

Running header: Disassortative mating in *Narcissus papyraceus*

Disassortative mating prevails in style-dimorphic *Narcissus papyraceus* despite low reciprocity and compatibility of morphs

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Abstract

Evolution to reduce inbreeding can favor disassortative (inter-morph) over assortative (intra-morph) mating in hermaphroditic sexually polymorphic plant species. Heterostyly enhances disassortative pollination through reciprocal placement of stigmas and anthers of morphs and appropriate pollinators. Styler dimorphism in which there is not reciprocal anther placement may compromise disassortative mating, particularly when there is not intra-morph incompatibility. Variable rates of disassortative mating along with differential female fecundity or siring success among floral morphs could lead to variation in morph ratio. We investigated mating patterns, female fecundity and siring success of style-length morphs in *Narcissus papyraceus*, a self-incompatible but morph-compatible species with dimorphic (long- and short-styled) and monomorphic (long-styled) populations in central and north regions of its range respectively. We established experimental populations in both regions and exposed them to ambient pollinators. Using paternity analysis, we found similar siring success of morphs and high disassortative mating in most populations. Female fecundity of morphs was similar in all populations. Although these results could not completely explain the loss of dimorphism in the species' northern range, they provided evidence for the evolutionary stability of styler dimorphism in *N. papyraceus* in at least some populations. Our findings support the hypothesis that prevailing inter-morph mating is key for the maintenance of styler dimorphism.

Introduction

Sexual polymorphisms have arisen multiple times during the evolution of flowering plants in response to selection favoring outcrossing among other factors (Darwin 1877, Barrett 2002). Dioecy is an extreme sexual polymorphism that enforces outcrossing, but hermaphrodites can also manifest outcrossing through physiological self or cross incompatibilities (Castric and Vekemans 2004), contrasting phenology (Renner 2001) and variable positioning of sex organs in different floral morphs (Barrett *et al.* 2000). The outcome of such processes is the presence of mating morphs within populations, whose maintenance depends on the prevalence of mating between morphs, i.e. disassortative, over mating within morphs, i.e. assortative (Eckert *et al.* 1996, Pannell *et al.* 2005). Disassortative mating is a major mechanism of negative frequency-dependent selection that increases the mating opportunities of the less frequent morph in a population, leading to the equilibrium ratio of floral morphs (Fisher 1930, Heuch 1979, Barrett *et al.* 2004). On the other hand, many sexual polymorphisms in hermaphroditic plants do not avoid assortative mating completely and this may lead to variation in population morph ratio. It is expected that an increase in assortative mating in one morph would raise its frequency in a population, which in turn would eventually drive that population towards monomorphism (Baker *et al.* 2000a). It has also been suggested that transitory differences in female fecundity or siring success among floral morphs could be the factors contributing to variation in morph ratio (Eckert and Barrett 1995, Baker *et al.* 2000a, Hodgins and Barrett 2008, Pérez-Barrales and Arroyo 2010).

Heterostyly is a sexual polymorphism in which two or three morphs differ in the position of sexual organs, which are placed reciprocally in the different floral morphs. In this way, there is increased pollen transfer between morphs through precise pollen delivery and deposition by pollinators (Darwin 1877, Barrett and Shore 2008). Typically, this morphological polymorphism is accompanied by a physiological heteromorphic incompatibility system which impedes self- and within-morph mating success (Ganders 1979), although exceptions occur (Casper 1985, Eckert and Barrett 1994, Ferrero *et al.*

2012). Styler dimorphism, a condition similar to heterostyly, consists of the occurrence of two floral morphs that differ in the position of the stigma (above or below the anthers), while the anthers maintain a constant position across floral morphs. Thus, style-dimorphic plants lack the reciprocity between sex organs displayed by typical heterostylous species. Most style-dimorphic species also lack a heteromorphic incompatibility system (Barrett 1992). The evolutionary models of Charlesworth and Charlesworth (1979), and later Lloyd and Webb (1992) propose that styler dimorphism represents an intermediate step in the evolution from monomorphism to heterostyly. In particular, Lloyd and Webb (1992) suggested that floral morphs are maintained in populations if disassortative mating results in greater reproductive success than assortative mating, i.e.

$$\frac{1}{2} (q_{LS} + q_{SL}) > q_{LL} \quad \text{Eq. 1}$$

$$\frac{1}{2} (q_{LS} + q_{SL}) > q_{SS} \quad \text{Eq. 2}$$

under pollen-limited conditions, or

$$q_{LS} > q_{LL}$$

Eq. 3

$$q_{SL} > q_{SS}$$

Eq. 4

under non pollen-limited conditions. In these equations, q represents reproductive success, and the subscripts L and S represent long-styled and short-styled floral morphs respectively, the maternal morph being in first position. Hence, q 's with the same subscript represent assortative mating and different subscripts mean disassortative mating, so the four possible types of crosses are included in the equations. Note that estimating q values in a population entails to identify the morph of both parents for each successful reproductive event. Due to the lack of reciprocity in spatial placement of sexual organs and absence of heteromorphic incompatibility, stylar dimorphism might not meet the requirements above, and the polymorphism would be unstable. In fact, stylar dimorphism, which has been reported in six families of angiosperms, is much less common than heterostyly, which has been reported in 28 families (Barrett and Shore 2008).

In spite of its overall rarity, stylar dimorphism is widespread in the Mediterranean genus *Narcissus* (Barrett and Harder 2005). This observation seems to contradict the model proposed by Lloyd and Webb (1992) and might result from the effectiveness of stylar dimorphism in promoting disassortative mating in the genus in the absence of heteromorphic incompatibility (Cesaro and Thompson 2004). On the other hand, population morph ratios show considerable variation in style-dimorphic *Narcissus* species (Arroyo and Dafni 1995, Barrett *et al.* 1996, Baker *et al.* 2000b, Arroyo *et al.* 2002, Thompson *et al.* 2012, Santos-Gally *et al.* 2013). In order to explain morph ratio variation in *Narcissus*, some studies have

investigated the association between morph ratio and differential maternal fitness between floral morphs (Baker *et al.* 2000a, Pérez-Barrales and Arroyo 2010). However, total fitness also includes a paternal component and a full understanding of selection driving morph ratios requires that both maternal and paternal fitness are taken into account. Moreover, to date the assessment of the relationship between morph ratio and mating patterns in *Narcissus* is mostly based on indirect procedures (Thompson *et al.* 2003, Cesaro and Thompson 2004, Pérez-Barrales and Arroyo 2010). However, the increasing use of paternity analysis to investigate patterns of reproductive success in natural populations (e.g. Meagher 1986, Smouse *et al.*, 1999, Wright and Meagher 2004, Kitamoto *et al.* 2006, Hodgins and Barrett 2008, Rosas and Domínguez 2009, see also Kulbaba and Worley 2012) provides a means to obtain a more complete picture of the relationship between reproductive success and morph ratio variation, and it is indeed the only straightforward way to estimate q values in Lloyd and Webb equations.

Due to the widespread occurrence of stylar dimorphism in *Narcissus*, this genus represents a good system for investigating the relationship between stylar dimorphism and reproductive success. However, no study to date has dealt with all of the factors involved: female fitness, male fitness, mating patterns and their relationship with population morph ratio in a style-dimorphic plant. In order to develop a more comprehensive view on the evolution of stylar dimorphism, the present study focuses on *Narcissus papyraceus* Ker-Gawler, a winter-flowering, style-dimorphic geophyte whose floral morphs present very low reciprocity between stigma and anthers placement. The flowers present two stamen whorls (upper and lower) and stigmas in different morphs occur at different heights, above or below the lower stamen whorl. In long-styled (L-) flowers, the lower stamen whorls are slightly shorter than those of short-styled (S-) flowers (Fig. 1; Pérez-Barrales and Arroyo 2010). Both morphs co-occur in populations around the Strait of Gibraltar, but in the northern limit of the species range (Guadalquivir Basin, in SW Spain) populations are monomorphic for the L-morph (Arroyo *et al.* 2002). The species has a late acting self-incompatibility system of

imperfect action (Simón-Porcar *et al.* 2015a) similar to that reported in other species in the genus (Dulberger 1964, Sage *et al.* 1999) that permits either between- and within-morph fertilization (Arroyo *et al.* 2002). Flowers of this species have a long and narrow floral tube with nectaries at the bottom that suggests an adaptation to pollination by long-tongued insects (Pérez-Barrales *et al.* 2007). In spite of this apparent floral specialisation, a variety of pollinators visit flowers of *N. papyraceus*, including long-tongued butterflies and moths and short-tongued hoverflies and bees (Pérez-Barrales *et al.* 2007, Santos-Gally *et al.* 2013). There is some evidence that short-tongued pollinators are more common in the monomorphic region in southwest Spain (Santos-Gally *et al.* 2013), which might have led to the disappearance of the S-morph by reducing their female fecundity or increasing assortative mating in the L-morph. This happens because short-tongued pollinators cannot reach the stigma of the S-morph, placed inside the narrow floral tube, and so they only contact the stigma of the L-morph (Pérez-Barrales and Arroyo 2010, Simón-Porcar *et al.* 2014). The maintenance of the stylar dimorphism in populations around the Strait of Gibraltar could be due to higher rates of disassortative mating in that region owing to the prevalence of long-tongued pollinators (Santos-Gally *et al.* 2013, Simón-Porcar *et al.* 2014). The alternative hypothesis of genetic drift or founder events as a cause of this pattern has been recently discarded (Simón-Porcar *et al.* 2015b).

We report on an experiment to assess the maternal and paternal fitness of each floral morph and mating patterns in the dimorphic and monomorphic natural regions of *N. papyraceus*. We compared the maternal and paternal fitness of floral morphs in terms of female fecundity and siring success and estimated levels of assortative and disassortative mating to evaluate Lloyd and Webb's model for the maintenance of stylar dimorphism. We also compared progeny performance (survival and growth) at early stages of development as an additional component of fitness of floral morphs (i.e. post-dispersal fitness). Our approach included experimental populations with genotyped plants, which increased the probability of paternity assignment while allowing us to test the effect of different morph

ratios on mating patterns. Ultimately, we aimed to determine whether stylar dimorphism, despite limited reciprocity of sex organs, can lead to disassortative mating, and whether variation in the extent of disassortative mating accounts for the absence or presence of stylar dimorphism in the monomorphic and dimorphic regions of *N. papyraceus*.

Methods

Source material and parental genotyping

In December 2008, we collected 150 *Narcissus papyraceus* individuals from a single population close to Tarifa, South Spain (36.1° N, 5.73° W). This population is located in the center of the distribution range of the species and is isoplethic (equal proportions of L- and S-morph), large (thousands of plants) and harbors a high genetic diversity ($H_s = 0.75$; Simón-Porcar *et al.* 2015b). We collected individuals separated by at least two meters from each other to ensure collecting different genets, given the vegetative reproduction but low bulb dispersal of the species. Individuals were collected after the end of the flowering period to diminish plant stress for subsequent flowering. Thus, we determined the morph from the wilted flowers to collect equal numbers of the two style morphs. Plants were labeled, potted and moved to a glasshouse at the University of Seville. Plants were watered at levels consistent with natural conditions until the end of winter and kept in darkness during summer. This procedure was repeated for two years until massive flowering occurred in 2010–2011. In November 2010, we collected leaf samples from each individual, which were immediately frozen at -80°C. DNA was isolated following Bernartzky and Tanksley's (1986) protocol without mercaptoethanol, and each individual was genotyped for four specific microsatellite markers which had high genetic variability and good amplification rates (A116, A121, B104 and B112; Simón *et al.* 2010). Polymerase Chain Reactions (PCR) were performed in 25 μ L of reaction mixture containing 50 ng of template DNA, 1 \times PCR buffer, 1.5 mM MgCl₂, 0.1 μ M fluorescently labeled (6-FAM™, VIC®, NED™ and PET® dyes) forward primer, 0.1 μ M reverse primer, 0.05 mM each dNTP and 1.25 U Taq polymerase.

PCRs were performed in a Biometra Gradient Thermal Cycler (Biometra, Göttingen, Germany), with an initial 5 min of denaturation at 94°C, 45 cycles at 94°C for 30 s, annealing at 58°C (markers A116 and A121) or 59°C (markers B104 and B112) for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. Polymerase chain reaction products were analyzed on an ABI 3130 × 1 Genetic Analyzer and sized using GeneMapper v.4.0 (Applied Biosystems, Foster City, USA) and GeneScan™ 500 LIZ size standard (data available in the Dryad depository doi: xx.xxxx/dryad.xxxxx).

Field experiment

The field experiment was performed during the flowering season 2010-2011. We set up a total of 15 artificial populations in two locations within the dimorphic and the monomorphic natural distribution regions of the species but without nearby natural populations (Fig. 2a). In the dimorphic region, experimental plots were located at Finca de la Alcaidesa (36.3° N, 5.4° W; Alcaidesa hereafter), 16 km from the closest known naturally occurring population. In the monomorphic region, experimental plots were located at Pinares de Hinojos (37.3° N, 6.4° W; Hinojos hereafter), 8 km from the closest known naturally occurring population.

Three types of populations were established at each site: Isoplethic, L-biased and S-biased populations, with proportions of 1:1, 3:1 and 1:3 of L- to S-styled plants, respectively (Fig. 2b). Each population was composed of 8–12 individuals, with morphs alternating in a circular pattern in order to ensure regular distances between individuals (Fig. 2b). Plants were selected to ensure synchronic flowering and high multilocus microsatellite diversity for marker-based paternity assignment within populations. For each experimental population, the number of alleles with the four markers ranged from 24 (Population 5) to 47 (Population 11). The experimental populations were set up to be synchronous with the natural flowering period of natural populations at each region to ensure similar pollinators to them (but see Discussion). At Alcaidesa, three replicates of each experimental population type were

maintained from 23 December 2010 until 21 January 2011. The means of maximum and minimum daily temperatures during this period were 16.9°C and 9.7°C, respectively, and the mean daily rainfall was 35.8 mm (Spanish National Meteorological Agency 2012). At Hinojos, two replicates of each experimental population type were maintained from 11 until 31 January 2011 with mean maximum and minimum temperatures of 14.9°C and 6.3°C, respectively, and mean daily rainfall of 2.7 mm (Spanish National Meteorological Agency 2012). These values were within the normal ranges of climatic conditions in the flowering period of each region in a period of 50 years excepting significantly higher rain rates at Alcaidesa during the experiment (mean daily max.temp/min.temp/rainfall: Alcaidesa: 15.9°C / 8.2°C / 4.2 mm; Hinojos 14.3°C / 6.4°C / 2.2 mm; Climatic Data from WorldClim database, Hijmans *et al.* 2005). Populations within each site were located at least 300 meters apart from each other to prevent pollen flow between them (Barthelmess *et al.* 2006). We counted the number of flowers per individual 2–4 times in each population to estimate the total number of flowers of each morph during the experiment. Populations were exposed to natural pollinators at each site. Plants were removed from the field after flower withering and kept in the greenhouse until fruit maturation.

Three populations were damaged by wild boars: one isoplethic and one L-dominant replicate at Alcaidesa, and one L-dominant replicate at Hinojos. These populations were removed from the study leaving 12 populations in our final analysis. Some flowers and fruits were also grazed by insects or snails (0.04% of total number of flowers) and were discarded for the female fecundity analysis. Individuals affected by herbivory in half or more of the flowers (0.05% of total number of individuals) were not included in the female fecundity analysis, but all individuals were included in the paternity analysis, as they could have sired seeds prior to be grazed.

Female fecundity

In March 2011, we collected all matured fruits. For each maternal individual we counted fruits and seeds per fruit and estimated fruit set, the average number of seeds per fruit and total seed production per plant as female fecundity measures (data available in the Dryad depository doi: xx.xxxx/dryad.xxxxx). Fruit and seed production were strongly dependent on flower position. Seed production of the first flower was statistically indistinguishable from that of the second, third and fourth flowers ($F_{7, 948} = 8.01$; $P > 0.27$; ANOVA and Tukey HSD), but was significantly different from that of the fifth to eighth flowers (average number of seeds set per flower \pm s.d.: first-fourth flower, $N=714$, 3.4 ± 5.9 ; fifth-eighth flower, $N=242$, 0.9 ± 2.2 ; $P < 0.04$). Overall, 92% of seeds were produced in the first four flowers of each plant. Because plants varied in the number of flowers and this variation was originated in the greenhouse, irrespective of morphs, we used the four first flowers per inflorescence to calculate female fecundity so that values were comparable between morphs and regions. We performed Generalized Linear Models (GLM) for the fixed effects of floral morph (L-morph and S-morph), morph ratio (isoplethic, L-biased and S-biased) and region (Alcaidesa and Hinojos) on fruit set, the average number of seeds per fruit and total seed production. We employed a binomial distribution of errors to analyze fruit set and Gaussian distribution of errors to analyze the average number of seeds per fruit and total seed production. Analyses were conducted with the package *stats* of the software R 2.13.1 (R Core Team 2011).

Progeny germination, growth and genotyping

Seeds were stored at 4°C in darkness until the next season. In November 2011, we sowed a total of 2,566 seeds from 315 fruits in trays (30 × 50 cm) with a mixture of peat and vermiculite grade 2 (3:1; PROJAR, Málaga, Spain), which were watered to field capacity twice a week for 18 weeks until germination and growing was deliberately stopped in March 2012, similarly as in field conditions. Fruits from each individual were distributed haphazardly

in trays, whose position was changed weekly to homogenize growing conditions. Seed germination, seedling height, anomalous growth (i.e. distorted or discolored leaves) and survival were monitored weekly during the entire experiment. Only successful seedlings (those that germinated and grew healthy) were considered in the rest of our study. This excludes mating events lost due to biparental inbreeding or other genetic incompatibilities and therefore, the term cross refers only to legitimate crosses hereafter. To ensure enough DNA material, we selected the three tallest seedlings of each fruit ($N = 748$ seedlings). Since germination percentage and maximum height did not differ between maternal style morphs or mating combinations (see Results), sampling bias was regarded as negligible. The aboveground fractions of seedlings were dried in silica gel, and DNA was extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Chatsworth, CA, USA). Seedling genotyping for the four markers (A116, A121, B104 and B112) was performed under the same conditions as for parents but with an annealing temperature of 50°C (data available in the Dryad depository doi: xx.xxxx/dryad.xxxxx). Over the course of our study we discovered that a much lower annealing temperature allowed higher amplification rates. Comparison of the same genotypes with the two annealing temperatures indicated that this change does not affect marker results. Including parental individuals, we genotyped 40 individuals twice to estimate genotyping error per locus, which was $1.4 \pm 0.2\%$.

Paternity analysis and deviations from random mating

We analyzed the deviation of the observed number of crosses of each type in each population from the expected number under the null model of random mating. The observed number of crosses of each mating combination in each population was determined with paternity exclusion analyses. In order to maximize precision and percentage of assignment, we combined direct and probabilistic assignments of seedlings. First, progeny and parental genotypes were compared to categorically assign a single paternal parent to all possible seedlings in each population. Autogamy was considered as some flowers set seeds by selfing (Arroyo *et al.* 2002). From the 748 genotyped seedlings, we could assign directly a

total of 545 seedlings (73%) to a single paternal parent. Seedlings that remained unassigned were subjected to exclusion analysis with the software CERVUS 3.0 (Marshall *et al.* 1998), which assigned categorically the most probable paternal parent to each seedling with 80 – 95% of confidence based on a log-likelihood ratio (Meagher 1986). We ran separate analyses for each population, simulating 10,000 offspring with 100% candidate paternal parents sampled and allowing for selfing. The mistyping error rate was set as the estimated genotyping error (0.014), and a minimum of two loci typed was required. CERVUS assigned 68 and 38 seedlings to a single father with the 80 and 95% of confidence, respectively (total seedlings assigned 106; 14%). Hence, in this study we assigned a total of 651 seedlings (87%) with an overall assignment probability of 97.6%. Correlated paternity, calculated as the proportion of seedlings sired by the same individual, was $37 \pm 23\%$ (average \pm SD) within maternal individuals, and $22 \pm 26\%$ within fruits, with no significant differences between maternal morphs or experimental sites (results not shown). Because each seedling has the same potential contribution to the morph ratio in the next generation of the population, we considered all the assigned seedlings as independent events in the following analyses. Some seedlings were self-fertilized; these seedlings were discarded for the analyses of mating patterns between floral morphs, which were performed on 18–91 assigned seedlings per population (N = 592 in total). Final contingency tables with the observed number of crosses of each mating combination in each population were obtained including the seedlings assigned directly and probabilistically to a single paternal parent.

The expected number of crosses between *i* and *j* morphs in the population *p* (E_{ijp}) under random mating was estimated as:

$$E_{ijp} = S_p \times \left(\frac{F_{iP} \times F_{jP}}{F_p} \right) \quad \text{Eq. 5}$$

were S is the number of seeds assigned to a paternal parent and F is the counted number of flowers, which in the case of random mating determines the frequency of crosses for each combination. In each population, we performed a X^2 test with 10,000 permutations to determine the goodness-of-fit between the observed and expected distributions and its significance. In addition, within each of four possible cross-types (regarding to each parental morph), we performed a sign test to determine significant directionality of the pattern of excess or deficiency of observed vs. expected crosses across the twelve populations (Sokal and Rohlf 1981). Analyses were performed with the package *stats* of the software R 2.13.1 (R Core Team 2011). Finally, to evaluate the model equations of Lloyd and Webb (1992) in each experimental population, we employed the observed number of seedlings from each cross combination as q values in equations 1, 2, 3 and 4. It is noteworthy that the occurrence of pollen limitation in *Narcissus papyraceus* is unknown, and our study cannot provide any insight about it. In other *Narcissus*, it was found that pollen limitation was very variable across pops and seasons (Baker *et al.* 2000c). Our aim was to explore trends in mating patterns, which are independent of the existence of pollen limitation. For this reason, we examined both scenarios using equations 1-4.

Siring success modelling

We used the software PatQuest 4.0 (Smouse *et al.* 1999, Meagher 2002) to directly estimate the effect of the parental morphs on mating success in each population. The software employs maximum likelihood to estimate the relative siring success of each individual male (λ_i , $i=1, \dots$, number of males), and the effect of phenotypic characters (β_k , $k=1, \dots$, number of phenotypic characters evaluated) and maternal-paternal interactions (γ) such as distance between mating pairs, using univariate or multivariate log-linear models (Smouse and Meagher 1994, Smouse *et al.* 1999, Wright and Meagher 2004). Univariate models were

employed to investigate (1) the variation in λ_i among individuals and (2) the effect of the spatial distance between mating pairs (γ_s). Multivariate models were applied to assess (3) the joint effect of the total number of flowers per individual (β) and the interaction between mating-pair morphs (γ_m), and (4) the joint effect of the λ distribution and the interaction between mating-pair morphs (γ_m). Model (3) allowed us to investigate the effect of the interaction between mating-pair morphs while taking into account the different number of flowers of each morph in each population, as in the former approach (see equation 1).

The interaction between mating-pair morphs (γ_m) was evaluated using a morph distance between individuals, with a distance of 0 for individuals of the same morph and a distance of 1 for individuals of different morph. In all analyses but (4), we assessed the significance of the estimated parameters by feature-based permutations (Smouse *et al.* 1999) and by pedigree-based bootstrap (Morgan and Conner 2001) with 1,000 iterations. As we did with female fecundity measures, we performed a GLM for the fixed effects of floral morph (L-morph and S-morph), morph ratio (isoplethic, L-biased and S-biased) and region (Alcaidesa and Hinojos) on siring success of individuals (λ), using Gaussian distribution of errors with the package *stats* of the software R 2.13.1 (R Core Team 2011).

Progeny performance

We analyzed the variation in the performance of seedlings from different maternal morphs and mating combinations. Some seedlings exhibited anomalous growth (2.4% overall) or died (1% overall) so these seedlings were excluded from the following analyses. From the remaining number of seeds sown ($N = 2,492$) we estimated percentage of germination and average germination time of seedlings for each of the 89 maternal sibships (range 1–104 seedlings per maternal sibship; data available in the Dryad depository doi: xx.xxxx/dryad.xxxxx). We analyzed the effect of the maternal morph (L-morph and S-morph) with GLMs with Gaussian-distributed errors for each germination variable. Next, we adjusted a logistic model with a spline to the growth curves of the seedlings as implemented in the R

package *Grofit* (Kahm *et al.* 2010). We extracted the four growth curve parameters Λ (length of lag phase), μ (maximum growth rate), A (maximum growth) and I (the integral) from the models for each seedling. We performed GLMs with Gaussian-distributed errors for the fixed effect of the maternal morph (L-morph and S-morph) on each of the four growth curve parameters, including maternal identity as random factor and germination week as covariate in the models. With the subset of the seedlings that were assigned to a paternal parent we also analyzed the effect of both maternal and paternal morphs and their interaction on the four growth curve parameters (Λ , μ , A , I), performing different GLMs with Gaussian-distributed errors as before.

Results

Female fecundity

Floral morph, morph ratio and region and their interactions did not have a significant effect on fruit set, average number of seeds per fruit and total seed production ($F_{1/2, 85/113} < 3.13$; $P > 0.077$). Only the interaction among floral morph, morph ratio and region had a significant effect on fruit production ($F_{2,102} = 3.87$; $P = 0.021$). The mean (\pm SD) number of fruits produced per individual was 1.6 ± 1.3 , the average number of seeds per fruit was 9.0 ± 6.2 and total seed production per plant was 14.5 ± 14.9 (Table S1).

Paternity analysis and deviations from random mating

Exclusion analyses revealed that a total of 59 seedlings (9%) from 29 maternal parents were self-fertilized, being 41 (69%) seedlings from S-morph mothers. The highest rates of self-fertilization occurred in populations 10 and 11 (Hinojos), which accounted for 39 self-fertilized seedlings. Overall, there were 34 and 13 selfed seedlings of the S- and the L-

morph in Hinojos, and 7 and 5 selfed seedlings of the S- and the L-morph in Alcaidesa, respectively.

Nine out of 12 populations showed significant departures from random mating (Table 1). In all these cases excepting one population the observed number of disassortative mates was higher than expected by chance for the S-morph. Two different patterns were repeated across experimental populations: populations 1, 5 and 8 showed more disassortative crosses and less assortative crosses for both morphs observed than expected by chance, and populations 2, 3, 9 and 11 showed more S×L crosses and less crosses of the other types than expected by chance. When comparing morphs, disassortative mating was higher than expected under random mating in seven populations for the L- morph, which was a statistically non significant trend ($P=0.774$; sign test), and in 10 populations for the S-morph, which was a statistically significant trend ($P=0.039$; sign test). Meanwhile, assortative mating was higher than expected under random mating only in two populations for the L-morph ($P=0.039$; sign test) and in three populations for the S-morph ($P=0.146$; sign test; Table 1). Based on the observed number of seedlings from each mating combination, four populations, all of them S-biased, satisfied the Lloyd and Webb conditions meaning prevalence of disassortative mating to the L-morph both in pollen-limited and non pollen-limited conditions (Equations 1 and 3; Table 1). Three populations in Alcaidesa, none of them S-biased, satisfied the Lloyd and Webb conditions representing prevalence of disassortative mating to the S-morph in pollen-limited and non pollen-limited conditions (Equations 2 and 4; Table 1). Two populations differing in site and morph ratio satisfied equations 1, 2 and 4. Finally, three populations in Hinojos and none in Alcaidesa met all of the Lloyd and Webb conditions (Equations 1–4) for maintenance of stylar dimorphism.

Siring success modelling

The univariate log-linear regression for the γ_s parameter showed that only four populations (Populations 5, 8, 10 and 11) had a significant negative effect of spatial distance between mates based on pedigree bootstrapping (Table 2), indicating that the distance between individuals within these experimental populations had an impact on mating, whereas remaining eight experimental populations were panmictic. The multivariate analyses of β and γ_m revealed a significant positive effect of the number of flowers per individual (β) on paternal success in populations 1 and 3 (Alcaidesa), and 9 and 10 (Hinojos), based on pedigree bootstrapping (Table 2). The interaction between parental morphs (γ_m) was positive and significant in populations 5 and 8 (one from each region, Alcaidesa and Hinojos), indicating increased mating success between individuals of different morph (Table 2). Populations 6 (Alcaidesa) and 9, 10 and 11 (Hinojos) showed a negative γ_m parameter (Table 2). For the joint estimate of γ_m and λ , every population except 9 and 10 (Hinojos) had a positive value for γ_m . The siring success was highly variable among individuals, with λ values ranging from 0 to 0.32 (Table S1). Floral morph, morph ratio and region did not have a significant effect on siring success ($F_{2,119} < 2.4$; $P > 0.1$).

Progeny performance

There were no significant differences between maternal morphs in the percentage of germination ($F_{1,88} = 0.293$, $P = 0.59$). The overall percentage of germination was 84.4%. The progeny from S-morph maternal parents germinated significantly earlier than those from L-morph maternal parents (mean \pm SD: S-morph progeny: 7.5 ± 1.6 weeks; L-morph progeny: 8.3 ± 1.5 weeks; $F_{1,86} = 6.422$, $P = 0.013$). The logistic models yielded average (\pm SD) parameters $\mu = 49.0 \pm 9.3$ (mm / week), $\Lambda = 7.3 \pm 1.4$ (week), $A = 189.1 \pm 40.6$ (mm), and $I = 1267.6 \pm 436.6$ (mm \times week). Germination week and maternal identity had a high effect over all the growth curve parameters ($F > 5.942$, $P < 0.016$). The growth curve of the progeny from S-morph maternal parents had significantly higher A and Integer and lower Λ

parameters than progeny from L-morph maternal parents (Table 3, Fig. 3). No parameter differed significantly among progeny from different paternal morphs or parental morph combinations (Table 3).

Discussion

In this study we found evidence of frequent disassortative mating in the style-dimorphic *Narcissus papyraceus*, thus one of the key conditions for the evolutionary stability of stylar dimorphism in this species was met. We validated results from previous studies in this and other species of *Narcissus*, which were based on indirect approaches to incorporating paternal success in style-dimorphic plants (Pérez-Barrales and Arroyo 2010, Thompson *et al.* 2003, Cesaro and Thompson 2004) as well as on direct paternity analysis of heterostylous *N. triandrus* (Hodgins and Barrett 2008). Specifically, our results showed increased rates of disassortative mating in the S-morph. This trend may well reflect the more accurate correspondence between the L-morph lower stamens and the S-morph stigma, which could lead the S-morph to receive a greater proportion of pollen from the opposite morph than the L-morph does (Cesaro and Thompson 2004).

Effects of morph ratio variation

Our different approaches concurred in indicating a prevalence of disassortative mating, but they showed different patterns in regard to the higher occurrence of disassortative mating in the dimorphic and the monomorphic regions. According to the Lloyd and Webb model disassortative mating prevailed in most populations in Hinojos, while estimates of γ_m showed a trend to increased disassortative mating in Alcaidesa. Overall these results indicate that, in contrast to our hypothesis, disassortative mating occurred independently of the site. Hence,

our results on mating patterns of *N. papyraceus* cannot explain the disappearance of the S-morph in the northern limit of the species range. In addition, we found similar female fecundity and siring success of both floral morphs in both regions, which can neither account for the disappearance of the S-morph in the northern range. According to the observed patterns of pollination efficiency in floral morphs of *N. papyraceus* (Simón-Porcar *et al.* 2014), seed production in the S-morph depends on the action of long-tongued pollinators that, in contrast to the observed trends in previous studies (Pérez-Barrales *et al.* 2007, Santos-Gally *et al.* 2013), were probably as frequent as short-tongued pollinators during our experiment in Hinojos. Inter-annual variability in pollinator activity might be determined by inconsistent weather conditions. However, during our experiment there were exceptional rainfalls in Alcaidesa but the weather in Hinojos matched the average conditions in the season. Hence, we cannot attribute our results contrary to our expectations in Hinojos to this reason, and other factors related to the oscillation of the insect populations should be involved. The only advantage reported for the S-morph in the dimorphic region was its higher assortative mating in one population at Alcaidesa, a result in concordance with female fecundity of experimental S-monomorphic populations in that region (Pérez-Barrales and Arroyo 2010). In contrast to our study, the previous work by Pérez-Barrales and Arroyo (2010) found a lower female fitness of the S-morph in the monomorphic region than in the dimorphic region. Their study included monomorphic populations of L- and S-morphs and isoplethic populations but not anisoplethic populations, which apart from weather may also explain the different results. Even though our experiment has not been able to detect differences between the monomorphic and dimorphic regions, the assessment of mating patterns among populations of different morph ratios sheds light into the maintenance of biased populations and the reversion to monomorphism.

According to the model of Lloyd and Webb (1992), populations should meet the conditions for increased disassortative mating in both morphs (i.e. satisfy equation 1 or 3 for the L-morph, and equations 2 or 4 from the S-morph, depending on the pollen limited conditions) to reach an equilibrium morph ratio. In our study, the four equations were satisfied in three populations in Hinojos, two isoplethic and one S-biased. Populations 2 (Alcaidesa, Isoplethic) and 10 (Hinojos, L-biased) satisfied equations 1, 2 and 4, while the rest of populations met two conditions (either equations 1 and 3, or equations 2 and 4). In such cases the model equations 1 and 3 for disassortative mating prevailing to the L-morph were never satisfied in L-biased populations, while equations 2 and 4 for disassortative mating prevailing to the S-morph were never satisfied in S-biased populations (Table 1). According to the theory on negative frequency-dependent selection, this result indicates that assortative mating prevailed in the dominant morph in each population. Given the lack of heteromorphic incompatibility in *N. papyraceus*, this should lead to maintenance of biased morph ratios. This could account for the stability of biased populations and the reversion to monomorphism in the northern range of *N. papyraceus*, though the consistent loss of the S-morph would require additional explanations. One such explanation comes from the putative inheritance system in *Narcissus* (Dulberger 1964), according to which assortative mating in the S-morph (heterozygote for the diallelic gene of stylar polymorphism) would lead to a percentage of L-morph individuals (homozygote recessive). This could account for the absence of S-biased populations in the wild, though the genetic base of stylar dimorphism needs to be explored. Second, mating disadvantages of the S-morph under the action of prevailing short-tongued pollinators could also account for the observed pattern (Santos-Gally *et al.* 2013, Simón-Porcar *et al.* 2014). Finally, in case of no differences in assortative mating but with disassortative mating consistently higher than assortative mating in the S-morph but not in the L-morph, like the trend shown, the L-morph would have greater siring success than the S-morph, and therefore a fitness advantage which could lead to its fixation in populations.

Effects of parental morphs on progeny performance

In our study, we found some distinct features in the progeny from different parental morphs that could potentially have implications for the occurrence of floral morphs in the wild, and in particular for the maintenance of the S-morph. First, we attributed a higher number of seedlings from S-maternal parents to self-fertilization, as shown from previous hand pollinations (Arroyo *et al.* 2002). Two factors acting in concert may account for the higher probability of selfing in the S-morph: the late-acting self-incompatibility system of imperfect action of *N. papyraceus*, which is particularly weak in the S-morph, and the reduced dichogamy of the S-morph (Simón-Porcar *et al.* 2015a), as previously reported for *N. assoanus* (Cesaro *et al.* 2004). It is generally agreed that increased rates of self-fertilization have been proved to attain the maintenance of floral morphs in other style-polymorphic species (Barrett *et al.* 1989). In the case of *N. papyraceus* this advantage seems to be insufficient, maybe due to the associated negative effects of ovule discounting in late-acting self-incompatibility (Dulberger 1964, Sage *et al.* 1999).

The second distinct feature of the S-morph was the better performance of the progeny of S-morph maternal parents, which germinated earlier than progeny of L-morph maternal parents. This result contradicts expected effects of increased pollen competition in long styles (Mulcahy and Mulcahy 1975, Mulcahy *et al.* 1983, McKenna 1986, Armbruster 1996), and may derive from genetic differences between floral morphs. Quantitative genetic approaches would be an effective means by which to explore the mechanisms underlying this difference, such as pleiotropic effects of the alleles codifying stylar morphs (Eckert and Barrett 1995). Alternatively, the concealing of the S-stigmas could permit their longer receptivity due to lesser rate of successful pollination events, which would allow to the S-morph to mate with a higher number of males increasing progeny fitness due to sexual

selection (Simmons 2005) or resource partitioning (Barton and Post 1986). Intra-fruit paternity analyses would be a useful way to test this hypothesis.

Disassortative mating in Narcissus

Theory on the evolution of sexual polymorphism emphasizes the role of disassortative mating in the evolutionary maintenance of different floral morphs in populations. In the evolution of heterostyly, enhanced inter-morph mating should be important for the establishment of intermediate stylar dimorphism (Lloyd and Webb 1992). In this study, we have directly estimated mating patterns for the first time in a stylar dimorphic plant. Our experimental design has also allowed us to obtain replicates for explicit testing of this question while increasing the percentage of paternal assignation up to 87%, something difficult to achieve in studies of natural populations. We have provided direct experimental evidence for the potential of stylar dimorphism in promoting disassortative mating in *N. papyraceus*, supported by similar results from goodness-of-fit tests, siring success modelling and the fulfillment of at least two Lloyd and Webb's (1992) model conditions in each experimental population. Taken together, our results help explain the commonness of stylar dimorphism in *Narcissus* and confirm previous studies on the genus using other methods (Pérez-Barrales and Arroyo 2010, Thompson *et al.* 2003, Cesaro and Thompson 2004, Hodgins and Barrett 2008).

Disassortative mating is obligate in most heterostylous species due to their heteromorphic incompatibility system. The finding of disassortative mating in *Narcissus* style-polymorphic species, where both inter- and intra-morph crosses can succeed, reveals the central role of morphological reciprocity of sexual organs between floral morphs in promoting disassortative pollination, even when such reciprocity is imperfect. Though no overall differences have been found in the rates of disassortative mating between regions with prevalent either long- and short-tongued pollinators, disassortative pollination is

probably conducted by long-tongued pollinators (Santos-Gally *et al.* 2013, Simón-Porcar *et al.* 2014). The incompatibility system in *Narcissus* is probably shared with ancestors of the genus and is unrelated with the promotion of disassortative mating. Hence, our results could provide the basis for the maintenance of stylar polymorphisms in other taxa which also lack a heteromorphic incompatibility system, such as some Boraginaceae (Dulberger 1970, Philipp and Schou 1981, Schou and Philipp 1983, Ferrero *et al.* 2012), and support the independence of sexual polymorphism and physiological heteromorphic incompatibility.

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Data Archiving

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Figure 1. Diagram of the long (L) and short (S) styled morphs of *Narcissus papyraceus* in longitudinal sections of flowers.

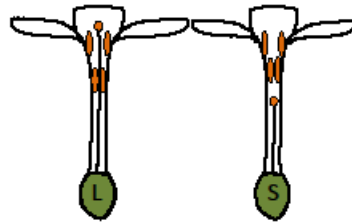


Figure 2. (a) Main distribution range of *Narcissus papyraceus* and experimental sites in the monomorphic and dimorphic regions of the species distribution range. (b) Arrangement of experimental population types with different morph ratios. L, L-morph; S, S-morph.

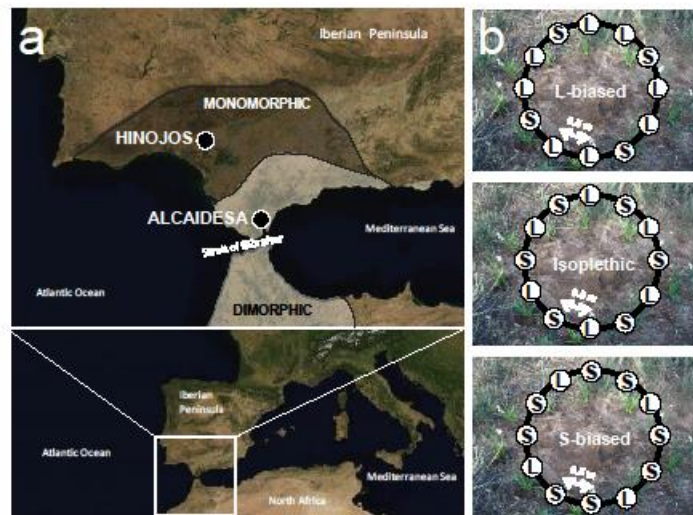


Figure 3. Growth of the progeny of each maternal morph throughout 16 weeks in a greenhouse experiment. Each data point is the average height of siblings with 95% confidence intervals.

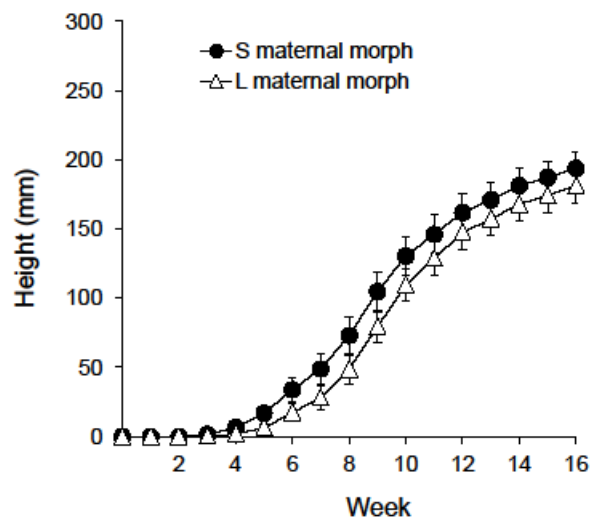


Table 1. Observed / expected number of seedlings from each possible mating combination in each experimental population (maternal morph in first position). Results of the goodness-of-fit test between both distributions are given. The last column indicates the equations of the Lloyd and Webb (1992) model that were fulfilled in each population (Eqns.1 and 3: sufficient disassortative mating to maintain L-morph in populations with pollen-limited and non pollen-limited conditions, respectively; Eqns. 2 and 4: sufficient disassortative mating to maintain S-morph in populations with pollen-limited and non pollen-limited conditions). Significance: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; *ns*, non significant.

Region	Morph Ratio	Pop	N	LxL	LxS	SxS	SxL	χ^2 -value	L-W Eqs.
Alcaidesa	Isoplethic	1	73	28 / 33.6	21 / 15.9	2 / 7.6	22 / 15.9	8.94 *	2,4
	Isoplethic	2	18	4 / 4.8	3 / 4.5	2 / 4.2	9 / 4.5	6.31 <i>ns</i>	1,2,4
	L-biased	3	44	19 / 20.7	8 / 9.5	0 / 4.4	17 / 9.5	10.67 *	2,4
	L-biased	4	38	21 / 15.6	11 / 8.7	0 / 4.9	6 / 8.7	8.18 *	2,4
	S-biased	5	91	0 / 1.8	20 / 11.1	38 / 67.1	33 / 11.1	65.16 ***	1,3
	S-biased	6	40	1 / 4.6	9 / 8.9	25 / 17.5	5 / 8.9	7.7 <i>ns</i>	1,3
	S-biased	7	49	0 / 2.8	10 / 8.9	30 / 28.3	9 / 8.9	3.05 <i>ns</i>	1,3
Hinojos	Isoplethic	8	72	7 / 17.3	28 / 18	8 / 18.7	29 / 18	24.56 ***	1,2,3,4
	Isoplethic	9	26	1 / 5.2	6 / 6.4	7 / 7.9	12 / 6.4	8.38 *	1,2,3,4
	L-biased	10	45	11 / 20	7 / 10	6 / 5	21 / 10	17.25 ***	1,2,4
	S-biased	11	65	0 / 0.6	5 / 5.8	39 / 52.8	21 / 5.8	44.13 ***	1,3
	S-biased	12	31	5 / 2.4	11 / 6.2	5 / 16.2	10 / 6.2	16.53 **	1,2,3,4

Table 2. Results of univariate and multivariate models performed with PatQuest for the factors affecting siring success of individuals in each experimental population. A LLR test of heterogeneity in λ_i is followed by presentation and statistical testing of: γ_s : effect of the spatial distance between mating pairs (univariate model); $\beta_{(\gamma_m)}$: effect of the number of flowers per individual estimated in a multivariate model jointly with the interaction between mating-pair morphs, $\gamma_m(\beta)$. $\gamma_m(\lambda)$: interaction between mating-pair morphs estimated jointly with the λ distribution. Log-likelihood (LLR) test statistics and significance based on feature-based permutations (Smouse *et al.* 1999; left) and based on pedigree-based bootstrap (Morgan and Conner 2001; right) are given. ***, $P < 0.005$; **, $P < 0.01$; *, $P < 0.05$; ns, non significant.

Site	Morph Ratio	Pop	N	LLR		γ_s	LLR		$\beta_{(\gamma_m)}$	LLR		$\gamma_m(\beta)$	LLR		$\gamma_m(\lambda)$
Alcaidesa	Isoplethic	1	85	34.87	-/***	0.050	0.08	ns/ns	0.022	5.22	ns/*	0.291	2.00	ns/ns	0.688
	Isoplethic	2	30	19.76	-/*	-0.738	3.15	ns/ns	0.076	1.03	ns/ns	0.250	0.33	ns/ns	0.244
	L-biased	3	47	31.21	-/***	0.031	0.01	ns/ns	0.077	14.09	ns/***	0.394	3.76	ns/ns	1.744
	L-biased	4	47	22.02	-/ns	-0.150	0.31	ns/ns	0.029	1.73	ns/ns	0.463	3.22	ns/ns	1.638
	S-biased	5	107	49.71	-/***	-0.295	3.80	ns/*	0.050	0.03	ns/ns	0.925	18.79	ns/***	0.909
	S-biased	6	54	24.11	-/***	-0.144	0.35	ns/ns	0.029	0.88	ns/ns	-0.306	1.02	ns/ns	0.044
	S-biased	7	56	27.73	-/**	0.188	0.50	ns/ns	-0.014	0.13	ns/ns	0.297	1.42	ns/ns	1.475
Hinojos	Isoplethic	8	83	11.76	-/ns	-0.584	9.65	ns/***	-0.008	0.03	ns/ns	1.075	25.96	***/**	1.144
	Isoplethic	9	30	23.43	-/***	-0.438	2.30	ns/ns	0.406	14.33	*/***	-0.431	3.68	ns/ns	-0.525
	L-biased	10	71	33.64	-/***	-1.081	24.26	***/**	0.172	15.45	*/***	-0.156	1.65	ns/ns	-0.041
	S-biased	11	108	16.23	-/ns	-1.363	49.59	***/**	0.026	2.30	ns/ns	-0.075	0.91	ns/ns	0.766
	S-biased	12	38	12.95	-/ns	0.463	2.30	ns/ns	-0.215	2.32	ns/ns	0.500	2.33	ns/ns	0.263

Table 3. Results of the progeny growth experiment. a) Results of the Generalized Linear Models testing the effect of maternal morph on four growth curve parameters measuring the performance of all germinated siblings (i), and the effect of both parental morphs and their interaction on the performance of siblings assigned to a paternal parent (ii). Degrees of freedom (d.f.) and *F*-values are given. Significance: *, $P < 0.05$; *ns*, non significant. b) Mean (\pm SD) of four parameters that define the growth curves of the progeny from different parental morphs. N, number of siblings; λ , length of lag phase (weeks); μ , maximum growth rate (mm/week); A, maximum growth (mm); I, integral (mm*week). L, L-morph; S, S-morph.

a	Fixed effect	d.f. / d.f. error	μ	λ	A	I
i	Maternal morph (M)	1 / 2014	0.12 <i>ns</i>	331.81 ***	45.33 ***	146.61 ***
ii	Maternal morph (M)	1 / 591	2.34 <i>ns</i>	46.77 ***	1.04 <i>ns</i>	19.43 ***
	Paternal morph (P)		0.22 <i>ns</i>	2.26 <i>ns</i>	3.59 <i>ns</i>	0.08 <i>ns</i>
	M \times P		0.11 <i>ns</i>	0.00 <i>ns</i>	0.25 <i>ns</i>	0.27 <i>ns</i>
b	Parental morphs	N				
i	L maternal parent	874	50.5 \pm 14.4	7.6 \pm 2.6	182.9 \pm 57.9	1195.2 \pm 636.5
	S maternal parent	1231	50.3 \pm 14.5	7.1 \pm 2.8	192.4 \pm 59.3	1335.7 \pm 719.7
ii	L \times L cross type	97	56.6 \pm 14.1	6.6 \pm 2.2	225.3 \pm 47.3	1578.8 \pm 573.9
	S \times S cross type	162	54.8 \pm 11.8	6 \pm 2.3	222.9 \pm 48.1	1692.9 \pm 670.4
	L \times S cross type	139	57 \pm 13.2	6 \pm 2	222.5 \pm 55	1706.0 \pm 640.3
	S \times L cross type	194	55.8 \pm 11.7	5.7 \pm 2	229.4 \pm 44.9	1784.7 \pm 610.5