



# Geographic variation of flower traits in *Narcissus papyraceus* (Amaryllidaceae): do pollinators matter?

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## ABSTRACT

**Aim** The geographic clinal variation of traits in organisms can indicate the possible causes of phenotypic evolution. We studied the correlates of flower trait variation in populations of a style-dimorphic plant, *Narcissus papyraceus* Ker-Gawl., within a region of high biogeographical significance, the Strait of Gibraltar. This species shows a geographic gradient in the style-morph ratio, suggested to be driven by pollinator shifts. We tested whether parallel geographic variation of perianth traits also exists, concomitant with vegetative trait variation or genetic similarity of plant populations.

**Location** The Strait of Gibraltar region (SG hereafter, including both south-western Iberian Peninsula and north-western Morocco).

**Methods** We used univariate and multivariate analyses of flower and vegetative traits in 23 populations. We applied Mantel tests and partial Mantel correlations on vegetative and flower traits and geographic locations of populations to test for spatial effects. We used Moran's autocorrelation analyses to explore the spatial structure within the range, and performed the analyses with and without the Moroccan samples to test for the effects of the SG on spatial patterns. Amplified fragment length polymorphism data were used to estimate the genetic distance between populations and to ascertain its relationship with morphometric distance.

**Results** There was high variation between and within populations in both flower and vegetative traits. Mantel correlations between geographic and morphometric distances were not significant, but the exclusion of Moroccan populations revealed some distance effect. Partial Mantel correlation did not detect a significant correlation between flower and vegetative morphometric distances after controlling for geographic distance. There were opposite trends in spatial autocorrelograms of flower and vegetative traits. The genetic distance between pairs of populations was directly correlated with geographic distance; however, flower morphometric and genetic distances were not significantly correlated.

**Main conclusions** The SG had some influence on phenotypes, although the causes remain to be determined. The opposite trend of variation in flower and vegetative traits, and the lack of correlation between genetic distance and dissimilarity of flower phenotypes favour the hypothesis of pollinator-mediated selection on flower morphology, although this may affect only particular traits and populations rather than overall phenotypes. Although stochastic population processes may have a small effect, other factors may account for the high flower variation within and between populations.

## Keywords

AFLP, ecogeography, ecotypic differentiation, flower biology, genetic distance, morphometry, pollinators, spatial autocorrelation, Strait of Gibraltar, style dimorphism.

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## INTRODUCTION

The geographic variation of organisms was one of the main sources of inspiration for Darwin in his formulation of the theory of evolution by means of natural selection (Darwin, 1859). Virtually all landmarks in evolutionary theory have involved critical assumptions and implications about the geographic range of organisms (e.g. Mayr, 1963; Gould & Johnston, 1972; Endler, 1977; J.N. Thompson, 2005). Geographic variation can occur at several scales and present some sub-structuring that might mask its detection. Thus, ascertaining geographic patterns at different scales and delimiting zones of sharp change require spatially controlled methods (Sokal & Oden, 1978a,b).

When geographic patterns of phenotypic variation are detected, they can appear in a clinal or a mosaic fashion and can be correlated with abiotic factors (Wolf *et al.*, 2000; Franiel & Wieski, 2005), biotic factors (J.N. Thompson, 2005), or both (Hampe, 2003). In animal-pollinated and -dispersed plants, it might be expected that abiotic factors tend to mould vegetative rather than reproductive traits; however, biotic factors can affect both reproductive (e.g., flowers: Boyd, 2002; fruits: Hampe, 2003; but see Traveset *et al.*, 2004) and vegetative (Berenbaum & Zangerl, 1998; Snyder & Linhart, 1998; Karkkainen *et al.*, 2004) traits. Pollinator–flower interactions are among the most studied of biotic interactions in an evolutionary context (Stebbins, 1970; Fenster *et al.*, 2004). This interaction is frequently correlated with clines of variation in flower traits at the species and population levels (e.g. Robertson & Wyatt, 1990; Boyd, 2002; Silva-Montellano & Eguiarte, 2003).

Currently, there is much evidence that the geographic variation of abiotic and biotic selective agents has a profound effect in shaping the evolution of plants in the Mediterranean basin, particularly for reproductive traits (J.D. Thompson, 2005). *Narcissus papyraceus* Ker-Gawl. (Amaryllidaceae) is a Mediterranean geophyte dimorphic for female organs of the flowers, which can be either long-styled (L) or short-styled (S). Populations around the Strait of Gibraltar (SG) region (south-western Iberian Peninsula and north-western Morocco) show a geographic cline in both morph ratio and population size (Arroyo *et al.*, 2002). Populations close to either side of the SG are very large and isoplethic (equal proportion of style morphs, L:S). In the surrounding areas the S morph is progressively scarcer, populations being L-biased, whereas in peripheral areas most populations are monomorphic for L. These marginal populations are consistently smaller.

Arroyo *et al.* (2002) proposed two non-mutually exclusive hypotheses to explain the geographic pattern of the loss of S. The genetic hypothesis is based on the morph inheritance (L is recessive homozygous and S is heterozygous; Dulberger, 1964), and the lack of linkage between both incompatibility and style length loci. Any new population founded by L plants will produce only L progeny. In contrast, a population founded by S plants will produce both L and S plants. This hypothesis is thus related to migration and founder events, and is conse-

quently associated with stochastic processes and does not assume any selective advantage of any morph (see Pannell *et al.*, 2005, for a review). Under this scenario, a few founder events by L plants would render populations fixed for this morph as they spread. The ecological hypothesis underlines the role of pollinators in pollen transfer between style morphs (Lloyd & Webb, 1992). The L plants have exposed stigmas and consequently are likely to receive pollen from pollinators, even if they are short-tongued. The S plants have the stigma concealed deep in the flower tube, and require long-tongued insects for pollination. Thus, this hypothesis assumes that different pollinator arrays act in populations with different morph ratios.

Pérez-Barrales *et al.* (2007) found different pollinator assemblages on L-monomorphic and dimorphic isoplethic populations of *N. papyraceus*, operating in functionally different fashions. The principal pollinators are nectar-seeking moths for isoplethic populations, whereas they are pollen-feeding syrphid flies for L-monomorphic populations. This conspicuous shift in pollinators provides a unique opportunity to test different potential effects on other flower traits and, therefore, whether directional selection is modifying their population mean values (Endler, 1986) in the SG region. If morph ratio is driven mainly by pollinator groups with contrasting behaviour, a change in population means of flower traits across the range should be expected, coupled with the variation in morph ratio. This analysis cannot completely discard the effect of migration and isolation and associated processes (drift and founder events). Assessing these effects requires appropriate population genetic data or some measure of within-population phenotypic variability, which would be smaller in small and isolated populations, regardless of population means.

A further insight into the possible effect of pollinators on flower traits comes from a comparative analysis of the geographic variation of vegetative traits, which should not be directly subjected to selective pressures by pollinators (Berg, 1960; Armbruster *et al.*, 1999). If both vegetative and flower traits show a parallel trend of variation, the hypothesis of pollinator-mediated selection would not be favoured, instead it would indicate a side effect of vegetative variation on flower traits.

In this paper we use correlative evidence to check whether geographic patterns are more consistent with the ecological or with the genetic hypothesis. The geographic analysis of phenotypic variation is carried out in a multi-approach manner. First, we globally analyse the relationship between phenotypic and geographic distance between populations. Second, we use partial correlations to discern the association between vegetative and flower traits, while controlling for geographic distance. Third, we use a spatial autocorrelation approach to search for distance and neighbourhood effects on the phenotypic variation across the range. All these analyses are undertaken both with and without North African populations, to account for the possible effect of the SG. Finally, we analyse within-population variability in relation to

population size, isolation and morph ratio gradient, and the relationship between genetic distance of populations and their morphometric (flower or vegetative) and geographic distances, to control for possible effects related to gene flow (by means of seed or pollen).

## MATERIALS AND METHODS

### Study area

The present study was carried out in the SG region (south-western Iberian Peninsula and north-western Morocco; Fig. 1). After several openings and closings, the SG has remained open since the early Pliocene, which has influenced plant migration and differentiation across the region (see Rodríguez-Sánchez *et al.*, 2008, and references therein). The climate is mild Mediterranean, with frost-free winters (the mean minimum temperature of the coldest month is 5°C) and frequent rainfall (annual mean > 1000 mm), whereas inland areas are usually drier (annual mean ~ 500 mm) and more seasonal (SINAMBA (Sistema de Información Ambiental de Andalucía, 1997).

### Population sampling

Twenty-three populations were selected along the range of *N. papyraceus* in the region, including isoplethic, L-biased and L-monomorphic populations (Arroyo *et al.*, 2002; see Fig. 1 and Table 1). Populations of *N. papyraceus* are frequent only in lowlands, and thus elevation was not included as a component of spatial variation. In each population, 11–17 complete plants were randomly collected for vegetative measurements. A maximum of 100 flowers (one per plant, the first to open) per population were collected and kept in 70% ethanol for flower measurements. In small populations (< 100 plants) flowers were collected from all blooming plants. All

plants collected for vegetative traits were also used for flower measurements.

### Morphometric measurements

#### Flower morphometry

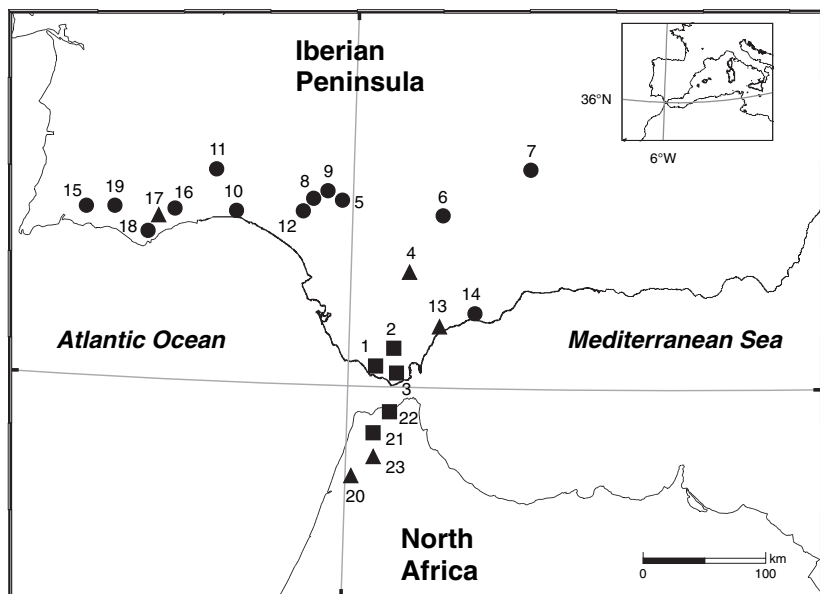
Six flower measurements were taken to the nearest 0.1 mm by digital calliper: flower width, outer tepal length, corona width and length, and flower tube width and length from the point of joining to the ovary (Fig. 2).

#### Vegetative morphometry

Ten vegetative traits were measured in each individual plant: bulb perimeter, number of bulbils (as a result of vegetative reproduction), leaf width and length, inflorescence sheath length, number of flower stalks, major and minor axes of stalk section at its middle height, spathe length, and number of flowers in the inflorescence. Inflorescence characteristics were considered as vegetative traits because they are more related to plant size than to putative direct pollinator selective pressures assumed in the study (see Armbruster *et al.*, 1999, for a similar treatment). All variables were recorded to the nearest 1 mm, except for leaf width and stalk section, which were to the nearest 0.1 mm.

### Population genetic data

To estimate genetic distances between populations we used an amplified fragment length polymorphism (AFLP) data set generated for phylogeographical purposes in *N. papyraceus* in the SG region (Rodríguez-Sánchez *et al.*, 2008). The data set consisted of 111 individuals scored for 287 dominant AFLP loci, with two to five individuals per population from a total of 26 populations, some coincident with populations analysed



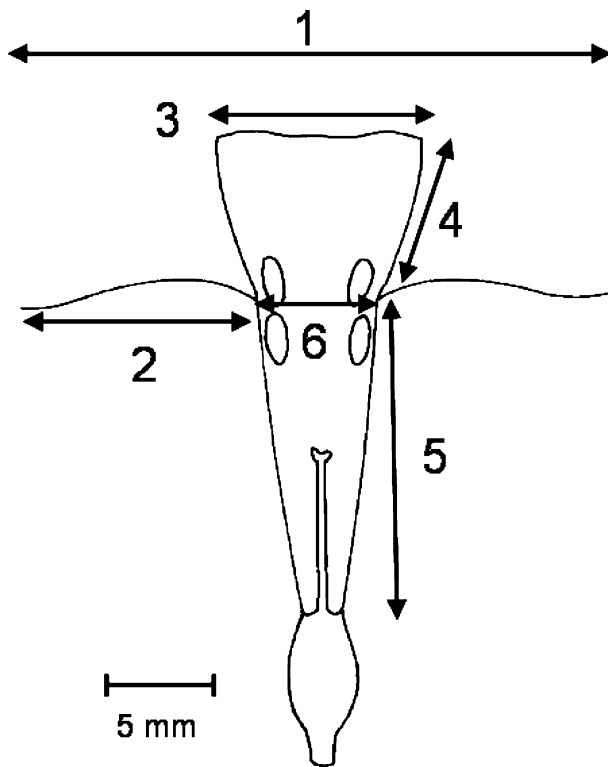
**Figure 1** Locations of the studied populations of *Narcissus papyraceus* in the region of the Strait of Gibraltar. Population numbers correspond to those in Table 1. Squares, isoplethic populations; triangles, anisoplethic populations; circles, L-monomorphic populations.

**Table 1** Populations of *Narcissus papyraceus* sampled for analysis of flower and vegetative morphology.

Population	Pop. no.	Coordinates	Elevation (m)	Approx. population size	Sample size (no. of flowers) for flower morphology	Sample size (no. of plants) for vegetative morphology	L morph (%)	Sample size (no. of plants) for genetic analysis
Spain: Cádiz, Tarifa, Bolonia*, †	1	36°7' N, 5°44' W	100	1,000,000	100	16	50	5
Spain: Cádiz, Los Barrios, Ahojiz-La Granja*, †	2	36°13' N, 5°35' W	100	250 000	100	16	50	5
Spain: Cádiz, Algeciras, El Palanca*	3	36°5' N, 5°33' W	400	5000	–	15	50	5
Spain: Cádiz, El Bosque*, †	4	36°46' N, 5°30' W	400	100	48	16	66	5
Spain: Sevilla, Aznalcázar†	5	37°16' N, 6°14' W	50	1000	98	–	100	5
Spain: Sevilla: Pruna–Algámitas*	6	37°1' N, 5°10' W	700	250	–	13	100	–
Spain: Córdoba, Carcabuey, Valdecañas*, †	7	37°28' N, 4°21' W	700	1000	100	14	100	4
Spain: Huelva, Hinojos, arroyo La Parrilla*, †	8	37°12' N, 6°26' W	50	1000	100	16	100	3
Spain: Huelva, Hinojos, El Caoso*, †	9	37°15' N, 6°23' W	50	100	24	16	100	–
Spain: Huelva, Cartaya, El Rompido*	10	37°13' N, 7°7' W	10	1000	–	16	98.6	–
Spain: Huelva, Villanueva de los Castillejos*, †	11	37°29' N, 7°15' W	150	1000	100	11	100	–
Spain: Huelva, Almonte, El Rocío†	12	37°9' N, 6°27' W	50	1000	100	–	98.5	–
Spain: Málaga, Casares–Mamilla*, †	13	36°26' N, 5°15' W	300	1000	100	–	87.4	2
Spain: Málaga, San Pedro de Alcántara†	14	36°30' N, 4°59' W	100	1000	68	–	100	–
Portugal: Algarve, Messines–Alte*, †	15	37°15' N, 8°15' W	300	1000	100	16	100	4
Portugal: Algarve, Tavira†	16	37°10' N, 7°39' W	100	500	100	–	100	–
Portugal: Algarve, Estoi, Barranco San Miguel†	17	37°6' N, 7°49' W	300	100	60	–	90.6	3
Portugal: Algarve, San Brass–Stia Catarina*	18	37°9' N, 7°49' W	300	100	–	17	100	5
Portugal: Algarve, Salir†	19	37°14' N, 8°1' W	300	1000	16	–	–	5
Morocco: Tetouan–Larache, Souk el Arba Ayacha†	20	35°25' N, 5°53' W	150	200	100	–	96.3	–
Morocco: Tanger–Tetouan, R'gaia†	21	35°41' N, 5°43' W	100	100	100	–	50	5
Morocco: Tanger–Tetouan, Oued Lediane*, †	22	35°50' N, 5°38' W	20	5000	100	16	50	–
Morocco: Tetouan–Larache, El Manzla-Dar Chaoui†	23	35°33' N, 5°42' W	300	–	8	–	–	5

\*Populations used for analysis of both vegetative and flower morphology.

†Populations used only for analysis of flower morphology (intensive sampling; see Materials and Methods). Morph ratios were obtained from Arroyo *et al.* (2002). Pop. no., population number.



**Figure 2** Schematic diagram of a flower of *Narcissus papyraceus*; for simplicity, only a short-styled (S) flower with two out of three stamens per whorl is shown. Flower measurements recorded in this study were: (1) flower width, (2) outer tepal length, (3) corona width, (4) corona length, (5) flower tube length and (6) flower tube width.

for morphometry. Genomic DNA was isolated from young silica gel-dried leaves following the cetyl trimethyl ammonium bromide (CTAB) protocol (Doyle & Doyle, 1987). AFLP development followed the protocol of Vos *et al.* (1995) with slight modifications (Schönswetter *et al.*, 2004). Two fluorescently labelled selective primer combinations were chosen: FAM-*EcoRI*-ACT/*MseI*-CGA and HEX-*EcoRI*-AAC/*MseI*-CAA. Amplified labelled products were analysed on an automated sequencer ABI 3700 (Applied Biosystems, Foster City, CA, USA), and fingerprint profiles consisting of band presence (1) or absence (0) were generated with GENOGRAPHER 1.1.6 (<http://hordeum.msu.montana.edu/genographer/>) for statistical analysis.

### Statistical analyses

#### *Differences between population means of morphological traits*

We performed a multivariate analysis of variance (MANOVA) and univariate analyses of variance (ANOVAs) on both intensive flower sampling (up to 100 flowers per population) and vegetative sampling (up to 17 plants per population), to detect between-population differences in all traits, and for each trait independently. Vegetative variables were log-transformed for

standardization, given the wide variation in their dimensions. Flower variables were not transformed because they were normally distributed and of comparable dimensions. Analyses were performed with STATISTICA 6.0 (StatSoft, 2001).

#### *Searching for morphometric spatial patterns*

*Relationship between geographic and morphometric distances.* We performed Mantel tests on correlations between geographic distances and both flower and vegetative Euclidean morphometric distances. To detect any possible effect of the SG we repeated the analyses excluding all North African populations.

*Partial correlations.* Flower variation and its geographic expression may be dependent on vegetative traits, for example as an effect of resource allocation. To estimate the influence of vegetative traits and geographic distance on flower morphology, we performed two partial Mantel correlations. We used a matrix of geographic distances (D) and two matrices of Euclidean distances corresponding to vegetative (independent matrix, X) and flower (dependent matrix, Y) traits. We computed (1) the correlation between flower and vegetative trait matrices while controlling for the effect of geographic distance, and then (2) the correlation between flower and geographic matrices while controlling for the effect of vegetative traits. These analyses were also performed excluding North African populations. A partial Mantel test is a Mantel test applied to residuals resulting from fitting two regression models with a control matrix as the independent variable (Fortin & Gurevitch, 2001). For analysis (1) we considered the residuals of the model with Y as the dependent matrix and D independent, and for analysis (2) we considered the residuals of the model with X as the dependent matrix and D independent. Similarly, we also applied partial Mantel tests to estimate the association between flower morphology (Euclidean distance between all pairs of populations) and difference in style-morph ratio (i.e. the absolute difference in the proportion of L plants) between populations, controlling for geographic distance between them. Mantel statistics and their significance levels were determined by Monte Carlo methods (10 000 permutations) with the library *ade4* (Thioulose *et al.*, 2004) of the R 2.5.0 statistical package (R Development Core Team, 2004).

*Moran's I coefficient of spatial autocorrelation.* The spatial pattern of style-morph ratio was explored with a wider population sampling in the SG region (66 populations included in the study by Arroyo *et al.*, 2002; plus five additional populations; localities can be obtained from J.A. on request), and thus a conventional distance-based autocorrelation analysis was performed with PASSAGE ver. 1.1 (Rosenberg, 2004). We constructed 10 distance classes of unequal length, setting constant the number of population pairs being correlated. We constructed an autocorrelogram for every flower and vegetative trait, and selected a neighbourhood rather than a distance approach to avoid the low number of correlations typical with long distance classes. In this case,

spatial classes were defined by progressive levels of neighbourhood (where  $k$  is the rank order of closest neighbours). The analyses were repeated excluding North African populations. We show only two averaged correlograms, one for flower and one for vegetative traits, respectively, with box and whisker plots for each  $k$ , representing the median, quartiles 1 (25%) and 3 (75%), and maximum and minimum values of Moran's  $I$  for each  $k$ -value. Moran's  $I$  coefficients of spatial autocorrelation based on neighbourhood were calculated with the *moran.test* function of the library *spdep* (Biv *et al.*, 2004) of the R package. Significance levels, achieved by Monte Carlo methods, were supplied by the function *moran.ms* from the same library (999 simulations, default value).

#### *Within-population variability*

The relationship between variability in flower and vegetative traits and several ecogeographic variables was analysed by univariate and multivariate linear multiple regressions. The dependent variables were coefficients of variation (CVs) of traits (flower or vegetative) in each population, and independent variables were geographic distance to population no. 1 (Table 1), population size, and the number of populations within a radius of 50 km. These independent variables were selected to account for the distance to the largest isoplethic population, which is very close to the SG (see Table 1 and Fig. 1), stochastic population processes, and isolation, all of them supposedly related to within-population variability (Arroyo *et al.*, 2002). We performed single multiple regressions for each flower and vegetative trait, and multivariate multiple regressions for all flower and vegetative traits, respectively. We used the General Linear Model module of the SPSS ver. 12 statistical software (SPSS Inc., 2003) for model fitting by multiple regressions.

#### *Spatial genetic structure and correlation with morphometric distance*

Owing to the dominant nature of the AFLP markers, allele frequencies for each population cannot be computed directly. To estimate the allele frequencies we used the Bayesian approach of Zhivotovsky (1999), assuming non-uniform prior information among populations. Genetic differentiation ( $F_{ST}$ ) values between populations were calculated from allele frequencies assuming Hardy–Weinberg equilibrium within populations (i.e. assuming an inbreeding coefficient  $F_{IS} = 0$ ), supported by the obligate outcrossing nature of the species (Arroyo *et al.*, 2002). To test for the presence of a pattern of isolation by distance in *N. papyraceus* in the SG region we performed a Mantel test correlating the  $F_{ST}$  values and geographic distances between 26 populations with available data from Rodríguez-Sánchez *et al.* (2008). In addition, we selected the 11 populations that have joint morphometric and genetic information (see Table 1), and used them to explore the existence of relationships between morphometric (Euclidean) and genetic data through Mantel tests. Signifi-

cance levels were assessed by permutation procedures, setting 1000 replicates in each test. The pairwise  $F_{ST}$  matrix was computed with AFLP-SURV ver. 1.0 (Vekemans, 2002), and Mantel tests were performed with PASSAGE ver. 1.1 (Rosenberg, 2004).

## RESULTS

### Differences between population means

MANOVAS for all flower and vegetative traits showed significantly different mean values between populations (flower traits: Wilk's lambda = 0.228,  $P < 0.0001$ ; vegetative traits: Wilk's lambda = 0.032,  $P < 0.0001$ ). Independent ANOVAS for single traits showed significant differences between populations for all flower and vegetative traits (results not shown), thus providing a basis for the spatial exploration of each trait throughout the range.

### Spatial patterns of morphological variation

Correlations between flower morphometric and geographic distances between populations were not significant (Mantel test:  $r = 0.009$ ,  $P = 0.459$ ). However, vegetative morphometric distance showed a nearly significant geographic structure ( $r = 0.127$ ,  $P = 0.079$ ). When we excluded North African populations, flower traits showed some geographic structure ( $r = 0.161$ ,  $P = 0.063$ ), and this was clearly significant for vegetative traits ( $r = 0.198$ ,  $P = 0.031$ ), despite a lower number of populations. In all cases, morphometric distance increased with geographic distance.

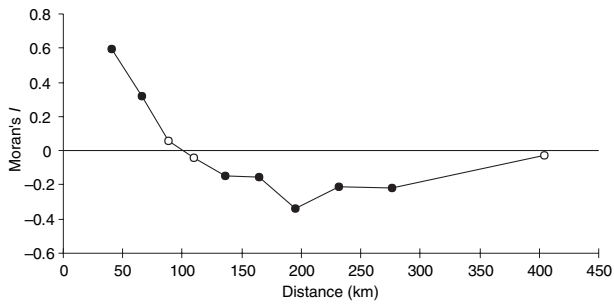
Multivariate flower morphology did not depend on vegetative traits when controlled for the effect of the geographic location of populations ( $r = 0.024$ ,  $P = 0.408$ ). Similarly, flower morphology was not related to geographic distance when controlled for vegetative morphology ( $r = 0.120$ ,  $P = 0.106$ ). When North African populations were excluded, there was no effect of vegetative morphology when controlled for geographic distance or vice versa ( $r = 0.031$ ,  $P = 0.388$ ;  $r = 0.058$ ,  $P = 0.308$ , respectively). Consequently, overall differences in flower morphology between populations were not dependent on either vegetative differences or distance between populations.

The relationship between perianth morphology (Euclidean distances) and morph ratio was also non-significant ( $r = 0.055$ ,  $P = 0.693$ ) when controlled for geographic distance.

### Spatial autocorrelation of morphology

Morph-ratio variation showed a clear typical distance-based pattern of decreasing similarity with increasing distance (Fig. 3). Neighbouring populations had similar morph ratios (i.e. were positively correlated), but at larger distances the correlations were negative.

Spatial autocorrelations of perianth traits at different neighbourhood classes tended to be higher for higher ranks



**Figure 3** Spatial autocorrelogram of Moran's  $I$  coefficients for style-morph ratio in *Narcissus papyraceus* populations along a distance axis. Distance classes were selected to maintain a constant number of population pairs being compared for morph ratio (see Materials and Methods for details). Filled symbols, coefficients significantly different from zero; open symbols, non-significant coefficients.

of neighbours (i.e. longer distances). This pattern was reinforced when North African populations were excluded from the analysis (12 vs. 27 significant coefficients; see Appendix S1 in Supporting Information). Thus, for Iberian populations the number of significant Moran's  $I$  coefficients for low-rank neighbours (i.e. short distances,  $k \leq 6$ ) was 3, whereas for high-rank neighbours (i.e. long distances,  $k \geq 7$ ) it was 24 (Fig. 4 and see Appendix S1).

The trend was opposite for vegetative traits. There were 32 significant Moran's  $I$  coefficients for the whole set of populations, and 23 when African populations were excluded (see

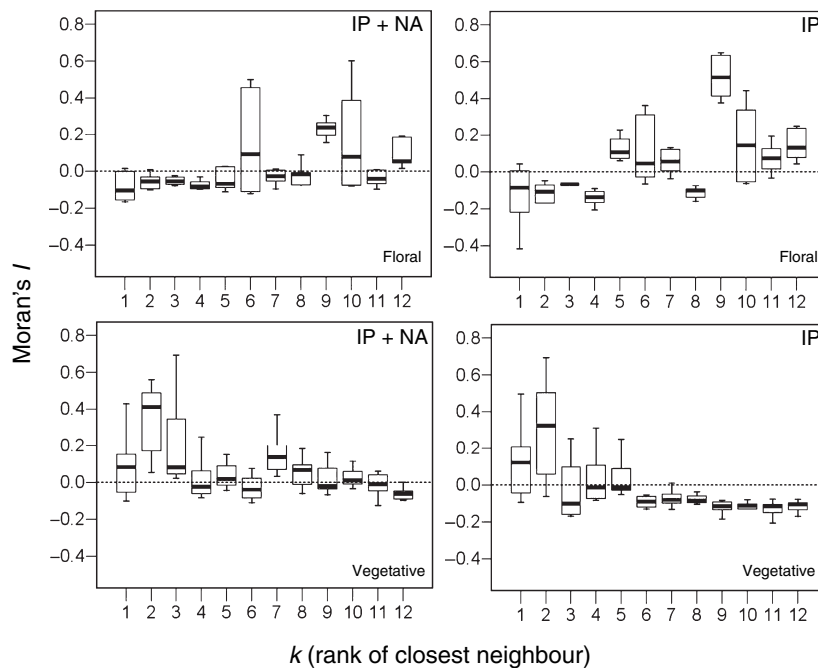
Appendix S1). The number of significant positive Moran's  $I$  coefficients was higher for low-rank neighbours ( $k \leq 6$ , 23 for the whole set of populations and 19 for Iberian populations only) than for high-rank neighbours ( $k \geq 7$ , 9 for all populations and 4 for Iberian populations) (Fig. 4 and see Appendix S1).

### Within-population variability

Populations had high variability for all morphological traits, either floral (average CV = 13–22% across populations) or vegetative (CV = 16–28%). The multivariate multiple regression models fitted for the CV of both flower and vegetative traits did not detect any significant relationship with any of the independent variables considered (Table 2). When traits were considered independently, only the CV of flower tube width was significant ( $F_{1,13} = 7.747$ ,  $P = 0.003$ ). In particular, this relationship was the result of the negative correlation between flower tube width variability and distance to population no. 1. Flower tube width was more variable in populations near the SG (isoplethic) than in peripheral populations (L-monomorphic).

### Spatial genetic structure and correlation with morphometric distance

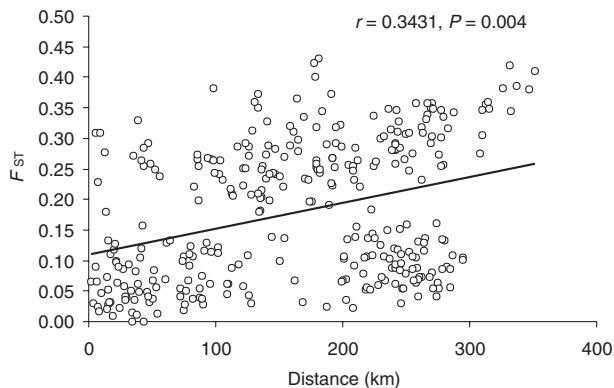
Genetic distance was positively and significantly correlated with geographic distance ( $r = 0.343$ ,  $P = 0.004$ ,  $n = 26$  populations; Fig. 5), fitting to an isolation-by-distance model. However, for the subset of populations in which both genetic



**Figure 4** Averaged spatial autocorrelograms (Moran's  $I$  coefficient) for flower and vegetative population mean traits of *Narcissus papyraceus* along a neighbourhood axis ( $k$ , rank order of neighbour populations). Analyses are carried out with the whole data set of populations (Iberian Peninsula and North Africa, IP+NA) and also excluding North African populations (IP). Box and whiskers include median, 25% and 75% quartiles, and maximum/minimum values of  $I$  coefficients for each  $k$  class from averaged autocorrelograms of traits.

**Table 2** Results of multivariate multiple regression models of three independent variables, related to isolation and morph-ratio gradient, on the within-population variability (coefficient of variation) of the ensemble of flower and vegetative traits in *Narcissus papyraceus*.

Flower traits	Lambda	$F_{6,8}$	$P$
Intercept	0.069	18.015	0.000
Distance to population no. 1	0.600	0.890	0.543
Population size	0.858	0.221	0.959
No. of populations in < 50 km	0.813	0.306	0.917
Vegetative traits	Lambda	$F_{12,1}$	$P$
Intercept	0.036	2.217	0.485
Distance to population no. 1	0.147	0.485	0.824
Population size	0.130	0.559	0.794
No. of populations in < 50 km	0.209	0.315	0.900



**Figure 5** Scatterplot of genetic differentiation ( $F_{ST}$ ) pairwise values against geographic distance for 26 populations of *Narcissus papyraceus*. Some data points are obscured owing to overlap.

and flower trait data were available, correlation between genetic distances and flower morphometric distances was not significant ( $r = -0.187$ ,  $P = 0.91$ ,  $n = 11$ ). The correlation between genetic distances and vegetative morphometric distances was positive but not significant ( $r = 0.189$ ,  $P = 0.097$ ,  $n = 11$ ). Unfortunately, the small population number available for joint genetic and morphometric data precluded partial Mantel and spatial autocorrelation analyses.

## DISCUSSION

The present study describes different morphological patterns of variation in flower and vegetative traits in *N. papyraceus* in one of the 25 major world biodiversity hotspots, the Mediterranean basin (Myers *et al.*, 2000). Within this region, the SG is an important centre for both species richness and ecological and evolutionary processes (Rodríguez-Sánchez *et al.*, 2008). Pérez-Barrales *et al.* (2007) found that L : S dimorphic and L-monomorphic populations differ in: (1) the array of

pollinators (long-tongued in L : S and short-tongued in L), (2) the population mean values of some flower traits directly related to pollinator activity, and (3) the patterns of phenotypic correlation among flower traits (i.e. integration). Therefore, pollinators seem to play a role in the flower phenotypic differentiation in the study region. In our study we show that differences in population means in both flower and vegetative traits present some contrasted spatial structuring, which in part supports this role of pollinators.

## Spatial patterns of variation

### Style-morph ratios

We confirmed previous reports on variation in the style-morph ratio of *N. papyraceus* (Arroyo *et al.*, 2002), with a larger population data set ( $n = 71$ ). The autocorrelograms depicted the distances at which similarity occurred. The morph ratio tended to be similar between populations, being either L-monomorphic, L-biased or isoplethic (L : S), at distances < 60 km. Clinal and disruptive morph ratios have been shown in other style polymorphic species, related to either stochastic processes (Eckert & Barrett, 1992, 1995, 1996; Agren & Ericson, 1996; Endels *et al.*, 2002; Kéry *et al.*, 2003) or morph-specific differences in pollination biology (Arroyo & Dafni, 1995; Baker *et al.*, 2000; Barrett *et al.*, 2004; Hodgings & Barrett, 2008).

### Flower (perianth) and vegetative traits

Although morphometric sampling was lower ( $n = 26$  populations) than morph-ratio sampling, those sampled populations were selected to cover the morph-ratio gradient over the SG range. The various methods used to detect spatial structure in morphometric trends showed that excluding North African populations strengthened the spatial patterns for flower traits, despite diminishing sample size. The SG probably represents some subtle change between otherwise nearby populations that reduces the Mantel correlation and the autocorrelation values. In contrast to the case for flower traits, correlations in vegetative traits decreased when North African populations were removed from the analyses, as would be expected under the influence of similar abiotic conditions on both shores of the SG (Ajbilou *et al.*, 2006). These opposite patterns may indicate different ecological factors operating on either side of the SG, influencing flower but not vegetative traits.

Another major finding of this study is that the geographic variation of flower traits is independent of variation in vegetative traits, as partial Mantel correlation analyses showed. This is probably related to the fact that these sets of traits belong to different plant modules, which are subjected to different environmental factors and selective agents (Berg, 1960; Pigliucci *et al.*, 1991; Armbruster *et al.*, 1999; Pérez-Barrales *et al.*, 2007), and are probably governed by unrelated gene complexes. Although vegetative trait patterns were not the main focus of this study, we can surmise that (1) the

positive Mantel correlation between morphometric vegetative distance and geographic distance, and (2) the higher positive autocorrelation with close neighbours are likely to be the direct consequence of simple environmental abiotic gradients. Edaphic conditions typical to *N. papyraceus* are very homogeneous across its range (clay–marl soils; Valdés, 1987), whereas climate has a clear gradient both in rainfall and temperature, from mild and humid conditions close to the SG, to colder and drier inland (SINAMBA (Sistema de Información Ambiental de Andalucía), 1997).

It is noteworthy that Mantel correlations and autocorrelation analyses produced somewhat different results for flower traits. The latter showed positive correlation at long distances, but the former showed no overall effect of geographic distance. These results could be the effect of, first, a less powerful analysis of Mantel correlations, and, second, different mean values for certain traits measured in isoplethic and L-monomorphic populations, which are located at the edges of the morph-ratio gradient (tepal length, corona width, and flower tube length and width; see Pérez-Barrales *et al.*, 2007). These traits were more similar in L-monomorphic populations, some of which were among the most distant of the SG range. These similarities increased the average of autocorrelations at long distances (Fig. 4). In a multivariate measure of flower similarities (Euclidean distances), as used in Mantel correlations, this pattern was apparently lost.

Our results do not strongly support directional selection on the overall mean of the flower phenotypes parallel to the geographical cline of the style-morph ratio (Figs 3 and 4). Instead, traits that specifically interact with pollinators (tube length and width, or corona width) and their correlation structure (phenotypic integration) in some populations at the extremes of the morph-ratio gradient (Pérez-Barrales *et al.*, 2007) seem to respond to directional and correlational selection (Endler, 1986), respectively. Studies on specialized plant–pollinator systems frequently report changes in mean values of flower traits involved in pollination, which have been interpreted as directional selection effects (Miller, 1981; Nilsson, 1988; Robertson & Wyatt, 1990; Johnson, 1997; Boyd, 2002; Silva-Montellano & Eguiarte, 2003), although not in other study cases (Herrera, 1990, 1996, 2005; Molina-Freaner *et al.*, 2003). An alternative explanation for a plastic response to local abiotic conditions is not plausible because variation in flower and vegetative traits are not coupled (cf. Herrera, 2005). In the particular case of style polymorphic species, correspondence between pollinator types, morph ratio and variation in perianth traits has rarely been addressed, and the results are mixed, both in favour of a pollinator-mediated effect on perianth variation (Barrett *et al.*, 2004; Pérez-Barrales *et al.*, 2007), and against it (Arroyo & Dafni, 1995).

### Migration and colonization effects

Alternatively, the geographic pattern of trait variation could be the result of migration and gene flow throughout the study region. This could be expected, since there was a positive

relationship between genetic and geographic distance (Fig. 5), according to a pattern of isolation by distance in populations of the study region (Hutchison & Templeton, 1999). For instance, *Calicotome villosa*, a species with a distribution across the SG and habitat similar to those of *N. papyraceus*, has a similar pattern of isolation by distance (Arroyo *et al.*, 2008). According to this pattern, the phenotypic similarity should match the genetic and geographic similarity of populations. This was not found for flower traits and is evidence against the migration hypothesis of flower phenotypic similarity. The lack of correlation could also be the result of stochastic processes (drift and founder events), which would lead to the random fixation of some population means of flower phenotypes (Mayr, 1942, 1963). The available genetic data set does not permit a powerful test to detect past bottleneck effects (e.g. Cornuet & Luikart, 1997) owing to both a small sample size and the dominant nature of AFLP markers. Besides, indirect evidence suggests a negligible effect on flower traits. Isolation and population size, typical variables used to test stochastic bottlenecks (Mayr, 1963), did not explain the within-population morphological variability. Surprisingly, flower variation was as high as vegetative variation, and flower tube width had a significantly higher CV in dimorphic populations, which were larger and more connected and thus less prone to stochastic processes. This higher CV is better explained by previously reported different mean values between style morphs within populations (Pérez-Barrales *et al.*, 2007).

As we used nuclear markers, the genetic similarity of neighbouring populations could be the result of either seed flow, required for colonization, or pollen flow. However, as long as seed and pollen dispersal distances across the region seem to be poor (C. Abarca, R. Pérez-Barrales & J. Arroyo, unpublished data), contemporary gene flow between populations is expected to be scarce, and genetic similarity should reflect historically steady migration processes (Hutchison & Templeton, 1999).

### CONCLUSIONS

The main results of the present study were the high phenotypic variation of vegetative and flower traits, both within and between *N. papyraceus* populations, and that the patterns of variation of these two trait types were not coupled. The phenotypic variation of the perianth was not clearly parallel to the morph-ratio cline or to the genetic relatedness of populations. All these results indicate a limited developmental side effect of vegetative traits and of genetic relatedness on flower phenotypes. The latter was proposed as one explanation for the morph-ratio gradient (Arroyo *et al.*, 2002). However, the main hypothesis posed in this study, that pollinators directionally affect variation in perianth population means, had mixed support when considering the flower phenotype as a whole, in contrast with previous reported effects of pollinators on means of particular flower traits (Pérez-Barrales *et al.*, 2007). Despite the lack of correlation between morph ratio and overall mean flower phenotype, the lack of coupling

between flower and vegetative traits and genetic relatedness makes it reasonable to argue for a role of selective agents (e.g. pollinators) on the whole flower phenotype of at least some populations. An unexpected result was the high within-population variability of flower traits, nearly as high as for vegetative traits, which leaves an open door to explore how this flower variation originated and is maintained, and its possible adaptive significance. For instance, it could result from past hybridization events and/or polyploidy processes, which have been frequent in *Narcissus* species (Fernandes, 1975; Díaz-Lifante & Andrés-Camacho, 2007; Marques *et al.*, 2007). Whether these diverse flower phenotypes have any selective advantage remains to be explored.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Appendix S1** Values of Moran's *I* coefficients for each flower and vegetative trait along classes of neighbourhood rank (*k*, rank of closest neighbour), considering the whole study area (Iberian Peninsula and North Africa, IP + NA) and also excluding North African populations (IP).

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## BIOSKETCH

**Rocío Pérez-Barrales** has a Postdoctoral Fellowship from the Spanish Ministerio de Educación y Ciencia (MEC) at the University of Portsmouth (UK), and was formerly at the University of Seville (Spain), where she completed her PhD on the evolution of style polymorphisms in *Narcissus* spp. She is interested in the evolutionary dynamics of sex polymorphisms in plants and plant–pollinator interactions.

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