

## RESEARCH PAPER

# Herkogamy and mate diversity in the wild daffodil *Narcissus longispathus*: beyond the selfing–outcrossing paradigm in the evolution of mixed mating

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## Keywords

Amaryllidaceae; anther–stigma separation; correlated paternity; mixed-mating system; multiple paternity; outcrossing rate; style length.

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## ABSTRACT

Spatial separation of male and female reproductive structures (herkogamy) is a widespread floral trait that has traditionally been viewed as an adaptation that reduces the likelihood of self-pollination. Here we propose that increased herkogamy may also influence another important aspect of plant mating: the diversity of pollen donors siring seeds within fruits. We test this hypothesis in *Narcissus longispathus*, a wild daffodil species with extensive variation in anther–stigma separation. To study the morphological basis of variation in herkogamy, floral measurements were undertaken in 16 populations of *N. longispathus*. We then quantified multilocus outcrossing rates and the correlation of outcrossed paternity in three of these populations sampled over several years. Mating system estimates were calculated for each population and year, and also separately for groups of plants that differed markedly in herkogamy within each population and year. In *N. longispathus* herkogamy was much more variable than other floral traits, and was more closely related to style length than to anther position. Averaged across populations and years, plants with high herkogamy had similar outcrossing rates (0.683) to plants with intermediate (0.648) or low herkogamy (0.590). However, a significant linear trend was found for correlation of outcrossed paternity, which increased monotonically from high herkogamy (0.221), through intermediate herkogamy (0.303) to low herkogamy (0.463) plants. The diversity of pollen donors siring seeds of high herkogamy *Narcissus* flowers was thus consistently greater than the diversity of pollen donors siring seeds of low herkogamy flowers. Results of this study contribute to the emerging consensus that floral traits can simultaneously influence several aspects of plant mating system in complex ways, thus extending the traditional focus centred exclusively on patterns and relative importance of self- and cross-fertilisation.

## INTRODUCTION

Since the pioneering work of Darwin (1862, 1876, 1877), evolutionary biologists have been fascinated by variation in floral traits and the contributions of floral design to mating patterns in flowering plant populations (Barrett 2003; Harder & Barrett 2006). One common trait of hermaphroditic flowers is the spatial separation of anthers and stigma (herkogamy), which is often interpreted as an adaptation reducing the likelihood of self-pollination (Webb & Lloyd 1986). This hypothesis has been supported by empirical studies that have found that lower selfing rates are often associated with increased herkogamy (Holtsford & Ellstrand 1992; Belaussoff & Shore 1995; Karron *et al.* 1997; Brunet & Eckert 1998; Motten & Stone 2000; Takebayashi *et al.* 2006; Herlihy & Eckert 2007), although exceptions to this functional expectation have also been reported (*e.g.*, Eckert & Barrett 1994; Medrano *et al.* 2005; Brunet & Sweet 2006). Much less attention has been

paid, however, to the possible ways whereby herkogamy could influence other mating patterns of hermaphroditic flowering plants. Webb & Lloyd (1986) proposed that herkogamy might promote more effective pollen export and receipt because of reduced interference between maternal and paternal functions. For example, herkogamy may enhance pollen export (*e.g.*, Fetscher 2001; Routley & Husband 2006; Quesada-Aguilar *et al.* 2008) in situations where it is mainly correlated with variation in stamen position (Harder & Barrett 1993; Kudo 2003; Conner *et al.* 2009). When variation in herkogamy is mainly due to differences in style length, however, higher levels of outcross pollen receipt could arise if accessibility of the stigma to pollinators is enhanced in the most herkogamous flowers (Herlihy & Eckert 2007; Forrest *et al.* 2011).

In situations where more herkogamous flowers capture proportionally more outcross pollen, there will also be an increased likelihood of microgametophyte competition, maternal filtering of male gametes, or both. Any of these

mechanisms may give rise to a direct association between herkogamy, on the one hand, and heterogeneity among pollen donors in seed siring success and/or pollen donor diversity within a maternal sibship, on the other (Mitchell 1997a,b; Shaner & Marshall 2003, 2007; Armbruster & Rogers 2004; Bernasconi *et al.* 2004; Ruane 2009). These effects are expected to be stronger when variation in herkogamy is mainly a consequence of variation in style length, since the length of the style may be an important component of the competitive environment faced by growing pollen tubes (*e.g.*, Nishihira *et al.* 2000; Lankinen & Skogsmyr 2001; Bernasconi *et al.* 2007). The hypothesis that herkogamy is related to mate diversity, however, remains relatively unexplored (but see Takebayashi *et al.* 2006), despite the fact that mating with multiple males can have important consequences for both maternal and offspring fitness (reviewed in Mitchell *et al.* 2005; Teixeira & Bernasconi 2007). For example, multiple paternity may enhance the phenotypic diversity of offspring in a clutch (Falconer 1981) and may lower the likelihood that all of the progeny in a fruit would have deleterious genetic effects. In addition, there have been suggestions that decreased genetic relatedness among siblings arising from increased number of mates may alleviate competitive interactions among developing seeds within fruits or among seedlings in the field (Karron & Marshall 1993; Bernasconi *et al.* 2004; Mitchell *et al.* 2005).

The main objective of this paper is to investigate whether herkogamy influences the diversity of pollen donors siring seeds in the wild daffodil *Narcissus longispathus* (Amaryllidaceae). This species is especially appropriate for this study because it is characterised by considerable, continuous variation in herkogamy (range = 0.5–10.4 mm; Medrano *et al.* 2005), and in almost all flowers the style is positioned above stamens (approach herkogamy). In addition, most plants only produce a single flower, so nearly all self-fertilisation results from within-flower pollen movement. Although these characteristics would suggest that herkogamy would be likely to play an important role in selfing avoidance, our earlier work failed to demonstrate a monotonic increase in outcrossing with increasing anther–stigma separation (Medrano *et al.* 2005). However, our 2005 study did not explore whether herkogamy influenced other aspects of the mating system. Here, we test the prediction that a greater diversity of mates will sire seeds of flowers with increased herkogamy. This would be especially likely if variation in herkogamy is explained primarily by variation in style length. Our study quantifies the relative degree of phenotypic variation in herkogamy in comparison with other floral traits across a broad sample of natural populations, and examines co-variation between herkogamy, style length and anther position to determine the morphological basis of herkogamy variation in *N. longispathus*. We then compare mate diversity and outcrossing rates for *N. longispathus* plants differing markedly in anther–stigma separation in three of these populations sampled over multiple years.

## MATERIAL AND METHODS

### Study species

*Narcissus longispathus* Pugsley (Amaryllidaceae) is a perennial herb restricted to a few mountain ranges in southeastern

Spain (Navarro 2009). This endemic large-flowered daffodil is a strict habitat specialist of poorly drained, deep soils around springs and permanent stream banks. The species is self-compatible, and has a mixed-mating system with intermediate to high levels of outcrossing (Barrett *et al.* 2004; Medrano *et al.* 2005). Flowering takes place in late winter–early spring, a period in which pollinator activity is frequently reduced. Flowers are primarily visited by small bees, especially *Andrena bicolor* (Herrera 1995), and occasionally by large bees such as *Bombus pratorum* queens and *Xylocopa violacea*.

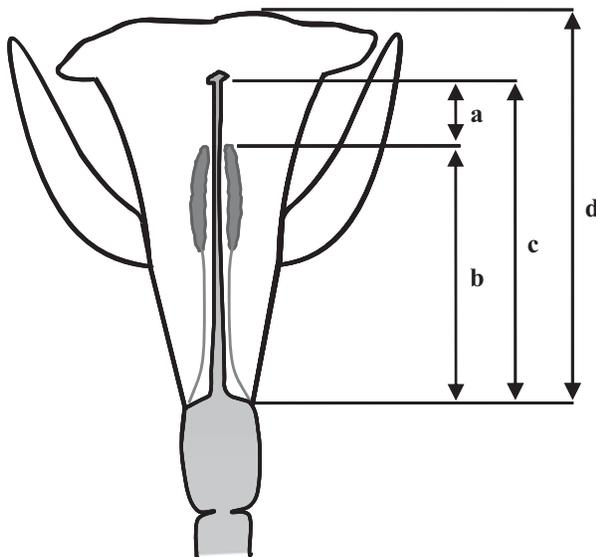
Despite low pollinator visitation rates, most flowers of *N. longispathus* are pollinated and fruit and seed production is only weakly pollen-limited in most populations and years (mean fruit set = 80.0% and 88.3% for flowers from natural and additional pollination, respectively; Herrera 1995; Table 1). Blooming plants generally produce a single hermaphrodite flower per inflorescence, with a greenish floral tube and large funnel-like yellow corona (see Fig. 1A in Herrera 2011). There is a conspicuous lack of dichogamy in this species, as stigma receptivity and anther dehiscence start simultaneously. At flower bud opening, the free part of the tepals curls backwards and the corona expands, leaving the style and stigma exposed to pollinators. In <1 h afterwards, all anthers begin to release pollen simultaneously, turning back and downwards towards the wall of the locule of each anther sac (M. Medrano, unpublished observations). The six anthers are presented in a single whorl around the style (see Fig. 1), and in the vast majority of flowers are positioned below the stigma (>99.5% of the *N. longispathus* flowers sampled for this study displayed positive herkogamy, so-called ‘approach herkogamy’). Although *N. longispathus* flowers may last up to 2–3 weeks, depending on weather conditions, anther–stigma separation does not change over the floral lifespan (see Medrano *et al.* 2005). The mean number of ovules per flower is 66.4 (SD = 16.4; range = 25–126; n = 333 flowers), and individual fruits contain 30 seeds on average (SD = 16.6; range = 1–95; n = 388 fruits), each between 6–9 mg. Fruit maturation and seed shedding take place in late May–early June. Seeds lack mechanisms for long-distance dispersal.

### Phenotypic variation in floral traits

To quantify natural levels of variation in herkogamy, and to assess the relative importance of variation in style length and stamen position as sources of herkogamy variation, flowers from 16 populations of *N. longispathus* located at the core of the species’ distribution range in the Sierra de Cazorla Mountains (Jaén province, southeastern Spain) were collected and measured in 2004 and 2005. Average distance between populations was 10.3 km, and they encompassed the whole range of elevations and population sizes for the species (see Medrano & Herrera 2008; for additional details on these populations). Within each population, flowers sampled for measurement were taken from widely spaced individuals and were at the same developmental stage (named ‘recently open’ hereafter), namely corolla with fully expanded corona and pollen sacs dehiscing, to rule out any possible effect of flower age-related variation in anther–stigma separation (see Medrano *et al.* 2005, however, for quantitative data showing that herkogamy remains constant along the lifespan of individual

**Table 1.** Variation in herkogamy, stamen height, style length and perianth length among the 16 populations of *Narcissus longispatus* studied in 2004 and 2005. The number of flowers measured per population (n), the mean, standard error (SE) and range (in mm) for each floral trait are presented. Population codes are the same used in Medrano & Herrera (2008), where additional information can be found. The three underlined population codes (CAB, CBE and GTN) denote populations included in the study of mating system parameters (referred to in the text as Cabrilla, Cuevas Bermejas, and Guadalupe, respectively).

year	population	n	herkogamy		stamen height		style length		perianth length	
			mean (SE)	range	mean (SE)	range	mean (SE)	range	mean (SE)	range
2004	<u>CAB</u>	35	4.7 (0.3)	1.4–9.3	28.0 (0.4)	23.3–34.3	33.4 (0.4)	33.4–39.1	39.7 (0.6)	32.7–47.0
	CAN	25	4.0 (0.4)	–0.9–7.3	28.9 (0.3)	26.1–32.7	33.7 (0.4)	33.6–37.5	41.0 (0.6)	36.6–45.9
	<u>CBE</u>	58	4.5 (0.3)	–1.5–9.2	28.4 (0.3)	23.2–33.0	33.8 (0.3)	33.8–39.2	40.4 (0.5)	31.4–47.7
	<u>GTN</u>	36	4.9 (0.2)	2.8–8.1	27.3 (0.3)	23.9–32.1	32.9 (0.4)	32.9–38.7	40.0 (0.5)	33.1–45.7
	NSP	31	5.9 (0.4)	0.1–13.6	26.4 (0.4)	21.1–30.1	32.9 (0.6)	32.9–42.0	38.7 (0.6)	31.8–45.5
	PER	30	3.8 (0.4)	0.4–7.3	29.5 (0.5)	24.2–33.7	33.8 (0.6)	33.8–39.7	41.0 (0.7)	31.4–49.7
	REI	35	5.1 (0.3)	2.3–9.8	28.4 (0.3)	24.4–33.7	34.2 (0.4)	34.2–38.8	40.8 (0.6)	33.5–50.6
	TGA	32	3.7 (0.3)	0.0–7.3	27.7 (0.4)	23.1–32.1	32.3 (0.4)	32.3–38.8	42.2 (0.6)	36.8–55.5
	VTF	33	6.0 (0.3)	1.7–9.2	26.0 (0.3)	22.7–30.9	32.7 (0.4)	32.7–38.3	40.2 (0.6)	33.4–46.6
	VTP	36	6.8 (0.2)	2.9–10.2	27.4 (0.3)	23.7–32.5	34.8 (0.4)	34.8–39.4	41.7 (0.4)	35.8–47.8
2005	CEN	35	4.5 (0.3)	0.2–8.2	28.3 (0.4)	20.4–32.5	33.4 (0.4)	33.4–37.2	41.2 (0.5)	32.5–48.1
	CLA	37	6.0 (0.3)	2.0–9.9	29.6 (0.3)	26.1–33.4	36.5 (0.4)	36.5–42.8	44.8 (0.7)	37.0–52.9
	HOR	34	5.6 (0.2)	3.5–7.9	26.9 (0.3)	24.1–30.1	33.2 (0.4)	33.2–37.8	40.4 (0.5)	33.9–48.2
	MCA	30	3.5 (0.3)	–1.4–8.0	28.7 (0.5)	24.2–35.6	32.9 (0.4)	32.9–37.5	41.6 (0.5)	36.9–48.6
	VAL	32	4.7 (0.3)	0.2–7.4	30.8 (0.4)	26.5–35.6	36.5 (0.4)	36.5–41.3	46.0 (0.6)	40.2–55.7
	VCU	30	5.6 (0.3)	1.8–10.2	27.8 (0.4)	24.4–31.7	34.2 (0.5)	34.2–40.4	42.7 (0.6)	37.9–50.4



**Fig. 1.** Schematic representation of a *Narcissus longispatus* flower (longitudinal view) showing the four floral traits measured: (a) herkogamy, minimum distance on a straight line between anthers and the rim of the stigma; (b) stamen height, maximum height reached by the highest anther, measured from the base of its filament; (c) style length, the distance between its base and the stigma; (d) perianth length, excluding the ovary and the pedicel. Note that only two of the six anthers of the flower are depicted.

flowers). Collected flowers were kept refrigerated in a portable cooler until returned to the laboratory.

Floral measurements were performed in a total of 549 flowers (all populations and years combined; mean  $\pm$  SD =  $34.3 \pm 7.03$ ; range = 25–58; Table 1). Accurate measurement of style length and stamen height required destructive sampling, since the base of the style and staminal filaments are deeply hidden inside the corolla. Flowers were split open

longitudinally, mounted on a paper sheet with a scale, and digitally photographed. Digital images were calibrated and analysed using SIGMA SCAN PRO (version 5.0; Systat Software Inc., San José, CA, USA). Four measurements were taken on each flower to the nearest 0.05 mm (Fig. 1): (i) minimum distance between anthers and the rim of the stigma ('herkogamy' hereafter); (ii) distance between the base of the style and the stigma ('style length'); (iii) maximum height reached by the highest anther from the base of its filament ('stamen height'); and (iv) total flower length ('perianth length'), which was used as a general index of flower size. All measurements were taken by the same person.

Levels of variability exhibited by the four floral traits considered were compared using a modification of Levene's median ratio test (Schultz 1985). Individual values for a floral measurement are first expressed as absolute relative deviations from the corresponding population median, and the population means of deviations for the different traits are then compared. Specifically, we compared the variability of herkogamy to that of style length, stamen height and perianth length using three separate paired-sample *t*-tests using the SAS TTEST procedure (SAS Institute 2005). Within- and among-population variance components of each floral trait were estimated using restricted maximum-likelihood, as implemented in the SAS MIXED Procedure (SAS Institute 2005). The relative importance of variation in style length, stamen height and perianth length as sources of herkogamy variation was evaluated by comparing the standard partial regression coefficients obtained from a multiple regression analysis with herkogamy as a dependent variable and the other three floral traits as predictors.

#### Herkogamy and mating system parameters

The relationship between herkogamy and mating system parameters was studied in three of the 16 populations for which quantitative information on herkogamy and style

length was available, hereafter named Cuevas Bermejas, Guadalentín and Cabrilla. Cuevas Bermejas was sampled in 2003 and 2005; Guadalentín was sampled in 2004, 2005 and 2006; and Cabrilla was sampled in 2005 and 2006. We randomly selected and tagged a total of 510 'recently open' (as defined earlier) flowers, all populations and years combined. Sample sizes in Cuevas Bermejas were 45 flowers in 2003 and 120 flowers in 2005. Sample sizes in Guadalentín were 55 flowers in 2004, 100 flowers in 2005 and 30 flowers in 2006. Sample sizes in Cabrilla were 130 flowers in 2005 and 30 flowers in 2006. Since accurate measurement of herkogamy was impractical without destructive manipulation of the corolla, marked flowers were classed in the field into six discrete herkogamy categories according to anther–stigma separation, defined as follows: 1 (0–2 mm), 2 (2–4 mm), 3 (4–6 mm), 4 (6–8 mm), 5 (8–10 mm) and 6 (>10 mm). Flowers were left exposed to natural pollination, and the fruits produced were collected at maturity in early June. Fruit set varied considerably between years and populations (range = 41–90%), but generally fell within the range typically observed for this species (Herrera 1995). Invertebrate predation caused the loss of some flowers, and some fruits matured and dispersed most or all seeds before collection. These plants were excluded from genetic analyses, which implied an important reduction of sample sizes in some herkogamy categories in some years and populations (see Table 2 for final sample sizes). For this reason, the six discrete herkogamy categories we initially used to characterise plants in the field were finally reduced to three (low, intermediate and high herkogamy) in our mating system analyses, as explained in detail below.

Mating patterns were studied by conducting allozyme analysis of progeny arrays. We assayed a total of 2952 seeds from 218 maternal families obtained from marked plants, with all populations and years combined (see Table 2 for details). A mean of 13.5 seeds were analysed per family (SD = 5.9; range = 4–38). Electrophoretic procedures, extraction, grinding and running buffers followed those described in Barrett *et al.* (2004), Medrano *et al.* (2005) and Medrano & Herrera (2008), with only minor modifications. A total of seven polymorphic allozyme loci that showed clear and genetically interpretable banding patterns were resolved: acid phosphatase (*ACP-1*), aconitase (*ACO-2*), aspartate aminotransferase (*AAT-3*), diaphorase (*DIA-1*), formate dehydrogenase (*FDH-1*), leucine aminopeptidase (*LAP-2*), phosphoglucosomerase (*PGI-2*). Five of these loci were polymorphic in Cuevas Bermejas, six of these loci were polymorphic in Guadalentín and four of these loci

were polymorphic in Cabrilla. All loci were biallelic in all populations, with the exception of *FDH-1*, which had three alleles, but one of them was rare and not found in all populations. Genotypes were inferred based on segregation patterns of either dimeric or monomeric co-dominant enzymes.

Mating system parameters were estimated in two ways: (i) for each population and year with all herkogamy classes combined, and (ii) analysing the low, intermediate and high herkogamy classes separately within each population and year. In all cases, we estimated both the multilocus outcrossing rate ( $t_m$ ) and the correlation of outcrossed paternity ( $r_p$ ) using the maximum likelihood procedures implemented in the program MLTR (ver. 3.4, revised September 2009, available at <http://genetics.forestry.ubc.ca/ritland/programs.html>; Ritland 2002). Correlation of outcrossed paternity is a reciprocal value of the effective number of pollen donors ( $N_{ep}$ ), which is the number of paternities weighted as if all males are represented equally within offspring arrays (Dudash & Ritland 1991). MLTR uses the methods of Ritland & Jain (1981) for multilocus maximum likelihood estimation of outcrossing rates, and implements Ritland's extensions to the mixed mating model to incorporate correlated mating (Ritland 2002). A Newton-Raphson iteration routine was used to find maximum likelihood estimates, as recommended by Ritland (1996). Estimations were performed with pollen allele frequencies constrained to equal ovule frequencies. Allele frequencies ( $p$ ) were within the range useful for estimating mating system parameters (*i.e.*,  $0.1 < p < 0.9$ ). Maternal genotypes were inferred from the progeny arrays by the maximum likelihood method of Brown & Allard (1970). At the population level, estimates of standard errors were calculated as the SD of 5000 bootstrap values generated with the progeny array as the unit of resampling. Ninety-five per cent confidence intervals (CIs) of the estimates were calculated as 1.96 times the standard error. Differences between population-level estimates of mating system parameters were tested for significance by the nonoverlap of their 95% CIs.

Population-level estimates of mating system parameters obtained using Ritland's maximum likelihood procedures are quite robust, especially when using multiple loci, but estimates for individual families using this or similar procedures (Cruzan & Arnold 1994; Hardy *et al.* 2004) are unreliable when based on small numbers of progeny per mother (Morgan & Barrett 1990; Cruzan 1998; Ivey & Wyatt 1999). An alternative is to compare mating system estimates for groups of plants differing in some focal trait (Brunet & Eckert

**Table 2.** Multilocus outcrossing rates ( $t_m$ ), correlation of outcrossed paternity ( $r_p$ ) and effective number of fathers ( $N_{ep}$ , calculated as  $1/r_p$ ) for three *Narcissus longispatus* populations sampled in multiple years.

population	year	sample sizes families (f)/ seeds (s)	number of polymorphic loci	multilocus outcrossing rate $t_m$ (SE)	correlation of outcrossed paternity $r_p$ (SE)	effective number of fathers $N_{ep}$
Cuevas Bermejas	2003	32f/637s	5	0.565 (0.032)	0.239 (0.062)	4.2
	2005	51f/497s	5	0.419 (0.033)	0.259 (0.107)	3.9
Guadalentín	2004	19f/368s	6	0.607 (0.073)	0.351 (0.092)	2.8
	2005	40f/396s	6	0.651 (0.035)	0.453 (0.080)	2.2
	2006	17f/306s	6	0.780 (0.035)	0.164 (0.055)	6.1
Cabrilla	2005	39f/401s	4	0.731 (0.053)	0.231 (0.075)	4.3
	2006	20f/347s	4	0.835 (0.034)	0.452 (0.069)	2.2

1998). We used this approach to explore how herkogamy influences the mating system. Due to the small sample sizes finally available for some of the six field herkogamy categories, and since no obvious natural discontinuity in the distribution of this trait was found, for these analyses plants were grouped into three equal-sized and increasing herkogamy classes: low herkogamy plants, including those whose flowers had anther–stigma separation <4 mm (field herkogamy classes 1 and 2); intermediate herkogamy plants, with anther–stigma separation >4 and <8 mm (field herkogamy classes 3 and 4); and high herkogamy plants, with anther–stigma separation >8 mm (field herkogamy classes 5 and 6). Despite the loss in resolution and decreased statistical power resulting from this new grouping scheme, however, results will still reveal significant differences between plants falling into different segments of the herkogamy distribution, which further reinforces our conclusions. Flowers with different herkogamy level were extensively intermingled in all studied populations and years, thus supporting the assumption that all families shared the same outcross pollen pool irrespective of their herkogamy class. This assumption was incorporated into the computations by running the MLTR program on the whole data set from each population and reproductive season, and treating plants with low, intermediate and high herkogamy flowers as a different group of families. In this way, a single value of  $t_m$  and  $r_p$  (and associated standard errors) was obtained for each of the three herkogamy categories in each population and year. Standard errors were calculated as the SD of 5000 bootstrap values generated with the progeny array as the unit of resampling.

To determine whether herkogamy was related to outcrossing rates ( $t_m$ ) and correlation of outcrossed paternity ( $r_p$ ), differences in estimated  $t_m$  and  $r_p$  between low, intermediate and high herkogamy groups within each population and year were evaluated statistically by calculating the proportional overlap between the bootstrap distributions of herkogamy groups. Estimates were considered significantly different if the overlap between the bootstrap distributions was <5% (Eckert & Barrett 1994). Consistency of the effect of herkogamy on mating system parameters ( $t_m$  and  $r_p$ ) over populations and years was assessed by fitting mixed models to the data using the SAS procedure MIXED (SAS Institute 2005). In these models herkogamy (low, intermediate, and high) was included as a fixed effect, and population and year nested within population as random effects. Note that as we are particularly interested in evaluating the shape of the relation between  $t_m$  and  $r_p$  and herkogamy, tests of significance of linear and quadratic contrasts between herkogamy classes were also performed. The accuracy of  $t_m$  and  $r_p$  estimates for the three herkogamy categories was expected to vary among the seven population–year combinations, because they differed widely in number of families and total number of progeny (Table 2). To correct for this effect, observations were weighted by the total number of seed families on which each estimate was based.

## RESULTS

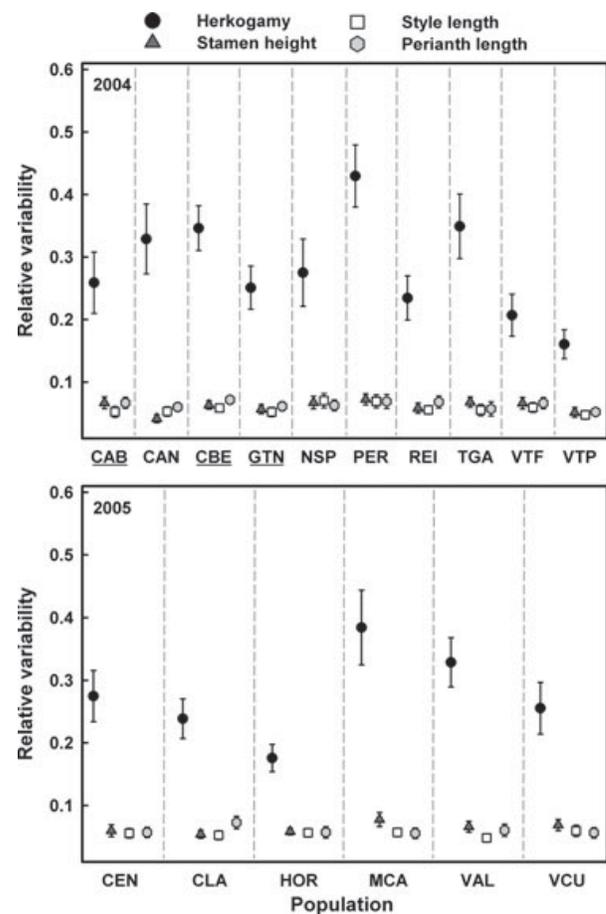
### Variation in floral traits

Variations within and among populations in herkogamy, style length, stamen height and perianth length are summarised in Table 1. Negative herkogamy (*i.e.*, anthers positioned above

the stigma) was recorded in only three of the 549 flowers measured. Floral variation was largely a within-population phenomenon. Most variance in herkogamy, style length, stamen height and perianth length was accounted for by differences among flowers of the same population (79.7, 82.3, 78.6 and 78.3% of total variance, respectively), and only secondarily by differences among populations (~20% of the total variance).

Irrespective of population and year, variability was far greater for herkogamy than for the other floral traits, all of which were remarkably homogeneous in their variability levels (Fig. 2). Paired *t*-tests clearly indicated that variability of herkogamy significantly exceeded that of style length ( $t = 12.41$ ,  $df = 15$ ,  $P < 0.0001$ ), stamen height ( $t = 12.35$ ,  $df = 15$ ,  $P < 0.0001$ ) or perianth length ( $t = 11.88$ ,  $df = 15$ ,  $P < 0.0001$ ).

A large proportion of observed variation in herkogamy was related to variation in style length, stamen height and perianth length, as revealed by regressing herkogamy on the other three floral traits ( $R^2 = 0.94$ ,  $n = 545$  flowers,  $P < 0.001$ , all



**Fig. 2.** Relative variability (= mean absolute deviations from the median) of herkogamy (black circles), stamen height (dark grey triangles), style length (white squares) and perianth length (light grey hexagons) in the 16 natural populations of *N. longispatus* studied. Symbols are population means ( $\pm 1SE$ ). Underlined population codes denote populations included in the study of mating system parameters (CAB = Cabrilla, CBE = Cuevas Bermejas and GTN = Guadalentín).

populations and years combined; similar relationships held true within individual populations, as revealed by separate analyses, results not shown). The relative importance of the three floral traits as herkogamy predictors differed widely, as denoted by differences in the absolute value of standard partial regression coefficients, which declined from style length (1.35), through stamen height (1.19) to perianth length (0.06). After statistically accounting for variation in flower size, therefore, variation in herkogamy was caused by differences in both style length and stamen height, the former variable being the most influential.

**Population mating patterns**

*Narcissus longispatus* had an intermediate outcrossing rate, which varied among populations and years (mean  $t_m = 0.655$ ; range = 0.419–0.835; Table 2). Outcrossing rates did not differ significantly between years in Cabrilla or Guadalentín, but the Cuevas Bermejas population exhibited significantly higher outcrossing rates in 2003 (95% CI of  $t_m = 0.495$ –0.622) than in 2005 (95% CI of  $t_m = 0.357$ –0.488).

Estimates for correlation of outcrossed paternity ( $r_p$ ) revealed that in *N. longispatus* an average 30% of outcrossed progeny in a fruit are full siblings (mean  $r_p = 0.307$ ; range = 0.164–0.453; Table 2). Although correlation of outcrossed paternity varied substantially across populations and years, a value of unity was never reached ( $r_p + 1.96 \text{ SE} < 1$ , in all populations and years), thus indicating that seeds in the same fruits are consistently sired by two or more outcross pollen donors. Only in the Guadalentín population did correlation of outcrossed paternity differ between years, being significantly lower in 2006 than in 2005 (95% CI of  $r_p = 0.057$ –0.271 and 0.295–0.609, respectively). Significant differences between years did not occur in either Cuevas Bermejas or Cabrilla. Between two and six different fathers typically sired the progeny within individual *N. longispatus* fruits (Table 2).

**Outcrossing rates in low, intermediate and high herkogamy flowers**

For all populations and years combined, outcrossing rates were roughly similar in the low, intermediate and high herkogamy classes (Table 3). When data were analysed separately for the different populations and years, statistically significant differences in  $t_m$  values between groups of individuals differing in herkogamy were detected in only two of the seven population–year combinations, and differences were of opposite signs in these two instances. When differences in outcrossing rates between low, intermediate and high herkogamy plant groups were tested, and possible differences between populations and years were accounted for, no significant trend towards high outcrossing rates in plants with higher anther–stigma distance was found (Fig. 3, Table 4).

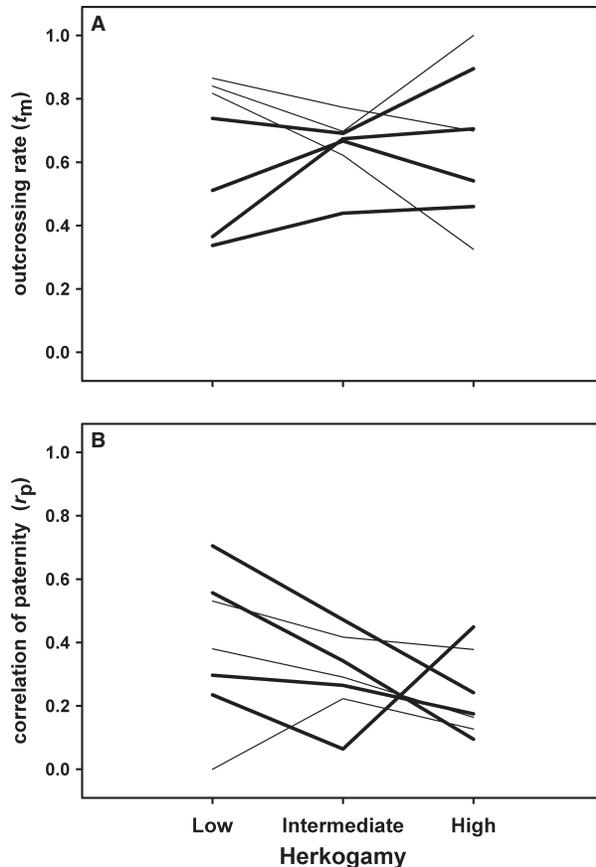
**Correlation of outcrossed paternity in low, intermediate and high herkogamy flowers**

Averaged across populations and years, the correlation of outcrossed paternity was substantially lower for high herkogamy flowers (mean  $r_p = 0.221$ ) than for either intermediate or low

**Table 3.** Multilocus outcrossing rates ( $t_m$ ), correlation of outcrossed paternity ( $r_p$ ) and effective number of fathers ( $N_{ep}$ ) for *Narcissus longispatus* plants with low herkogamy (LH), intermediate herkogamy (IH) and high herkogamy (HH). Estimates were calculated separately for low, intermediate and high herkogamy plants in each population–year sample. Values followed by different letters indicate significant differences ( $P < 0.05$ ) within each population and year.

population	year	sample sizes families (f)/seeds (s)			multilocus outcrossing rate $t_m$ (SE)			correlation of outcrossed paternity $r_p$ (SE)			effective number of fathers $N_{ep}$		
		LH	IH	HH	LH	IH	HH	LH	IH	HH	LH	IH	HH
Cuevas Bermejas	2003	7f/106s	12f/268s	13f/263s	0.511 (0.069)	0.667 (0.051)	0.541 (0.051)	0.235 (0.228)	0.064 (0.047)	0.449 (0.123)	4.26	15.63	2.23
	2005	12f/116s	22f/124s	17f/167s	0.337 (0.061)	0.439 (0.073)	0.460 (0.115)	0.557 (0.394)	0.342 (0.175)	0.095 (0.159)	1.80	2.92	10.53
Guadalentín	2004	5f/91s	7f/129s	7f/148s	0.817 <sup>a</sup> (0.059)	0.622 <sup>a</sup> (0.074)	0.325 <sup>b</sup> (0.053)	0.380 (0.134)	0.291 (0.179)	0.164 (0.182)	2.63	3.44	6.09
	2005	4f/41s	22f/216s	14f/139s	0.365 <sup>a</sup> (0.094)	0.674 <sup>b</sup> (0.051)	0.705 <sup>b</sup> (0.065)	0.705 (0.384)	0.472 (0.105)	0.242 (0.115)	1.42	2.12	4.13
Cabrilla	2006	3f/55s	11f/199s	3f/52s	0.865 (0.091)	0.773 (0.040)	0.698 (0.093)	−0.010 (0.099)	0.223 (0.071)	0.127 (0.157)	–	4.48	7.87
	2005	5f/50s	20f/204s	14f/147s	0.738 (0.113)	0.691 (0.075)	0.895 (0.069)	0.297 (0.179)	0.265 (0.148)	0.175 (0.101)	3.37	3.77	5.71
2006	3f/56s	13f/219s	4f/72s	0.840 (0.165)	0.697 (0.080)	1.109 (0.198)	0.531 (0.090)	0.417 (0.143)	0.378 (0.379)	1.88	2.40	2.65	
mean*				0.590	0.648	0.683	0.463	0.303	0.221	2.15	3.30	4.56	

\*Values are model-adjusted means obtained by fitting a linear mixed model to the data.



**Fig. 3.** Effects of herkogamy on mating system parameters. The three classes of herkogamy correspond to flowers with anther–stigma separation <4 mm (low herkogamy), between 4 mm and 8 mm (intermediate herkogamy) and >8 mm (high herkogamy). A: Influence of herkogamy on multilocus outcrossing rates. B: Influence of herkogamy on the correlation of outcrossed paternity. Each line connects values obtained for the three herkogamy classes in one of the seven population–year combinations. The thickness of each line is proportional to the weight of that group of data in analyses, and reflects the number of seed families in each sample.

**Table 4.** Summary of linear mixed models testing for the effect of herkogamy (fixed factor) on the response variables multilocus outcrossing rate ( $t_m$ ), and correlation of outcrossed paternity ( $r_p$ ) in *Narcissus longispathus*, once differences between populations and years nested within population (random factors) were controlled for. In addition to the main effects, contrasts involving linear and quadratic relationships were also tested. Significant P values ( $P < 0.05$ ) are in bold.

	response variable					
	$t_m$			$r_p$		
herkogamy effect	df	F	P	df	F	P
main effect	2,12	0.72	0.5053	2,11	4.86	<b>0.0307</b>
linear contrasts	1,12	1.42	0.2570	1,11	9.46	<b>0.0105</b>
quadratic contrasts	1,12	0.03	0.8665	1,11	0.34	0.5714

herkogamy flowers (mean  $r_p = 0.303$  and  $0.463$ , respectively; Table 3). The mean effective number of sires for high herkogamy plants (4.6 pollen donors) doubled the corresponding

figure for low herkogamy plants (2.1 pollen donors) (Table 3), whereas a mean of 3.3 different fathers sired seeds from intermediate herkogamy flowers. Not surprisingly, given the broad standard errors of estimates, within-population differences in  $r_p$  between low- intermediate and high herkogamy individuals did not reach statistical significance in individual population–year samples. However, after statistically accounting for variation among populations and years, an overall significant effect of herkogamy on outcrossed paternity was found (Table 4). More interestingly, the relationship between herkogamy and outcrossed paternity showed a consistent and monotonically decreasing pattern, as indicated by the significance of the linear contrasts and non-significance of the quadratic ones (Fig. 3, Table 4).

## DISCUSSION

Herkogamy occurs frequently in self-compatible flowering plants, and is widely viewed as a floral trait that reduces the likelihood of self-pollination. In addition to considering the traditional hypothesis linking anther–stigma separation with outcrossing rate, we have also explored here the relatively neglected possibility that herkogamy may affect other important components of mating as well, specifically pollen donor diversity within fruits. Although it has previously been suggested that polyandry may have significant effects on plant mating strategies (e.g., Armbruster *et al.* 2002; Teixeira & Bernasconi 2007), few studies have looked for relationships between mate diversity within fruits and natural variation in floral traits (but see Takebayashi *et al.* 2006; Lankinen & Madjidian 2011). Our results for different *N. longispathus* populations and sampling years demonstrate a remarkable spatiotemporal constancy in mating parameters, tend to rule out an effect of herkogamy on outcrossing rate and provide compelling support for the hypothesis postulating a role of herkogamy in determining sire diversity in single-fruit progeny.

### The mixed mating system of *N. longispathus*

Populations of *N. longispathus* consistently exhibited an intermediate outcrossing rate ( $t_m$  range = 0.42–0.83), and the values obtained in this study are very similar to those obtained in earlier investigations on this species. For six populations sampled in 1990, the  $t_m$  range reported in Barrett *et al.* (2004) was 0.54–0.77. Constancy of these figures over nearly two decades suggests that intermediate outcrossing rates are inherent to this species, irrespective of season or year, in contrast to the strong spatial and temporal fluctuations in outcrossing rates often found in other species (e.g., Kalisz *et al.* 2004; Brunet & Sweet 2006; Eckert *et al.* 2009).

Regarding the other component of mating system considered in this study, namely the effective number of sires per fruit ( $1/r_p$ ), our results have shown that a range of 2.2–6.1 different paternal genotypes were represented in single-fruit progeny of *N. longispathus*. These values for mate diversity within fruits are among the highest reported so far for bee-pollinated flowering plants (e.g., Sun & Ritland 1998; Mitchell *et al.* 2005; Kubota *et al.* 2008). The combination of relative constancy in outcrossing rates and a high level of multiple paternity might be attributed, among other causes,

to the extended lifespan of *N. longispathus* flowers (12–20 days), since floral longevity will increase the likelihood of stigmas receiving outcross pollen from multiple visits by different pollinators (Dudash & Ritland 1991; Marshall *et al.* 2010).

#### Herkogamy and mating parameters

Broad continuous variation in herkogamy levels is a distinctive feature of *N. longispathus*, which, in combination with self-compatibility and a mixed mating system, render this species a particularly favourable system for studying the implications of that floral feature (Barrett *et al.* 2004; Medrano *et al.* 2005). We have shown in this paper that most variation in herkogamy occurred between individuals within populations, and that anther–stigma separation was considerably more variable than the other floral traits considered (style length, anther height and perianth length). As expected from the composite nature of the trait, herkogamy variation was related to variation in both style length and anther position. The finding that herkogamy was more closely related to style length than to anther position suggests that in *N. longispathus*, as in other species (*e.g.*, Herlihy & Eckert 2007), herkogamy variation should possibly be best considered a proxy for style length variation. This is clearly supported by the fact that, in our *N. longispathus* sample, stamen height alone explained only 6.9% of observed variation in herkogamy, yet style length alone accounted for 21.5%.

This study corroborates and expands our previous research on *N. longispathus* (Medrano *et al.* 2005) by showing, for a broader sample, that herkogamy variation has little, if any, effect on outcrossing rate in this plant. But, on the other hand, the present study also revealed that the relative outcrossing advantage of *N. longispathus* flowers with intermediate herkogamy observed in our previous work on a single population is not consistently maintained across different populations and years. Relatively few studies have explored spatial and temporal effects of herkogamy on mating system parameters (but see *e.g.*, Herlihy & Eckert 2007; Eckert *et al.* 2009 for *Aquilegia*). Studies on *Aquilegia canadensis* have shown, for example, that although flowers with more pronounced herkogamy generally experienced less self-fertilisation, the effect of herkogamy on selfing rates varied among populations and between years within populations, and these fluctuations were related to spatial and temporal differences in levels of outcross pollen transfer (Eckert *et al.* 2009). The absence of a linear relationship between herkogamy and outcrossing rate in *N. longispathus* could perhaps be due to our mating system estimates referring to the flowering peak, when most plants are simultaneously in bloom and pollinators are most abundant. It could be speculated that earlier or later in the season outcrossing rates might be lower and related to levels of herkogamy. The possibility cannot be ruled out, however, that failure to detect a significant relationship between herkogamy and outcrossing rate reflects Type II error due to the intrinsically low statistical power of these estimates (see *e.g.*, Ivey & Wyatt 1999; Medrano *et al.* 2005).

In contrast to our inability to detect a relationship between herkogamy and outcrossing rate, a significantly linear, monotonically decreasing trend was found between herkogamy and

outcrossed paternity. This means that on average fruits of *N. longispathus* plants characterised by higher herkogamy were sired by significantly more outcross pollen donors than those of plants with lower levels of herkogamy. To our knowledge, the relationship between anther–stigma separation and correlation of outcrossed paternity has previously been studied only in the annual *Gilia achilleifolia* (Takebayashi *et al.* 2006). In this species, plants with high herkogamy showed a high correlation of outcrossed paternity ( $r_p$ ), while plants with low herkogamy  $r_p$  did not depart significantly from zero, although the mechanism(s) involved remained unknown. In the case of *N. longispathus*, two different mechanisms acting in concert could account for observed patterns, as hypothesised below. These hypotheses attach causal roles to both the parentage diversity of the pollen arriving to the stigma and to possible genotype filtering processes occurring along the style, from pollen grain germination to ovule fertilisation.

A parsimonious mechanism accounting for the higher mate diversity of fruits from high herkogamy flowers is that stigmas of these flowers may have the opportunity to capture genetically more diverse pollen, especially if low and high herkogamy flowers differ in pollinator composition. Pollinators of *N. longispathus* include small (*e.g.*, *Andrena*), medium-sized (*e.g.*, *Anthophora*) and large (*e.g.*, *Bombus*) bees (C.M. Herrera and M. Medrano, unpublished data). Differently sized pollinators differ in the way they interact with the stigma of flowers of different herkogamy levels and in pollen carryover (see *e.g.*, Forrest *et al.* 2011). Since some theoretical models predict a relationship between variance in pollen carryover and pollen donor diversity (Galen & Rotenberry 1988), herkogamy-dependent variance in the diversity of pollinators visiting individual flowers could eventually lead to variation in pollen donor diversity. Studies are currently underway to test the possibility that differently sized bees may impose heterogeneous selection pressures on the flowers of *N. longispathus*, as shown for other species (*e.g.*, Fenster *et al.* 2004; Kulbaba & Worley 2008).

Another mechanism contributing to the higher mate diversity in fruits from flowers with high herkogamy may stem from the connection between style length and non-random mating (reviewed by Ruane 2009). As shown in this study, herkogamy variation in *N. longispathus* is mainly due to variation in style length, with the most herkogamous flowers also tending to have the longest styles. Longer styles may enhance the opportunities for sorting amongst pollen donors, giving maternal plants a better opportunity to determine the fertilisation success of different pollen genotypes by enhancing gametophyte competition through forcing pollen tubes to travel greater distances to fertilise ovules (Willson & Burley 1983). Also, longer styles may allow more pollen grains from later pollinator visits to successfully fertilise ovules (Spira *et al.* 1996; Snow *et al.* 2000), since pollen deposited during later visits could ‘catch up’ to pollen deposited during earlier ones (Karron *et al.* 2006). The data currently available do not allow us to discriminate amongst these possibilities.

Irrespective of the proximate mechanisms that could account for observed patterns, the monotonic increase in mate diversity with increasing anther–stigma separation consistently maintained across multiple populations and years will likely translate into directional selection favouring

increased herkogamy in *N. longispathus*. Our results, however, do not support this hypothesised scenario, since extensive variation in herkogamy takes place within all populations studied. This seeming contradiction perhaps reflects that other selective forces may be involved in the evolution of herkogamy. For instance, we cannot exclude the possibility that if pollen export is also strongly affected by the relative position of anthers and stigma (see Harder & Barrett 1993), this effect may help to explain the high levels of phenotypic variation in this character, especially if the strength of selection through male function varies spatiotemporally. Another possibility would be that other selective agents (like herbivores) may perhaps counteract the beneficial effect of higher pollen donor diversity, if flowers with longer styles and higher herkogamy were more likely to be eaten, as has been demonstrated for some distylous species (Leege & Wolfe 2002).

Direct experiments manipulating the paternal and/or genetic diversity of outcross pollen loads on stigmas with differing levels of herkogamy (and associated variation in style length) are required to fully understand the implications of herkogamy for realized mate diversity in natural populations of *N. longispathus* and the causal mechanisms involved. Results of the present study, based on data from different populations and years and nearly 3000 genotyped seeds, pro-

vide some guidance for setting up these manipulative experiments, and also contribute to an emerging consensus that floral traits might influence several aspects of plant mating, extending the traditional focus on patterns of self- and cross-fertilisation (Barrett 2003; Bernasconi 2004; Lankinen & Märdjian 2011).

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