig. 4) that we surveyed. Hence, unlike most other tristylous species, female fertility differences among the morphs appear to be a common feature of *N. triandrus* populations.

Female fertility and variation in M-morph frequency

Narcissus triandrus populations display wide variation in frequency of the M-morph (0–0.63; Barrett *et al.*, 1997, 2004). A central goal of our study was to identify if differences in female fertility among morphs could contribute to this pattern. The results of our pollen-transfer models revealed that a reduction in female fertility of the M-morph via either ovule discounting or pollen limitation could result in a decline and loss of this morph from populations. When mating asymmetries occur in the manner predicted for *N. triandrus*, even slight reductions in M-morph fertility resulted in very low-equilibrium M-morph frequencies and the loss of this morph from populations (Fig. 2h). Thus a positive correlation between M-morph frequency and the fertility of the M-morph is predicted if population morph ratios are in equilibrium. Alternatively, a negative correlation is predicted if populations are not at equilibrium and morph ratios are evolving towards an isoplethic equilibrium by frequency-dependent selection (Ågren & Ericson, 1996). Negative correlations between fitness

components and morph ratios have been demonstrated in other plant species with polymorphic traits under frequency-dependent selection (McCauley & Brock, 1998; Gigord *et al.*, 2001; Thompson *et al.*, 2003; Olson *et al.*, 2005).

Our empirical results support the prediction that a female fertility disadvantage of the M-morph of *N. triandrus* may play a role in its loss from populations. In the three populations with the lowest M-morph frequency, the M-morph had significantly lower proportional seed set than the other two morphs (Fig. 5). Moreover, as predicted, M-morph frequency was positively correlated with both the proportional and relative seed set of the M-morph (Fig. 6a). However, as pollination is often a stochastic process and *N. triandrus* is a perennial plant, evaluation of this pattern across multiple years would be important in order to support this hypothesis. Differences among *N. triandrus* populations in local environmental conditions that influence the female fertility of the M-morph probably contribute to the variation in its frequency. Identifying the proximate ecological mechanisms represents a major challenge.

Female fertility in plant populations can be influenced by both pollen quality (e.g. self vs outcross pollen) and the amount of pollen transferred to stigmas (reviewed by Wilcock & Neiland, 2002). In *N. triandrus*, prior self-pollination reduces outcrossed seed set by up to 74%, indicating that there could be significant fertility costs associated with self-pollination and ovule discounting (Barrett *et al.*, 1997). Elsewhere, it has been demonstrated that the M-morph in tristylous species experiences significantly higher selfing rates than the L- and S-morphs (Kohn & Barrett, 1992), presumably because of its particular morphology. Mid-level stigmas are sandwiched between two stamen levels, and this arrangement is likely to promote higher levels of self-pollination (Charlesworth, 1979). Our theoretical work indicates that morph-specific differences in ovule discounting can influence morph ratios, and it is possible that such effects could play a role in explaining the positive association between M-morph frequency and fertility that we observed (Fig. 6a). In dimorphic species of *Narcissus*, ovule discounting has also been implicated as a

factor involved in morph-frequency variation (Barrett *et al.*, 1996; Cesaro *et al.*, 2004).

Modifications to sex-organ position in tristylous populations have the potential to influence patterns of outcrossed siring success and therefore morph ratios (Weller, 1986; Barrett *et al.*, 2004). Our pollen-transfer model also demonstrated that, in pollen-limited conditions, such morphological changes affect female fertility because of differences in pollen receipt among morphs. In these situations, morph frequencies will be determined by both negative frequency-dependent selection and selection via female fertility. We found that pollen receipt of the M-morph declined as the upper-level stamens of the L-morph corresponded more closely in position to stigmas of the L- rather than the M-morph. With pollen limitation (represented by decreasing α), this resulted in reduced fertility of the M-morph compared with the other morphs, and the frequency of the M-morph declined rapidly as Q increased (Fig. 3b,c). Once the M-morph is lost from populations, the L-morph will go to fixation when assortative mating in this morph reaches 1/2 (Baker *et al.*, 2000b). Therefore, under pollen-limited conditions, the anomalous positioning of the upper-level stamens of the L-morph of *N. triandrus* could cause reductions in the fertility of the M-morph. This effect, combined with ovule discounting resulting from self-pollination typical of pollen-limited situations, may be sufficient to cause repeated loss of the M-morph from tristylous populations.

Pollen limitation has been identified as an important ecological factor that can influence the evolution of polymorphic sexual systems (Charlesworth & Charlesworth, 1979b, 1979c; Washitani *et al.*, 1994; McCauley & Taylor, 1997). Our model demonstrates that, if pollen limitation occurs in tristylous populations, it could lead to the evolution of dimorphic populations. Dimorphic populations of *N. triandrus* are of lower density and smaller size than tristylous populations (Hodgins & Barrett, 2006). These demographic characteristics often make populations more prone to pollen limitation (Ågren, 1996; Groom, 1998; Ward & Johnson, 2005). However, levels of pollen limitation in *N. triandrus* were not significantly different between dimorphic and trimorphic populations, and no dimorphic populations exhibited pollen limitation. Proportional seed set was actually 13% higher in dimorphic compared with trimorphic populations. Evolutionary changes to floral morphology, such as an increase in flower size (Hodgins & Barrett, 2006), may have occurred in dimorphic populations, facilitating more proficient pollen transfer and mitigating pollen limitation.

There were no significant differences among morphs in pollen limitation, even in populations with significantly lower proportional seed set of the M-morph. This suggests that pollen quantity was not responsible for the fertility differences we observed. However, the two populations with the lowest frequency of M-morph were pollen-limited and exhibited low M-morph fertility. More extensive studies of morph-specific differences in pollen limitation in trimorphic populations

having low frequencies of the M-morph are necessary to determine whether morph-specific differences in pollen limitation cause the extensive variation in frequency of the M-morph.

The L-morph bias and stability of the S-morph

There was no evidence that differences in female reproductive success could account for the L-biased morph ratios that characterize populations of *N. triandrus*. Unlike the M-morph, the frequency of the L-morph among populations was not correlated with either its proportional or relative seed set. This strongly suggests that significant assortative mating in the L-morph, facilitated by the elevated position of its upper-level stamens, is largely responsible for its predominance in both trimorphic and dimorphic populations. High levels of assortative mating by the L-morph are also implicated as the major cause of L-biased morph ratios in *Narcissus* species with stigma-height dimorphism (Barrett *et al.*, 1996; Baker *et al.*, 2000a, 2000b; Arroyo *et al.*, 2002).

Our theoretical results indicate that, with assortative mating in the L-morph, both M- and L-morphs can easily be lost from populations when seed set is reduced in these morphs (Fig. 2g,h). The fact that no *N. triandrus* populations lack the L-morph or show a deficiency of this morph indicates that, unlike the M-morph, the L-morph does not commonly experience a female fertility disadvantage in populations. In *N. triandrus*, the pendulous flowers and long-level stigma located above the upper-level stamens probably restrict opportunities for self-pollination and ovule discounting. Moreover, as all three morphs possess long-level stamens targeting stigmas of the L-morph (Fig. 1), the relative fertility of this morph is unlikely to suffer in environments with reduced pollinator service.

In tristylous populations of *N. triandrus*, the S-morph often has the lowest representation (average frequency 0.22) and exhibits strikingly less variation in frequency among populations compared with the L- and M-morphs (Barrett *et al.*, 2004). Our models demonstrate that the frequency of the S-morph is not influenced by changes in pollen transfer between the L- and M-morphs (increased Q), but remains at a stable equilibrium frequency of 1/3 (Fig. 2f,i). Ovule discounting in the S-morph can contribute to a reduction in its frequency to below 1/3 (Fig. 2c,f,i). In contrast to the L- and M-morphs, mating asymmetries resulting from assortative mating in the L-morph do not hasten decline in frequency of the S-morph with reduced female fertility. This is because of the S-morph's equal interdependence on both L- and M-morphs for pollen import and export. Therefore the mating patterns of the L- and M-morphs do not influence the frequency of the S-morph, because these morphs are interchangeable as maternal parents of seeds sired by the S-morph. Because of the distinct combination of stamen levels in the S-morph, in comparison with the shared stamen positions of the L and M-morphs (Fig. 1),

the S-morph should experience more intense frequency-dependent selection. This probably explains the remarkable stability in frequency of the S-morph among populations of *N. triandrus*.

Floral morphology, female fertility and geographical variation in morph frequencies

Geographical variation in the floral morphology of *N. triandrus* is correlated with morph-frequency variation (Barrett *et al.*, 2004). In large-flowered populations in the north-western parts of the Iberian Peninsula, the M-morph is either at low frequency or absent. In contrast, in small-flowered populations further south, the M-morph occurs at higher frequencies. This flower-size variation is associated with contrasting allometric relations among sex organ positions, particularly mid-level organs, with consequences for pollen transfer. Our data revealed a negative correlation between flower size and proportional seed set of the M-morph (Fig. 6b). Significantly, this association was not evident in the other morphs. The mechanism(s) causing this reduction in fertility of the M-morph in *N. triandrus* are unclear. Geographical changes in the behaviour and size of the major flower visitors (*Anthophora* vs *Bombus*), and their effectiveness in promoting cross- vs self-pollination when interacting with the different floral morphologies of the style morphs, may be involved.

The ability to predict the frequency of the M-morph in *N. triandrus* populations based on the flower size and female fertility of this morph suggests that local ecological conditions play an important role in governing morph-ratio evolution. The association between heterostyly and a self-incompatibility system that permits assortative mating results in complex mating asymmetries not evident in heterostylous species with heteromorphic incompatibility. In *N. triandrus*, interactions between floral morphology and pollinators visiting flowers largely govern the character of frequency-dependent selection on morph ratios. These interactions are dependent on regional context, particularly climatic gradients, because *N. triandrus* is widely distributed in the Iberian Peninsula. Accordingly, morph-frequency variation exhibits predictable geographical patterns not often observed in polymorphic species. This variation provides a valuable spatial template for future studies of the causes and consequences of floral evolution for morph-ratio dynamics in heterostylous plants.

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