Geographic variation in floral morphology and style-morph ratios in a sexually polymorphic daffodil[1]

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Narcissus triandrus is a tristylous daffodil from the Iberian Peninsula that has striking geographical variation in floral morphology and style-morph ratios. Here, we investigate the relation between this variation and ecological factors to understand mechanisms governing morph ratios. We estimated morph ratios in 124 populations throughout the range of *N. triandrus* and measured 13 morphological traits in 35–78 populations. Sampling of morph ratios conducted over 2–10-yr intervals demonstrated strong temporal stability. Variation in floral and vegetative traits enabled statistical prediction of morph-frequency variation among populations. Latitudinal gradients in precipitation and temperature were correlated with plant and flower size, with larger flowers in northern populations associated with bumblebee visitation and stylar dimorphism. Flowers of the L- and M-morphs differed significantly from the S-morph in several size-related characters, unlike other tristylous species. This pattern and the similarity in anther positions of the L- and M-morphs suggest that the M-morph of *N. triandrus* originated through genetic modifiers that shortened styles of the L-morph. Our findings support the hypothesis that geographical variation in style-morph ratios is largely governed by climatic gradients in the Iberian Peninsula, which influence the floral morphology and pollination biology of *N. triandrus* populations.

Key words: floral morphology; geographic variation; heterostyly; *Narcissus*; pollination; style-morph ratios.

Microevolutionary investigations of intraspecific variation in floral traits are important for identifying the role of adaptive evolution in floral diversification. Placing these studies in a spatial context can elucidate the genetic and ecological factors underlying evolutionary modifications to floral traits and mating strategies (reviewed in Barrett et al., 2001; Herrera et al., 2006). A geographical perspective is particularly important for species with broad distributions because they are likely to experience a wide variety of environmental conditions. Populations in portions of a species' range may undergo changes in life history, demography, and pollination environment with consequences for floral traits and sexual-system evolution (e.g., Costich, 1995; Dorken and Eckert, 2001; Case and Barrett, 2004; Weller et al., 2007). However, few investigations in plant reproductive biology have examined landscape-level patterns of floral diversification. Moreover, the role of geographical variation in environmental conditions on pollination and mating is generally poorly understood (Herrera et al., 2006).

Polymorphic sexual systems, such as dioecy and heterostyly, provide valuable opportunities for investigating ecological and evolutionary processes in plant populations (reviewed in Barrett, 1992; Geber et al., 1999). These polymorphisms are maintained in populations by negative frequency-dependent selection, where the fitness of a morph depends on its frequency relative to the frequency of partners with which it can mate. The ease with which sexual morphs can be identified and their simple pattern of inheritance permit the development of models concerning their evolution and maintenance (Lloyd, 1974; Charlesworth and

Charlesworth, 1979; Heuch, 1979; Lloyd and Webb, 1992a, b). Sampling of morph ratios in natural populations of heterostylous plants often reveals patterns of variation that deviate from theoretical expectations (reviewed in Weller, 1992; Barrett, 1993). Investigation of this variation in a geographic context can provide insights into how polymorphic sexual systems evolve in response to environmental conditions and the role of deterministic and stochastic forces in governing morph-ratio variation.

Heterostylous species have two (distyly) or three (tristyly) style morphs that differ reciprocally in the placement of their anthers and stigmas (Darwin, 1877; Barrett, 1992). The polymorphisms function primarily as mechanisms that promote cross-pollination resulting in disassortative mating among the morphs (Darwin, 1877; Lloyd and Webb, 1992a, b). Most heterostylous species possess heteromorphic incompatibility, in which intramorph mating is prevented physiologically. This type of incompatibility ensures symmetrical mating and typically results in equal morph ratios within populations (isoplethy—Fisher, 1941; Charlesworth and Charlesworth, 1979; Heuch, 1979; Barrett and Hodgins, 2006). However, some heterostylous species lack heteromorphic incompatibility (reviewed in Barrett and Cruzan, 1994), and this characteristic can provide opportunities to study the direct influence of floral morphology on mating patterns and morph ratios.

Narcissus triandrus is a wide-ranging, heterostylous wild daffodil from the Iberian Peninsula that possesses extensive geographical variation in style-morph ratios (Fernandes, 1965; Barrett et al., 1997, 2004). The species is tristylous with three floral morphs (long-, mid- and short-styled morphs, hereafter referred to as L-, M-, and S-morphs) and, in contrast to most heterostylous species, possesses a self-incompatibility system that permits both inter- and intramorph mating. High outcrossing rates are reported in populations of *N. triandrus* (Barrett et al., 1997; Hodgins and Barrett, 2006b). Surveys of morph ratios in *N. triandrus* have revealed three atypical features: (1) most populations are L-biased; (2) there is a negative relation between the frequencies of the L- and M-morphs among tristylous

populations; and (3) some populations are dimorphic and lack the M-morph. The frequency of the M-morph declines in the northwest portion of the range, with dimorphism in central Portugal and northwest Spain and trimorphism throughout the rest of the range (Barrett et al., 2004). This variation in morph ratios is correlated with an increase in flower size accompanied by changes in the positional relations of sex organs. The ecological factors that may contribute to this variation, including climatic gradients and differences in the types of pollinators visiting flowers, have not been investigated in detail. Preliminary observations suggested that plants in populations in the southern portion of the range, in drier Mediterranean climates, were smaller in size and were visited by different pollinators than those in northern populations, where plants and flowers were larger. A goal of this study was therefore to investigate in detail associations among environmental factors, geographical variation in floral morphology and morph ratios in *N. triandrus*.

Here, we use a broad geographic sampling of variation in floral and vegetative morphology and style-morph ratios in populations of *N. triandrus* to address the following specific questions. (1) What are the spatial and temporal patterns of style-morph frequencies throughout the geographical range? Temporal stability in morph ratios would support the role of deterministic processes, such as negative frequency-dependent selection, as the predominant evolutionary force shaping morph ratios. (2) Is there evidence for geographic differentiation in floral and vegetative morphology, and is this associated with variation in climate and variation in the composition of the pollinator fauna visiting populations? Here we wanted to confirm our preliminary observations through an extensive sampling of populations throughout the geographical range. (3) Can geographical variation in vegetative and floral traits be used to predict among population variation in style-morph frequencies? Correlations among these traits would support a functional association between morphology and morph ratios. (4) Do the style morphs differ in floral attributes that may be informative with respect to the evolution of tristyly in *N. triandrus*? We predicted that, because of the similarity in stamen position in the L- and M-morphs (see Fig. 1C in Barrett et al., 2004), these morphs may differ from the S-morph in other traits. If this were the case, it would support the hypothesis that the evolution of tristyly involves the origin of the M-morph through shortening of style length in plants of the L-morph (Barrett and Hodgins, 2006).

MATERIALS AND METHODS

Narcissus triandrus L. (Amaryllidaceae) is a widespread and abundant geophyte found in the central and northern regions of the Iberian Peninsula. Populations begin flowering during late February at the southern margin of the range and continue into early May at higher elevations in central and northern Spain and Portugal. Flowers of *N. triandrus* have a narrow floral tube that expands into a wider corona with reflexed tepals. Nectar-feeding, long-tongued bees, primarily *Anthophora* and *Bombus* species, enter the corona and insert their proboscis into the floral tube to access nectar produced at the top of the ovary. *Narcissus triandrus* flowers produce short-level sex organs within the floral tube, midlevel organs at or beyond the transition from the tube to the corona, and long-level organs at or beyond the corona mouth. Individual flowers last for one to two weeks, depending on temperature and pollen receipt.

Several intraspecific taxa have been recognized in *N. triandrus* (reviewed by Blanchard, 1990), and here we focus on the two common varieties: *N. triandrus* var. *cernuus* (Salisb.) Baker and *N. triandrus* L. var. *triandrus*. These varieties differ in a range of morphological traits and are genetically differentiated at chloroplast and microsatellite loci (Hodgins and Barrett, 2007). *Narcissus triandrus* var. *cernuus* occurs in the central and southern parts of the species' range (Fig. 1, filled symbols) and is characterized by small stature, pale lemon

flowers, and usually one or two flowers per inflorescence. In contrast, *N. triandrus* var. *triandrus* occurs in northern Portugal and northwestern Spain (Fig. 1, open symbols), is taller with larger white-to-cream flowers that are often produced in greater numbers per inflorescence (Hodgins and Barrett, 2007). Dimorphic populations, all of which lack the M-morph, have only been identified in var. *triandrus*.

Variation in style-morph ratios—During 2002–2004, we sampled 124 populations to determine the frequencies of style morphs in each population and to investigate geographical and temporal patterns of variation. The sample included 36 populations of var. *cernuus* and 88 populations of var. *triandrus* (40 dimorphic and 48 trimorphic). We resampled 33 of these populations in two different years (2002 and 2004) to determine the temporal stability of morph ratios. In addition, 20 populations sampled in a previous study during 1991–1997 (Barrett et al., 2004) were resampled. Most populations were separated by at least 5–10 km, and we sampled throughout the species range in relation to its overall abundance. In each population, we obtained a random sample of flowering stems and classified them according to style morph. We sampled a minimum of 100 individuals, unless population sizes were smaller than this, in which case we sampled all individuals where possible. The mean number of plants sampled per population was 92.6 (range 11–286). Because *N. triandrus* does not propagate clonally, the sampling of genets is unambiguous. Detailed localities and data on style-morph ratios are available from the first author upon request.

Geographical variation in vegetative and floral traits—In a subset of populations that were sampled for morph ratios, we randomly sampled flowering plants and measured several morphological characters. We measured plant height and leaf length with a ruler and stem and bulb diameter, flower size, corona length, tube length, corona width at the corona mouth, the maximum width of the corona, tube width at the junction of the tube, and at the base of the tube, and tepal length with digital calipers. We also recorded leaf number for each individual. In total, the eight floral attributes were measured in 78 populations (52 var. *triandrus* and 26 var. *cernuus*, mean = 53.9 individuals per population; range 9–198), while the five vegetative traits were measured in 45 of these populations (20 var. *triandrus* and 25 var. *cernuus*).

Climate data and pollinator observations—To investigate the relation between climate and geographical variation in plant traits, we obtained average annual precipitation and temperature data from the WorldClim version 1.4 database (http://www.worldclim.org/) for each population where morphology was measured. WORLDCLIM provides interpolated climate surfaces for global land areas at a spatial resolution of 1 km, derived from data for the period 1950–2000 (see Hijmans et al., 2005). However, it is important to note that the interpolation cannot reflect all of the variation occurring at a 1-km scale, especially precipitation in mountainous regions.

To determine the composition of the pollinator fauna of *N. triandrus*, we recorded the number of bees that we observed pollinating flowers (based on observation of contact with the sex organs) during measured time spans in 82 of the 124 populations surveyed (13 populations of var. *cernuus*, 34 dimorphic and 40 trimorphic populations of var. *triandrus*). The time spent in each population varied, but averaged 3.3 h per population. Only visitors that we concluded were contributing to cross-pollination were included in our analyses.

Statistical analysis—*Variation in style-morph ratios*—All statistical analyses were carried out in SAS version 9.1 (SAS Institute, Cary, North Carolina, USA). We used likelihood-ratio (G) tests to explore variation in style-morph frequencies. We compared morph ratios to the isoplethic equilibrium (1:1:1) with heterogeneity G-tests (Sokal and Rohlf, 1995) using sequential Bonferroni adjustment to control for the overall Type I error rate ($\alpha = 0.05$; Rice, 1989). We also compared morph ratios across years with likelihood-ratio (G) contingency tests with sequential Bonferroni correction ($\alpha = 0.05$; Rice, 1989). We compared morph frequencies of dimorphic and trimorphic populations with previous surveys by Barrett et al. (2004) using G-tests.

Principal components analysis—Due to the number and the multicollinearity of the traits we measured (results not presented), we used principal components analysis (PCA) to summarize the patterns of correlation among vegetative and floral traits in *N. triandrus*. We included five vegetative traits—leaf length, leaf number, stem diameter, plant height, and bulb diameter—and performed PCA on the correlation matrix of these traits using population means. For all analyses, due to nonhomogeneity of variance and nonnormality, we either
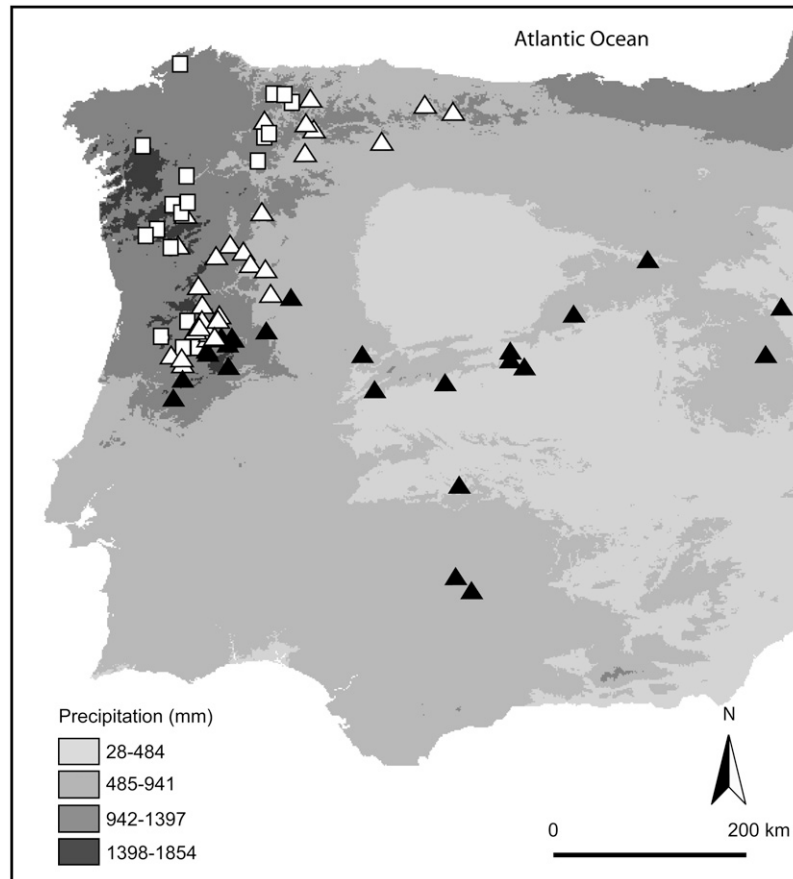
Fig. 1.   The geographical distribution of 78 populations of *Narcissus triandrus* that were sampled for floral morphology in this study. Triangles represent trimorphic populations and squares represent dimorphic populations. The different shades of gray represent average annual precipitation. Open symbols represent *Narcissus triandrus* var. *triandrus*, and closed symbols represent *Narcissus triandrus* var. *cernuus.*

square-root or ln-transformed the data where appropriate. We performed a similar analysis on the following eight floral traits—flower length, tube length, corona length, corona width at the mouth, corona width at the maximum, tepal length, tube width at junction and tube width at base—and performed PCA on the correlation matrix of these variables.

Morphological characteristics of dimorphic and trimorphic populations— We used general linear models (GLM procedure, SAS) to determine if there was a difference between dimorphic and trimorphic populations in floral and vegetative traits. We determined the influence of sexual system on the first two principal components of floral and vegetative traits. Sexual system (dimorphism vs. trimorphism) was treated as a fixed effect. We also included latitude in the model as a covariate. We removed nonsignificant interactions between latitude and sexual system. We analyzed all populations and then repeated the analysis using only var. *triandrus* populations to remove any potential influence of taxonomic variety on the analysis.

Comparisons of floral morphology among the morphs—To compare floral traits of the style morphs, we used mixed effects, general linear models (MIXED procedure, SAS; Neter et al., 1996). For all analyses, we considered population as a random factor and used restricted maximum likelihood to estimate the associated variance component. We calculated denominator degrees of freedom for *F*-tests of fixed effects by Kenward and Roger's approximation (1997). We analyzed dimorphic and trimorphic populations separately. For the analysis of dimorphic populations, we considered population and the interaction of population and morph as random effects, while we considered morph as a fixed effect. In trimorphic populations, we included morph and variety, as well as their interaction as fixed effects. In addition, we included population nested within variety and the interaction between population (variety) and morph as random effects. We tested random effects using likelihood ratio tests.

Nonsignificant interactions were removed in a backward stepwise manner. Although our analyses considered transformed data, we present the results for back-transformed means and standard errors, which resulted in asymmetric standard errors.

Correlation between morphology and morph ratios—To determine if morphology varied predictably with morph ratios, we performed multivariate multiple regression analysis (GLM procedure, SAS; Aitchison, 1986) on data from 40 trimorphic populations for which we had measurements of floral and vegetative traits. We used population means for each trait, although because some floral attributes of the S-morph were significantly different from the other two morphs (described later), we did not include morphological data from this morph in the analysis. We simultaneously analyzed two dependent variables, the log-odds of the L morph relative to the S morph, $£L = \ln(fL/fS)$ and the log-odds of the M morph relative to the S morph, $£M = \ln(fM/fS)$, where *fL, fM,* and *fS* are the relative frequencies of the L-, M-, and S-morphs, respectively. The regression used backward elimination (Neter et al., 1996) to find the set of independent variables that explained significant proportions of the joint variation in both dependent variables, based on Wilks' lambda (see Barrett et al., 2004 for further details).

Geographic variation in climate and morphology—Larger flowers are found in the northwest portion of the range in populations where the M-morph is low or absent (Barrett et al., 2004; Hodgins and Barrett, 2006b). We hypothesized that this increase in flower size is associated with higher rainfall and more favorable climatic conditions for growth. To address this hypothesis, we used multiple regressions (REG procedure, SAS) to examine geographic variation in climate as well as in floral and vegetative traits. We considered variation in traits with respect to longitude (*E*) and latitude north (*N*) in decimal degrees. The initial model for each analysis assessed the effects of *E, N, E^2, N^2,* and *EN*. We

conducted these analyses across all populations and also within var. *triandrus*. We focused in particular on var. *triandrus* as much of the variation in M-morph frequency, including the loss of the M-morph, occurs within this variety. The analyses used floral PC1 and PC2 and vegetative PC1 and PC2 as well as annual mean precipitation and annual mean temperature. To assess if the climatic variables annual mean precipitation and temperature were associated with variation in morphological traits, we used multiple regressions (REG procedure, SAS) and focused specifically on variation in flower size (floral PC1) and plant size (vegetative PC1) in relation to temperature (T) and precipitation (P). The initial model for each analysis assessed the effects of P, T, P^2, T^2 and PT. For both analyses we used backward elimination ($\alpha = 0.05$) to select the final regression model (Neter et al., 1996). To determine if plant size can predict changes in flower size, we performed a regression (REG procedure, SAS) that considered variation in plant size (vegetative PC1) with respect to flower size (floral PC1).

Pollinator observations—Pollinators influence patterns of pollen transfer and changes in species composition could contribute to population differentiation in floral morphology and morph ratios. We performed *G*-tests to determine if pollinator composition differed between dimorphic and trimorphic populations both among all populations and within var. *triandrus*. In populations where pollinators were investigated, we calculated the visitation rate as the number of pollinators observed per hour for each population. Because we were unable to transform the data on pollinators and achieve normality, we used the Kruskal–Wallis nonparametric test and compared visitation rates of *Bombus* and *Anthophora* in dimorphic vs. trimorphic populations.

RESULTS

Variation in morph ratios—Overall, morph ratios differed significantly from 1:1:1 in trimorphic populations ($G_{pooled} = 2142.71$, 2 df, $P < 0.001$, $G_{het} = 3734.57$, 168 df, $P < 0.001$) and differed from 1:1 in dimorphic populations ($G_{pooled} = 570.1$, 1 df, $P < 0.001$, $G_{het} = 69.7$, 39 df, $P < 0.001$). For the 84 trimorphic populations, mean morph frequencies were 0.57, 0.23, and 0.20 for the L-morph, M-morph, and S-morph, respectively, while in dimorphic populations mean morph frequencies were 0.72 and 0.28 for the L-morph and S-morph, respectively. In 63 of the 84 (74.1%) trimorphic populations, the morph frequencies differed significantly from 1:1:1, while 24 out of 40 (60.0%) dimorphic populations differed from 1:1. Of the 124 populations sampled, the L-morph was the most common morph in 91% of populations. In trimorphic populations, the M-morph predominated in 13% of the populations (one var. *triandrus* and 11 var. *cernuus* populations). In none of the populations was the S-morph the most common morph.

For the 53 populations that were resampled between years, there were no significant changes in local morph frequency. In addition, global morph frequencies (i.e., the overall frequency of each morph) did not change significantly between 2002 and 2004 (dimorphic $G_2 = 5.39$, $P = 0.07$; trimorphic $G_2 = 2.93$, $P = 0.23$). The average morph ratios for this study and those reported in Barrett et al. (2004) were similar for both trimorphic and dimorphic populations (trimorphic populations L-morph = 0.58, M-morph = 0.19, and S-morph = 0.23; dimorphic populations L-morph = 0.71 and S-morph = 0.29; Barrett et al., 2004). Although morph ratios did not differ for dimorphic populations ($G_1 = 3.28$, $P = 0.07$), morph ratios differed significantly for trimorphic populations ($G_2 = 30.90$, $P < 0.001$). However, the difference between samples of tristylous populations was slight (percentage difference: L = −1%, M = 4%, S= −3%), and the significant difference probably reflects the large sample size and differences in the populations sampled between studies.

Principal components analysis of floral and vegetative traits—The analysis of vegetative traits yielded two factors that cumulatively explained 83.9% of the variation (Fig. 2A). We

extracted the scores from the first two factors (eigenvalue = 2.87 and 1.33) explaining 57.4% and 26.6% of the variation in the original five variables, respectively (Table 1). The first component (PC1) is related to plant size and was positively correlated with all variables ($P < 0.01$ in all cases). The second factor (PC2) was positively correlated with height and leaf length and negatively correlated with stem diameter and leaf number ($P < 0.01$).

The analysis of floral traits yielded two factors (eigenvalues = 4.78 and 1.21) explaining 59.7% and 15.2% (cumulative 74.9%), respectively, of the variation in the eight variables (Fig. 2B). As before, we obtained the value of the variable "flower
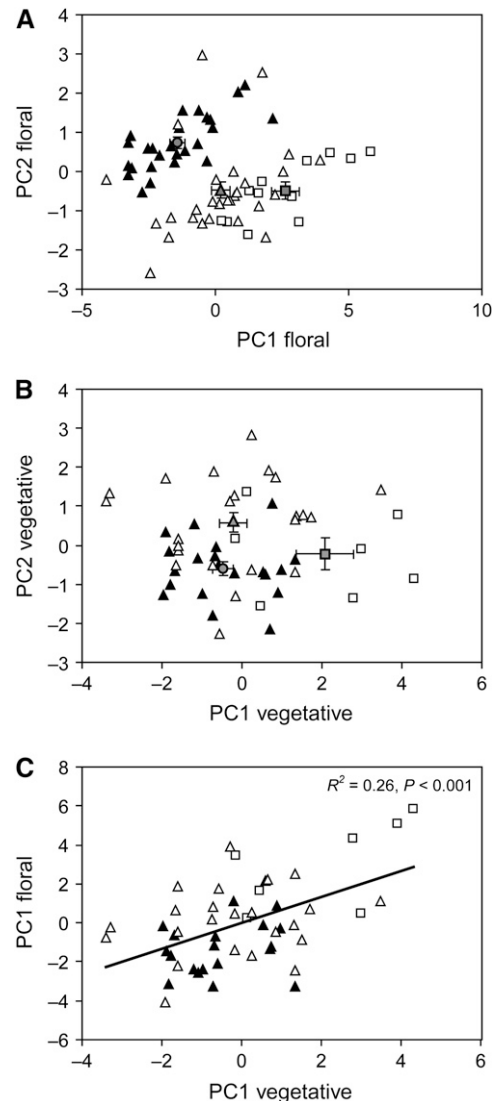

Fig. 2.   The first two principal components of eight floral and five vegetative traits of *Narcissus triandrus*. (A) The first and the second principal components of floral traits. (B) The first and the second principal components of vegetative traits. The first principal component was related to size for both floral and vegetative traits. The gray symbols represent means with the standard error bars of PC1 and PC2 for dimorphic (square), trimorphic var. *triandrus* (triangle), and trimorphic var. *cernuus* (circle) populations. (C) The correlation between the first principal components of floral and vegetative traits. Dimorphic populations are represented by squares and trimorphic populations by triangles. The open symbols represent var. *triandrus*, and closed shapes represent var. *cernuus*.

TABLE 1. Eigenvectors for the first two principal components of (A) floral and (B) vegetative traits in *Narcissus triandrus*. From a principal components analysis (PCA) of the correlation matrix of eight floral traits and a PCA of the correlation matrix of five vegetative traits. Asterisks indicate factors that are significantly correlated with the original variable. All analyses are based on population means.

(A) Floral trait	PC1	PC2
Flower length	0.43***	0.13
Tube length	0.37***	0.14
Corona length	0.42***	0.09
Tepal length	0.39***	−0.29**
Corona width (mouth)	0.31***	−0.27*
Corona width (max.)	0.38***	0.14
Tube width (junction)	0.15**	0.79***
Tube width (base)	0.30***	−0.39***
(B) Vegetative trait		
Bulb	0.48***	0.20
Height	0.45***	−0.48***
Leaf length	0.42***	−0.58***
Stem diameter	0.51***	0.35**
Leaf number	0.36***	0.52***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

size" (PC1), which positively correlated with all traits ($P < 0.01$), and the second factor "flower shape" (PC2), which positively correlated with tube width at the junction and negatively with tube width at the base of the flower, tepal length, and corona width at the mouth ($P < 0.05$). We used these floral and vegetative components in several of the following analyses, rather than each of the 13 traits that we measured.

Morphological characteristics of dimorphic and trimorphic populations—In general, populations that lacked the M-morph had larger floral and vegetative characteristics compared to tristylous populations (Fig. 2). The first principal component of floral traits and the first two principal components of vegetative traits differed between dimorphic and trimorphic populations among all populations and within var. *triandrus* populations even when latitude was incorporated into the model (Table 2). However, the second principal component of floral traits did not differ between the sexual systems, and the first principal component of vegetative traits was only marginally significant.

Comparisons of floral morphology among the morphs—The style morphs differed for a number of traits in both dimor-

phic and trimorphic populations. For trimorphic populations, four of eight floral attributes (flower length, corona length, corona width at the maximum and corona width at the mouth) differed among the style morphs (Table 3, Fig. 3). Independent contrasts revealed that flower length, corona length and corona width (maximum) of the L- and M-morphs were significantly larger than the S-morph ($\alpha = 0.05$, sequential Bonferroni adjustment). For corona width at the mouth, the interaction between variety and morph was significant. Independent contrasts identified that corona width at the mouth of the L-morph was significantly greater than the S-morph in var. *triandrus* but not in var. *cernuus* ($\alpha = 0.05$, sequential Bonferroni adjustment). The analysis of tube width at the junction identified a significant interaction between population (variety) and morph. For dimorphic populations, flower length and corona length were significantly larger in the L-morph than the S-morph (Table 4, Fig. 4).

Morphological correlates of morph-ratio variation in trimorphic populations—Morph ratios varied with population mean morphology among the 40 trimorphic populations and enabled statistical prediction of morph frequencies based on various trait combinations. The ability of the overall regression model to predict morph frequencies varied among the morphs and was strongest for the L- and M-morphs (Fig. 5A, B) and weakest for the S-morph (Fig. 5C). Following backward elimination, four morphological variables contributed significantly to the joint prediction of morph ratios: flower length (Wilks' lambda $= 0.67$, $F_{2,37} = 9.20$, $P < 0.001$), tube length (Wilks' lambda $= 0.70$, $F_{2,37} = 7.90$, $P < 0.01$), tepal length (Wilks' lambda $= 0.61$, $F_{2,37} = 12.07$, $P < 0.001$) and stem diameter (Wilks' lambda $= 0.67$, $F_{2,37} = 9.02$, $P < 0.001$).

The morphology of style morphs had contrasting effects on their frequencies within populations. The log-odds for the L-morph relative to the S-morph varied weakly with tepal length (partial regression coefficient $b = 0.69$ SE $= \pm 0.39$, $t_{37} = 1.74$, $P < 0.10$) but not with flower length, tube length or stem diameter (flower length $t_{37} = 0.32$, $P = 0.75$; tube length $t_{37} = 1.27$, $P = 0.21$; tepal length $t_{37} = 1.18$, $P = 0.25$, $R^2 = 0.50$). In contrast, the log-odds for the M-morph relative to the S-morph varied negatively with flower length and tepal length (flower length $b = -15.43$, SE $= \pm 3.60$, $t_{37} = -4.29$, $P < 0.001$; tepal length $b = -4.63$, SE $= \pm 1.03$, $t_{37} = -4.49$, $P < 0.001$), and positively with tube length and stem diameter (tube length $b = 16.09$, SE $= \pm 4.10$, $t_{37} = 3.92$, $P < 0.001$; stem $b = 1.43$, SE $= \pm 0.34$, $t_{37} = 4.23$, $P < 0.001$, $R^2 = 0.63$).

TABLE 2. General linear models of the first two principal components of floral and vegetative characteristics in dimorphic and trimorphic populations of *Narcissus triandrus* in all populations and var. *triandrus* populations. We treated sexual system as a fixed effect and included latitude as a covariate. Interactions between the covariate, latitude, and the main effect, sexual system, were tested and removed because they were nonsignificant.

Population	Variable	Sexual system	Latitude
All populations	PC1 floral	$F_{1,63} = 17.66$***	$F_{1,63} = 26.47$***
	PC2 floral	$F_{1,63} = 1.13$	$F_{1,63} = 2.31$
	PC1 vegetative	$F_{1,47} = 3.64$†	$F_{1,47} = 17.59$***
	PC2 vegetative	$F_{1,47} = 7.75$**	$F_{1,47} = 19.93$***
Var. *triandrus*	PC1 floral	$F_{1,37} = 12.75$**	$F_{1,37} = 10.03$**
	PC2 floral	$F_{1,37} = 0.00$	$F_{1,37} = 0.00$
	PC1 vegetative	$F_{1,27} = 3.29$†	$F_{1,27} = 21.41$***
	PC2 vegetative	$F_{1,27} = 5.67$*	$F_{1,27} = 4.33$*

†$P < 0.10$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

TABLE 3. Mixed model analyses of factors influencing floral variation in trimorphic populations of *Narcissus triandrus*. Random and fixed effects for each floral trait are listed. We tested the random effects population and morph × population (variety) with a likelihood ratio test.

Floral trait	Morph	Variety	Population (variety)	Morph × variety	Morph × population (variety)
Flower length	$F_{2,1538} = 7.71$***	$F_{1595} = 53.40$***	$G_1 = 10.3$**	$F_{2,1538} = 1.68$	$G_1 = 0.00$
Tube length	$F_{2,1544} = 0.75$	$F_{1785} = 46.96$***	$G_1 = 9.90$**	$F_{2,1544} = 0.23$	$G_1 = 0.00$
Corona length	$F_{2,1544} = 9.85$**	$F_{1780} = 33.61$***	$G_1 = 5.50$*	$F_{2,1542} = 0.68$	$G_1 = 0.10$
Tepal length	$F_{2,1532} = 0.75$	$F_{1,1218} = 359.36$***	$G_1 = 12.30$***	$F_{2,1532} = 2.21$	$G_1 = 0.00$
Corona width (mouth)	$F_{2,1479} = 18.84$***	$F_{1,1127} = 38.11$***	$G_1 = 7.60$**	$F_{2,1479} = 4.33$*	$G_1 = 0.00$
Corona width (max.)	$F_{2,1474} = 5.14$**	$F_{1931} = 2.95$	$G_1 = 5.80$*	$F_{2,1474} = 2.24$	$G_1 = 0.00$
Tube width (junction)	$F_{2430} = 12.61$***	$F_{1,1298} = 1.02$	$G_1 = 1.80$	$F_{2430} = 5.23$**	$G_1 = 10.30$**
Tube width (base)	$F_{2,1527} = 0.47$	$F_{1,76} = 14.21$***	$G_1 = 5.30$*	$F_{2,1527} = 0.77$	$G_1 = 0.00$

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Geographic variation in morphology and climate—Vegetative and floral morphology varied spatially throughout the range of *N. triandrus* (Figs. 1, 6). The most prominent feature of this variation was an increase in plant size from southeastern to northwestern portions of the range, which was accompanied by correlated changes in floral traits. Regression analysis revealed a positive relation between floral size and plant size ($b = 0.66$, $SE = ±0.16$, $R^2 = 0.26$, $F_{1,48} = 16.94$, $P < 0.001$; Fig. 2C). Analyses of the first and the second principal components for both floral and vegetative traits revealed correlated changes with geography among all populations and within var. *triandrus* (Fig. 6A, B). Flower size was significantly influenced by both latitude and longitude among all populations and within var. *triandrus* (all populations $R^2 = 0.52$, $F_{3,62} = 21.99$, $P < 0.001$; var. *triandrus* $R^2 = 0.57$, $F_{3,35} = 15.22$, $P < 0.001$). However, floral PC2 was not correlated with geography among all populations and only correlated with latitude within var. *triandrus* ($R^2 = 0.20$, $F_{2,36} = 4.49$, $P < 0.05$). Plant size was associated with both latitude and longitude among all populations and within var. *triandrus* (all populations $R^2 = 0.48$, $F_{2,47} = 21.28$, $P < 0.001$; var.

triandrus $R^2 = 0.62$, $F_{2,25} = 20.27$, $P < 0.001$), as was vegetative PC2 (all populations $R^2 = 0.39$, $F_{4,45} = 7.17$, $P < 0.001$; var. *triandrus* $R^2 = 0.37$, $F_{4,23} = 3.3$, $P < 0.05$).

Climate varied across the range of *N. triandrus*, and this variation was associated with changes in plant and flower size. Multiple regression analysis revealed that average annual precipitation and temperature correlated with latitude and longitude (precipitation $R^2 = 0.83$, $F_{3,76} = 120.61$, $P < 0.001$; temperature $R^2 = 0.63$, $F_{3,76} = 43.83$, $P < 0.001$). Precipitation generally increased from southeast to northwest, while temperature declined to the northeast because of the increased elevation of populations. Within the range of var. *triandrus*, precipitation increased in the west and temperature increased in the northwest (precipitation $R^2 = 0.65$, $F_{1,53} = 98.49$, $P < 0.001$; temperature $R^2 = 0.61$, $F_{4,50} = 19.49$, $P < 0.001$). Climatic variables were also correlated with flower size (all populations $R^2 = 0.27$, $F_{3,61} = 7.52$, $P < 0.001$; var. *triandrus* $R^2 = 0.65$, $F_{1,53} = 98.49$, $P < 0.05$). Temperature and precipitation were associated with flower size across all populations, but only precipitation explained a significant proportion of the variation in flower size within var. *triandrus*.
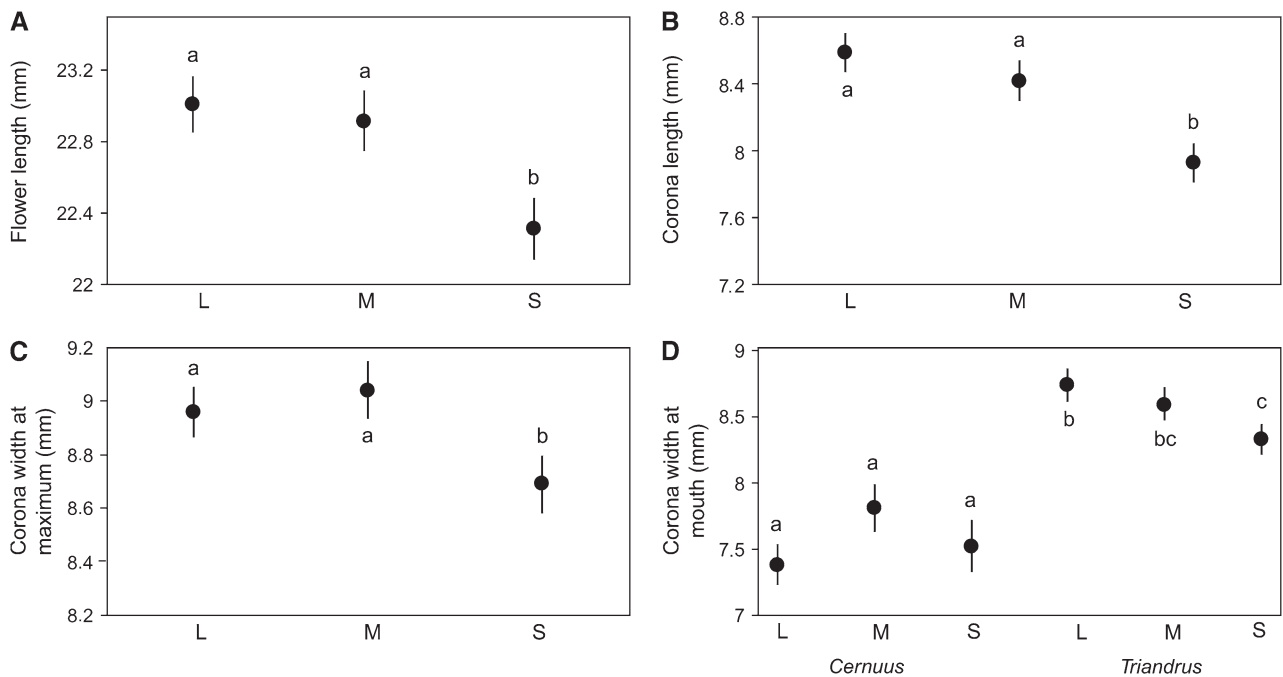

Fig. 3. Comparisons of floral attributes among the morphs for trimorphic populations of both varieties of *Narcissus triandrus*. (A) Flower length, (B) corona length, (C) corona width at the maximum, (D) corona width at the mouth for var. *cernuus* and var. *triandrus*. We present the results for back-transformed means and standard errors.

TABLE 4. Mixed model analyses of factors influencing floral variation in dimorphic populations of *Narcissus triandrus*. Random and fixed effects for each floral trait are listed. We tested the random effects population(variety) and morph × population(variety) with a likelihood ratio test.

Floral trait	Morph	Population (variety)	Morph × population (variety)
Flower length	$F_{1357} = 9.05**$	$G_1 = 0.58$	$G_1 = 0.00$
Tube length	$F_{1357} = 0.03$	$G_1 = 5.70*$	$G_1 = 0.00$
Corona length	$F_{1356} = 18.84***$	$G_1 = 0.00$	$G_1 = 0.00$
Tepal length	$F_{1356} = 0.63$	$G_1 = 2.90$	$G_1 = 0.00$
Corona width (mouth)	$F_{1354} = 1.77$	$G_1 = 1.20$	$G_1 = 0.00$
Corona width (max.)	$F_{1352} = 3.43$	$G_1 = 2.10$	$G_1 = 0.00$
Tube width (junction)	$F_{1356} = 2.58$	$G_1 = 0.00$	$G_1 = 0.00$
Tube width (base)	$F_{1356} = 0.04$	$G_1 = 3.33*$	$G_1 = 0.00$

$*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

However, precipitation was correlated with plant size across all populations ($R^2 = 0.13$, $F_{2,35} = 3.36$, $P < 0.05$), but not within var. *triandrus*.

Pollinator observations—In total we recorded 273.5 h of pollinator observations in 82 populations. All pollinators we observed were either solitary bees (*Anthophora*) or bumblebees (*Bombus* spp.). A few small flies and pollen-collecting bees were also observed visiting flowers but likely did not contribute significantly to cross pollination and were therefore not included in our analysis. We identified significant differences in pollinator composition among populations of *N. triandrus* (Fig. 7). Although pollinator visitation to *N. triandrus* was generally low, we observed 40 pollinators in total (0.15 pollinators per hour of observation). Solitary bees predominated in trimorphic populations (all populations 66%, $N = 27$; var. *triandrus* 57%, $N = 21$), whereas bumblebees were more common in dimorphic populations (92%, $N = 13$). Likelihood ratio tests revealed that dimorphic and trimorphic populations differed significantly in pollinator composition among all populations ($G_1 = 13.93$, $P < 0.001$) and among var. *triandrus* populations only ($G_1 = 9.50$, $P < 0.01$). Rates of pollinator visitation differed in the same manner because dimorphic populations had a higher rate of visitation by *Bombus* ($\chi^2_1 = 7.06$, $P < 0.01$, $N = 6$ populations), while trimorphic populations were visited more frequently by *Anthophora* across all populations, but not within var. *triandrus* (all populations $\chi^2_1 = 5.05$, $P < 0.05$, $N = 12$ populations; var. *triandrus* $\chi^2_1 = 0.59$, $P = 0.44$, $N = 8$ populations), suggesting that solitary bees were the predominant pollinators of var. *cernuus* populations.

DISCUSSION

Populations of *N. triandrus* display striking geographic patterns of morph-ratio variation unlike those reported in other tristylous species (reviewed in Barrett, 1993). The generally large population sizes and the temporal stability of morph frequencies suggest that this variation is unlikely to result from stochastic processes alone. Instead, the differentiation in morph ratios that we report among populations probably results from variation in patterns of mating and hence the nature of frequency-dependent selection acting throughout the range of the species. Specifically, we hypothesize that (1) climatic gradients in the Iberian Peninsula result in correlated changes to plant and flower size; (2) clinal variation in flower size influences the types of pollinators visiting populations; and (3) interactions between floral morphology and pollinator composition result in variation in patterns of outcrossed mating causing geographic variation in

style morph frequencies. In our study, we obtained evidence to support several of these hypotheses, although because our results are largely correlative in nature we cannot definitively establish the causal relations we hypothesize.

In our study, we established that climatic gradients and the local pollination environment were significantly associated with geographic variation in plant and flower size. Also, spatial variation in floral and vegetative traits was correlated with morph-ratio variation among populations. These results are consistent with the hypothesis that changes in environmental factors contribute to morph-ratio variation, including the decline and loss of the M-morph from populations. In addition, our morphological analysis of floral traits identified that the L- and M-morphs differed significantly from the S-morph in several features. As we discuss later, this novel result for a tristylous species provides insight into the evolutionary origins of the M-morph in *N. triandrus* and for understanding transitions between heterostylous sexual systems.
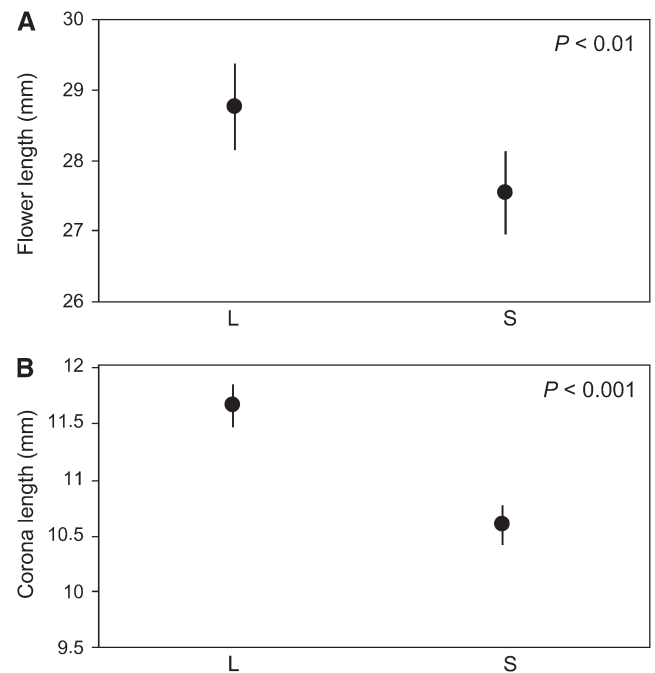

Fig. 4. Comparisons of floral attributes among the morphs for dimorphic populations of *Narcissus triandrus* var. *triandrus*. (A) Flower length, (B) corona length. We present the results for back-transformed means and standard errors.
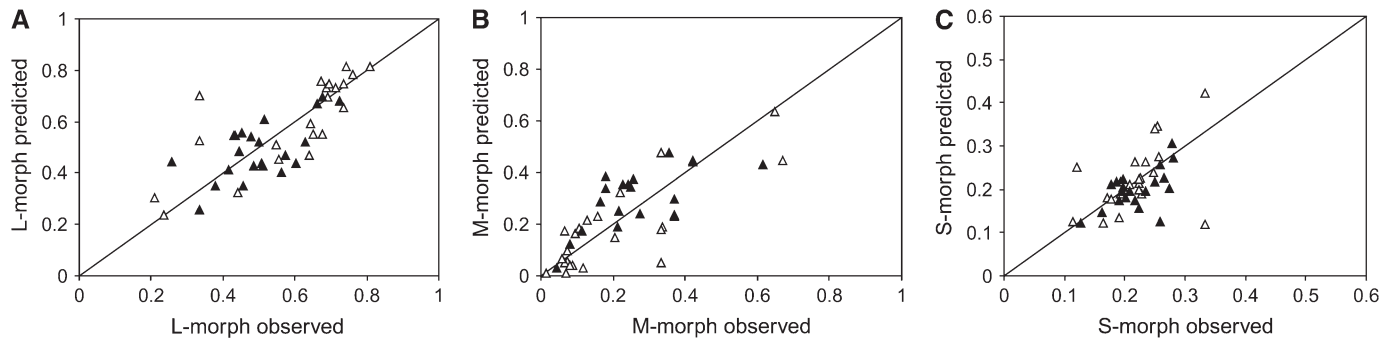
Fig. 5.   Associations between observed and predicted frequencies for the (A) L-morph, (B) M-morph, and (C) S-morph in 40 trimorphic populations of *Narcissus triandrus*. We derived predicted frequencies from a multivariate, multiple regression that considered the mean positions of flower length, tube length, tepal length, and stem diameter. The diagonal lines indicate equivalence of observed and predicted frequencies. Closed symbols depict var. *cernuus*, and open triangles indicate trimorphic populations of var. *triandrus*.

Geographic variation in morphology, climate, and pollinators—We demonstrated that patterns of geographic variation in morphology among *N. triandrus* populations were associated with environmental gradients. Most of the vegetative and floral traits varied spatially and the most significant feature of this morphological variation was an increase in flower size along a southeast–northwest gradient (Fig. 6A; and see Barrett et al., 2004). This variation was correlated with a corresponding increase in plant size (Figs. 2C, 6B) and is also in accord with patterns of variation in sex-organ length reported in an earlier study (Barrett et al., 2004). We also found that the climatic variables annual precipitation (Fig. 1) and temperature displayed geographic patterns across the species' range. These variables were associated with changes in vegetative and floral morphology, and, in particular, precipitation was correlated with an increase in plant and flower size. Plants of var. *cernuus* are more diminutive with smaller flowers, and populations occur in hot, dry Mediterranean environments in the southern portions of the range. In contrast, individuals of var. *triandrus* are larger in stature with bigger flowers and occupy cooler, wet Atlantic environments in central and northern regions of the Iberian Peninsula (Hodgins and Barrett, 2007; Figs. 1, 2). However, we were unable to detect an association between climatic variables and geographic variation in plant size among populations of var. *triandrus*. Other ecological factors, such as soil type or community composition, may be important in causing variation in plant size in this variety.

The types of pollinators visiting flowers of *N. triandrus* also varied geographically. Solitary bees pollinated flowers throughout the range of *N. triandrus*, although they were much less common in dimorphic populations, which are found in the northwest portion of the species' range (Fig. 7). In contrast, bumblebees were often found pollinating flowers of dimorphic populations, but were never observed visiting flowers of var. *cernuus*. The greater flower size of dimorphic populations likely permits the entry of the large-bodied *Bombus*, while these bees are probably excluded from the smaller-flowered var. *cernuus* populations. These patterns are unlikely to result from the direct influence of climate on the distribution of *Anthophora* and *Bombus* because both are common throughout the geographical range of *N. triandrus*. Experiments with both types of pollinators using arrays of *N. triandrus* would be required to assess whether variation in flower size plays an important role in determining the patterns we observed. Unfortunately, low pollinator visitation rates may make this difficult to implement.

Patterns of style-morph variation—Our surveys demonstrate that *N. triandrus* has biased style-morph ratios in contrast to the theoretical expectation of 1:1:1 morph ratios for tristylous species (Fisher, 1941; Barrett, 1993). The preponderance of the L-morph in populations of this species illustrates the importance of floral morphology in mating. Unlike other tristylous species, the L-morph possesses long-level anthers, which should promote pollen transfer among plants of the L-morph. Measurements of paternity in *N. triandrus* using microsatellite markers confirm that significant assortative mating occurs in the L-morph, resulting in asymmetrical mating (Hodgins and Barrett, in press). Theory predicts that asymmetrical mating of this type should result in L-biased populations (Barrett et al., 2004; Barrett and Hodgins, 2006). Indeed, 91% of the populations that we surveyed were dominated by the L-morph. We also observed considerable variation in M-morph frequency associated with its decline and loss from populations of var. *triandrus*, especially in northern parts of the range. The pattern of morph-frequency variation found here closely matches that reported in an earlier survey of *N. triandrus* (Barrett et al., 2004) and demonstrates that biased morph ratios are a pervasive and general feature of this species.

Our study of temporal patterns of morph-ratio variation revealed that average frequencies generally remained unchanged over different census periods. Global morph ratios were consistent between this study and an earlier study (Barrett et al., 2004). This result likely reflects the fact that both surveys included populations from throughout the geographical range and hence the sampling of morph-ratio variation was generally equivalent. Resampling of population morph ratios, where the intervals between samples ranged from 2 to 10 years, indicated temporal stability of local morph ratios. The only other large-scale survey of temporal variation in morph ratios in a tristylous species involved the annual aquatic *Eichhornia paniculata*, where Husband and Barrett (1992) detected significant temporal shifts in morph frequencies over 2–3 years and invoked stochastic forces to explain these changes. The temporal stability observed in this study, in conjunction with the predictable geographic pattern of morph-ratio variation, suggest that morph ratios, especially those in populations of large size, may be at equilibrium and are maintained by local processes influencing negative frequency-dependent selection.

Correlates and causes of morph-ratio variation—The most prominent feature of geographic variation in morph frequencies
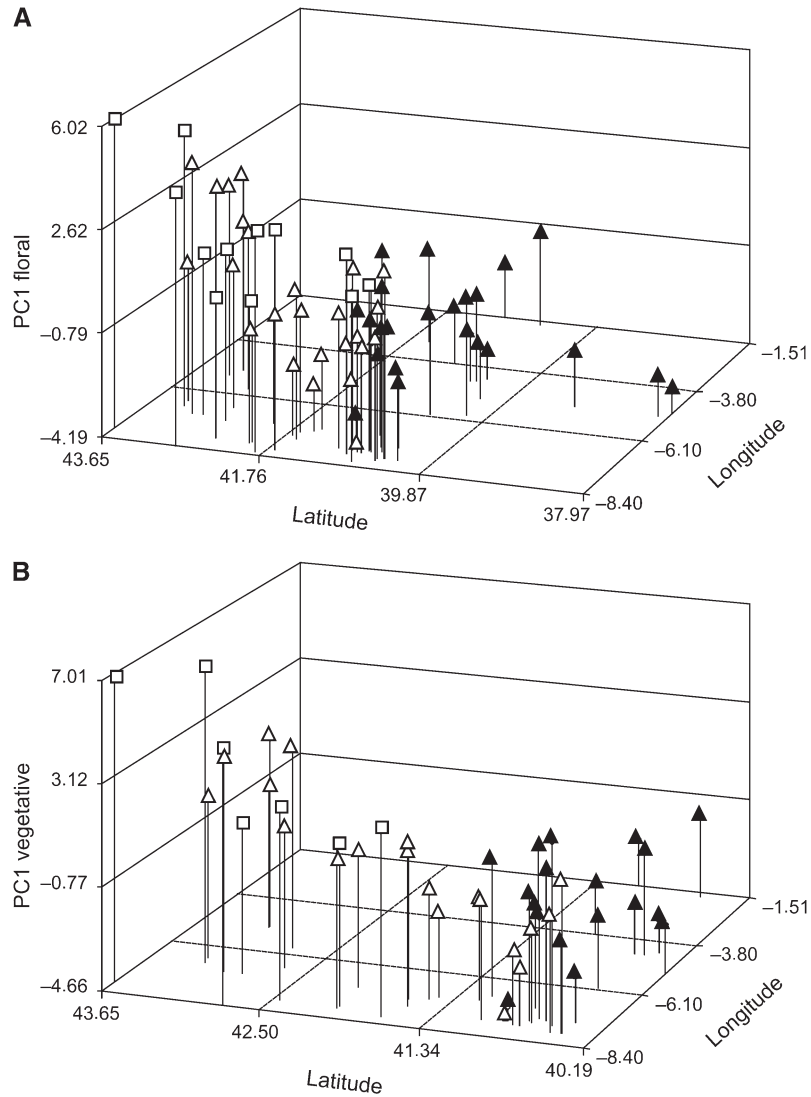
Fig. 6. The association between geography and the first principal components of eight floral and of five vegetative traits in *Narcissus triandrus*. (A) The correlation between latitude, longitude and flower size. (B) The correlation between latitude, longitude, and plant size. Dimorphic populations are represented by squares and trimorphic populations by triangles. The open symbols represent var. *triandrus*, and closed shapes represent var. *cernuus*.

in *N. triandrus* is the southeast to northwest decline in frequency of the M-morph (Barrett et al., 2004), which parallels a general increase in flower size. We found a negative correlation between flower length and the log odds of the frequencies of the M-morph relative to the S-morph in trimorphic populations. In addition, even when we accounted for variation in latitude and variety, flower and plant size were significantly larger in dimorphic compared to trimorphic populations. In contrast, there was no difference in flower shape between the two sexual systems.

Flower-size variation in *N. triandrus* could influence M-morph frequency through both female and male functions. Changes in flower length are correlated with unequal shifts in the relative position of midlevel organs with consequences for pollen transfer and mating among the morphs (Barrett et al., 2004). Theoretical models indicate that alterations in mating patterns, particularly a reduction in pollen transfer from the S-morph to the M-morph, could result in the loss of the M-morph

from populations (Barrett et al., 2004). A similar mechanism is proposed for the loss of the M-morph in *Oxalis alpina* owing to physiological modifications to the incompatibility relations between the L- and S-morphs (Charlesworth, 1979; Weller, 1979; Weller et al., 2007). A shift to bumblebee pollination is also evident in large-flowered dimorphic populations, which may have consequences for mating and morph frequencies. The larger and more variable sizes of *Bombus* individuals compared with *Anthophora* could influence the precision of pollen transfer in northern populations of *N. triandrus* var. *triandrus* and make it difficult to maintain the M-morph in populations. Theoretical models demonstrate that such an effect can cause the loss of the M-morph from tristylous populations (Charlesworth, 1979).

Pollen transfer models indicate that increased self-pollination in the M-morph or pollen limitation can also result in the decline and loss of the M-morph from tristylous populations
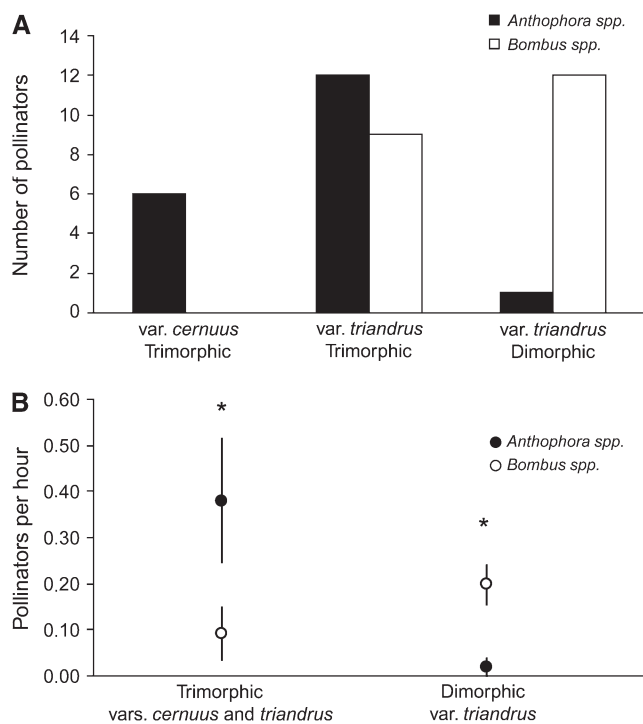
Fig. 7.   The pollinator composition of dimorphic and trimorphic populations of *Narcissus triandrus*. (A) The number of pollinators observed in 33 dimorphic (var. *triandrus*) and 49 trimorphic populations (var. *triandrus* and var. *cernuus*). (B) The number of pollinators per hour of observation in 18 populations where pollinators were observed for dimorphic (var. *triandrus*, 6 populations) and trimorphic populations (var. *cernuus*, 4 populations, and var. *triandrus*, 12 populations). Asterisks represent significant differences at $P < 0.01$.

(Charlesworth, 1979; Hodgins and Barrett, 2006a). Surveys of morph-specific fitness components in *N. triandrus* support these theoretical predictions, as the female fertility of the M-morph is positively correlated with its frequency in populations and declines with flower size (Hodgins and Barrett, 2006a). The shift in pollinator composition from trimorphic to dimorphic populations could result in insufficient or inferior pollination (sensu Harder and Barrett, 1996), both of which could lead to the loss of the M-morph. Elsewhere in *Narcissus*, pollinator shifts accompanied by modifications to floral morphology have been proposed as an important factor promoting changes in sexual systems among species of the genus (Graham and Barrett, 2004; Pérez et al., 2004; Barrett and Harder, 2005; Pérez-Barrales et al., 2006). The results of our study support the hypothesis that the pollination biology of *N. triandrus* populations plays an important role in the transition between trimorphism and dimorphism. However, the precise mechanisms that are involved are not known and await future enquiry.

Our statistical model using quantitative variation in four floral and vegetative traits independent of sex-organ position was able to predict morph ratios in trimorphic populations of *N. triandrus*. The model provided a more precise prediction of morph frequencies within populations, particularly of the S-morph, than a previous analysis using only variation in sex-organ position (Barrett et al., 2004), which suggests that factors other than stigma and anther height are also important in determining morph frequencies. These factors are likely to be aspects of floral morphology that govern the interaction with pollinators and

influence patterns of pollen transfer and mating as well as the character of frequency-dependent selection on morph ratios. It is unlikely that two of the traits we used in our model, stem diameter and tepal length, directly impact mating and morph ratios. Rather, these traits are likely to be correlated with other features which directly influence pollination and mating.

The origin of the M-morph and the evolution of tristyly— Our comparison of the floral morphology of mating types revealed that the L- and M-morphs were significantly larger in size than the S-morph for a number of floral traits. This finding would be unexpected in a typical tristylous species. The floral morphs of heterostylous species are commonly distinguished by a number of ancillary polymorphisms. These usually involve differences in the size of pollen and length of stigmatic papillae (Dulberger, 1992), but occasionally, other floral attributes are reported, such as contrasting flower and pollen color in the morphs of distylous *Linum pubescens* (Wolfe, 2001). Several examples of corolla size differences are known in distylous species, and in these cases, flowers of the S-morph are typically larger than the L-morph (e.g., *Jasminum fruticans*, Thompson and Dommée, 2000; *Amsinckia vernicosa*, *A. douglasiana*, Ganders, 1976; *Lithospermum caroliniense*, Levin, 1968; *Fagopyrum esculentum*, Darwin, 1877). However, prior to our study, significant differences in quantitative traits among the floral morphs, other than the sex organs themselves, have not been reported in tristylous species.

The morphological similarities between the L- and M-morphs provide insight into the evolutionary origins of tristyly in *N. triandrus*. In general, the evolutionary pathway(s) leading to tristyly in flowering plants is not well understood. However, it seems probable that the invasion of the M-morph is a key step in the establishment of trimorphism from dimorphism (Charlesworth, 1979; Barrett, 1993). Our findings indicate that the L- and M-morphs are similar in flower size and anther positions, whereas the S-morph possesses smaller flowers and midlevel anthers. The resemblance between the L- and M-morphs implies that the M-morph is likely derived from the L-morph, and according to this hypothesis, genetic modifiers in dimorphic populations that shorten the length of the style in long-styled plants result in midstyled variants. Under the appropriate pollination conditions, these may then spread to give rise to floral trimorphism.

Measurements of style-length variation in *N. triandrus* populations provide support for this scenario since the patterns in some populations involve near continuous variation (Barrett and Hodgins, 2006). This pattern indicates that the necessary standing genetic variation may be present in contemporary populations to facilitate the evolution of discrete style morphs. However, it is unclear what fitness benefits may accrue to phenotypes with midlength styles that enables them to spread in dimorphic populations. Although, our study of geographic variation in morphology, climate and pollinators provides a first step in identifying the ecological factors involved in this transition between sexual systems, the selective mechanisms favoring the gain or loss of the M-morph from *N. triandrus* populations remain a puzzle. Molecular evidence indicates that transitions between the sexual systems of *N. triandrus* occur quite often (Hodgins and Barrett, 2007). This evolutionary lability undoubtedly reflects the minor morphological differences that distinguish the L- and M-morphs and, unlike other tristylous species, the absence of an incompatibility system that enforces disassortative mating.

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