**Herkogamy and Mating Patterns in the Self-compatible Daffodil Narcissus longispathus**

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INTRODUCTION

The avoidance of inbreeding has been a major driving force shaping floral design and the evolution of plant mating systems (Darwin, 1877; Charlesworth and Charlesworth, 1987; Richards, 1997; Barrett, 2003). In self-compatible, animal-pollinated species, the spatial segregation of anthers and stigmas within the same flower (herkogamy) is usually considered an adaptive feature that functions to limit inbreeding by reducing the intensity of self-pollination. Indeed, there is some empirical evidence to support this assumption. Studies examining the relationship between the degree of herkogamy and outcrossing rate have generally found a monotonic increasing relationship both within (e.g. Nicotiana rustica, Breese, 1959; Mimulus ringens, Karron et al., 1997; Aquilegia caerulea, Brunet and Eckert, 1998; Datura stramonium, Motten and Stone, 2000) and among (e.g. Clarkia temblorensis, Holtsford and Ellstrand, 1992; Turnera ulmifolia, Belaoussoff and Shore, 1995) populations. Although in some species individual differences in anther–stigma separation may largely reflect plastic responses to variation in environmental factors (Elle and Hare, 2002; Weing, 2002), investigations on the genetic basis of variation in herkogamy have generally found moderate to high heritabilities, and a rapid response to artificial selection (Breese, 1959; Shore and Barrett, 1990; Holtsford and Ellstrand, 1992; Motten and Stone, 2000; Lendvai and Levin, 2003). Selection on herkogamy variation therefore provides a simple mechanism for adjusting mating patterns in plant populations.

However, the functional role of herkogamy in promoting outcrossing is not always straightforward. Many species that possess this trait are self-incompatible and are therefore already protected from the harmful effects of selfing. In such cases herkogamy may function primarily to reduce interference between female and male sexual functions and/or to enhance pollen export efficiency (Webb and Lloyd, 1986; Barrett, 2002; Cesaro et al., 2004). Moreover, herkogamy commonly occurs in self-compatible species with large floral displays and, in these situations, the trait may be ineffective as a means of avoiding inbreeding, because extensive selfing can occur as a consequence of pollen dispersal between flowers of the same plant (Robertson, 1992; Harder and Barrett, 1995; Brunet and Eckert, 1998; Eckert, 2000; Montaner et al., 2001; Williams et al., 2001; Elle and Hare, 2002; Karron et al., 2004). To isolate clearly the functional role of herkogamy in promoting outcrossing requires a self-compatible species with limited floral display that maintains considerable phenotypic variation in anther– stigma variation in natural populations.

Here the influence of natural variation in herkogamy on outcrossing rate in the self-compatible, mostly single-flowered daffodil Narcissus longispathus Pugsley (Amaryllidaceae) is examined. This species exhibits considerable within-population variation in anther– stigma separation and a previous study (Barrett et al., 2004) identified variable allozyme markers that can be used to estimate multilocus variation, Narcissus longispathus, outcrossing rates.

Key words: Allozymes, Amaryllidaceae, anther–stigma separation, floral design, herkogamy, intra- and interpopulation variation, Narcissus longispathus, outcrossing rates.
outcrossing rates. Moreover, this study also found that selfed progeny rarely survive to maturity because of high inbreeding depression. Therefore, any influence of anther–stigma separation on outcrossing rate is likely to translate into differential contributions to the next generation, providing evidence for natural selection on herkogamy. The present investigation of *N. longispathus* addressed three specific questions: (1) What is the magnitude of the variation in anther–stigma separation within and between populations? (2) To what extent do developmental changes in anther–stigma position contribute to variation in herkogamy? (3) Is there a monotonically increasing relationship between the degree of herkogamy exhibited by plants within a population and their outcrossing rates, as found for other species?

**MATERIALS AND METHODS**

**Study species**

*Narcissus longispathus* (Amaryllidaceae) is a bee-pollinated, perennial herb endemic to a few mountain ranges in southeastern Spain (Moreno Saiz and Sainz Ollero, 1992; Herrera et al., 1999). This large-flowered daffodil (corolla length approx. 50 mm) commonly produces a single flower per inflorescence and is self-compatible, producing equivalent amounts of seed following experimental self- and cross-pollinations (Herrera, 1995). This contrasts with other species in the genus, most of which are moderately to strongly self-sterile (reviewed by Barrett et al., 1996; Sage et al., 1999). In the Sierra de Cazorla mountains, where this study was conducted (see below), *N. longispathus* is represented by scattered populations confined to stream margins or poorly drained meadows around springs at 1000–1500 m. Flowering occurs from late February to mid-April, a period characterized by cool, rainy weather that frequently limits the activity of the species’ main pollinator (*Andrena bicolor*, Andrenidae). Despite low pollinator visitation, most flowers of *N. longispathus* are pollinated and seed production is only weakly pollen limited. The species has a mixed mating system producing significant amounts of both outcrossed and selfed seed [range of population mean outcrossing rates $(t_m) = 0.54-0.77$; $N = 6$ populations; Barrett et al. (2004)].

**Field methods**

The present study was conducted in six *N. longispathus* populations located in the Sierra de Cazorla mountain range, Jaén province, south-eastern Spain. Plants were sampled at the same (five sites), or very close to (one site), locations studied by Barrett et al. (2004), where further details of localities can be found. In March 2003, anther–stigma separation was measured in the field on 30–35 flowers (from as many individual plants) per population. Digital calipers were used and, after slitting the corolla, the shortest distance to the nearest 0.1 mm between the rim of the flat stigma and the nearest anther was measured. Given that in *N. longispathus* the stigma is always above the anthers, variation in herkogamy was positive in all cases. To minimize any possible effect of flower age-related variation in anther–stigma separation, flowers chosen for measurement were all of the same developmental stage: corolla with fully expanded corona, and pollen sacs undehisced or newly dehisced. In the latter case, only flowers with convex anthers that still presented most or all pollen grains were chosen for measurement.

Flowers of *N. longispathus* are long-lived, lasting for 16–50 days on average when exposed to natural pollination (Herrera, 1995). To assess variation in anther–stigma separation during the life of individual flowers, two different kinds of data were obtained in 2004. At the Cuevas Bermejas population, 25 newly opened flowers were individually marked on 12 March and scored using the following six classes of anther–stigma separation: 0–2 mm, 2–4 mm, 4–6 mm, 6–8 mm, 8–10 mm and >10 mm. One week later this procedure was repeated. In addition, seven bulbs with sprouting scapes bearing flower buds were excavated. They were potted with local soil, and kept outdoors until their flowers opened. The herkogamy class of each flower was then recorded daily from opening until the flowers withered or the anthers had most pollen removed.

To investigate the influence of herkogamy on outcrossing rate, 45 flowers in a population at Cuevas Bermejas were individually marked. Each flower was assigned to one of the six classes of anther–stigma separation indicated above. These discrete herkogamy classes, rather than direct measurements, were used because destructive manipulation of the corolla needed for accurate measurements would have most likely affected pollinator visitation and mating patterns. These flowers were left exposed to natural pollination, and the fruits produced (N = 32) collected at maturity in early June. Observed fruit set (71 %) fell within the limits commonly observed for this species (Herrera, 1995). After collection seed progenies were kept separate in paper bags at ambient temperature until electrophoretic analyses.

**Electrophoresis**

Variation was assayed at allozyme loci from the seed families (N = 32 families/637 seeds) using horizontal starch gel electrophoresis following methods detailed in Barrett et al. (2004). For each seed family genotypes were scored for 6–38 seeds (mean = 19.9). Seeds were prepared for electrophoretic analysis by soaking overnight in water, and homogenized in a 0.02 M Na2HPO4 buffer (pH 7.4) containing 2–dithiothreitol (1 mg ml−1). The crude extract was immediately absorbed onto chromatography paper wicks (Whatman 3MM™), and kept at −80°C until electrophoresis was performed on 11 % starch gels. Based on previous work (Barrett et al., 2004), six variable loci which were known to be polymorphic in the Cuevas Bermejas population were resolved. Two buffer systems were used: lithium-borate (pH 8.3) to resolve aspartato amino transferase, diaphorase and cytosol aminopeptidase; and histidine-citrate (pH 6.5) to resolve aconitate, acid phosphatase and phosphoglucone isomerase. All loci were diacletic. Genotypes were inferred based on segregation patterns of either dimeric or monomeric codominant enzymes. Polymorphic loci conformed to Mendelian expectations for segregation in the analysed progeny arrays.
Estimates of mating system and inbreeding

We estimated the multilocus outcrossing rate \(t_m\) and the parental inbreeding coefficient \(f\) for the Cuevas Bermejas population (all herkogamy classes combined) using the computer program MLTR for Windows by K. Ritland (version 2-4, November 2002, available from the Internet at http://genetics.forestry.ubc.ca/ritland/programs.html; see also Ritland, 1990a). This program uses the methods of Ritland and Jain (1981) for multilocus maximum likelihood estimation of outcrossing rates, and implements Ritland’s extensions (Ritland, 2002) to the mixed mating model to incorporate correlated mating. Estimates of \(t_m\) were also computed for each herkogamy class using the MLTR program. There was extensive spatial intermingling in the studied population of flowers in different herkogamy classes; hence it seemed reasonable to assume that all families shared the same outcross pollen pool irrespective of the herkogamy class they belonged to. This assumption was incorporated into the computations by running the MLTR program on the whole data set \((N = 32\) families) and treating each herkogamy class as a different group of families. In this way, a single \(t_m\) figure (and standard error, see below) was obtained for the entire set of progeny in each herkogamy class. This procedure was deemed preferable to estimating the outcrossing rate for each seed family and then relating these values to herkogamy using regression or analysis of variance approaches. The difficulties involved in obtaining accurate family-level estimates of outcrossing in naturally pollinated plants have been repeatedly emphasized in the literature, and Ivey and Wyatt (1999) went on to conclude that current family-level estimators for outcrossing are possibly inadequate. The different estimation methods available produce inconsistent estimates, involve very large estimation errors, fail to converge on reasonable estimates for some families, and lead to spurious correlations with environmental or other parameters (e.g. Morgan and Barrett, 1990; Cruzan et al., 1994; Leclerc-Potvin and Ritland, 1994; Cruzan, 1998; Ivey and Wyatt, 1999; Ritland, 2002).

Inbreeding depression \((\delta = 1 - \text{fitness of selfed progeny/fertility of outcrossed progeny})\) for survival from seed to reproductive maturity for the Cuevas Bermejas population was calculated following Barrett et al. (2004), by substituting the population estimates of multilocus outcrossing rate and parental inbreeding coefficient into Ritland’s equilibrium estimator of inbreeding (Ritland, 1990b). This estimator allows calculation of inbreeding depression when information is available from one generation, but requires an assumption of inbreeding equilibrium. Standard errors for all parameters were calculated by bootstrapping 5000 times over progeny arrays, using the \(N = 32\) families as the units of resampling.

RESULTS

Variation in herkogamy

Variation in anther–stigma separation over the life of individual \(N.\) longispathus flowers was relatively minor and statistically non-significant. In the set of 25 flowers scored twice for herkogamy class, mean anther–stigma separation \((\pm 1\text{ s.d.})\) changed from 4.8 ± 2.8 mm to 5.5 ± 2.0 mm over 1 week. This increase was not statistically significant \((P = 0.36, \text{randomization test for paired data on class midpoints})\). A similar result was obtained for the flowers of potted plants kept outdoors and monitored daily for a 10-d period. In this group of flowers, anther–stigma separation increased from 3.3 ± 1.8 mm at the time of flower opening to 4.7 ± 2.1 mm at flower withering or pollen exhaustion \((P = 0.38, \text{randomization test for paired data})\).

Anther–stigma separation varied considerably among flowers within the six populations studied, ranging from 1 to 10 mm (Table 1). In all populations, the range of variation observed was nearly one order of magnitude broader than the slight, statistically non-significant developmental variation recorded during the lifespan of individual flowers. There was significant heterogeneity among populations in mean anther–stigma separation \((F_{5,179} = 8.88, P < 0.0001)\), but variation among populations was relatively minor in absolute terms, accounting for only 20.8 % of the total variance. Most observed variation in anther–stigma separation occurred among flowers of the same population (79.2 % of total variance).

Population-level mating and genetic parameters

The mean multilocus outcrossing rate \((\pm 1\text{ s.e.})\) for the Cuevas Bermejas population in 2003, all herkogamy classes pooled, was \(t_m = 0.598 \pm 0.011\) (95 % range of bootstrap estimates = 0.504–0.699). The inbreeding coefficient of parent plants \((\pm 1\text{ s.e.})\) was \(f = -0.098 \pm 0.011\), which did not differ significantly from zero (95% range of bootstrap estimates = -0.200–0.009). The estimate of inbreeding depression obtained using Ritland’s equilibrium estimator \((Ritland, 1990b)\) was \(\delta = 1.265\), which was significantly >0 and did not differ significantly from unity (95% range of bootstrap estimates = 0.97–1.58).

Anther–stigma separation and outcrossing rate

Mean multilocus outcrossing rates for the different herkogamy classes are illustrated in Fig. 1. The number of families and seeds available for the smallest herkogamy class \((0–2\text{ mm})\) was insufficient for separate analysis, and this class was therefore combined with the contiguous one \((2–4\text{ mm})\) to form a single category \((<4\text{ mm})\). Standard

Table 1. Variation in anther–stigma separation within and among six Narcissus longispathus populations in the Sierra de Cazorla mountain range, south-eastern Spain

<table>
<thead>
<tr>
<th>Population</th>
<th>(N)</th>
<th>Range (mm)</th>
<th>Mean ± s.d. (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cuevas Bermejas</td>
<td>30</td>
<td>2.2–10.4</td>
<td>6.7 ± 2.2</td>
</tr>
<tr>
<td>Tornillos de Gualay</td>
<td>30</td>
<td>1.1–7.3</td>
<td>4.5 ± 1.6</td>
</tr>
<tr>
<td>Tornillos de Gualay</td>
<td>35</td>
<td>0.5–10.4</td>
<td>5.1 ± 2.2</td>
</tr>
<tr>
<td>Valdecuevas</td>
<td>30</td>
<td>3.5–10.3</td>
<td>7.1 ± 1.6</td>
</tr>
<tr>
<td>Valdetrillos – 1</td>
<td>30</td>
<td>1.5–9.5</td>
<td>6.1 ± 2.0</td>
</tr>
<tr>
<td>Valdetrillos – 2</td>
<td>30</td>
<td>1.8–7.7</td>
<td>5.1 ± 1.5</td>
</tr>
</tbody>
</table>

\(N\) = number of flowers measured.
Herkogamy is a character that is frequently subject to ontogenetic change during a flower’s lifespan, particularly in species in which anthers and stigmas mature at different times (Webb and Lloyd, 1986). Repeated measurement of anther–stigma separation on the same flowers of *N. longispathus* indicated that herkogamy increased with flower age. However, the temporal change in sex-organ separation was generally very small (0.72 ± 1.72 mm) and did not differ significantly from zero. This result clearly indicates that the within-population variation in anther–stigma separation that was observed mainly reflects differences between individual plants, rather than any heterogeneity of flower ages. Accordingly, it is valid to consider the ecological and evolutionary consequences of herkogamy variation in populations of *N. longispathus*.

Mating consequences of herkogamy variation

Individual variation in herkogamy was considerable in each of the six *N. longispathus* populations studied. The observed range is among the broadest so far reported for species with continuous herkogamy variation. In absolute terms, within-population ranges of anther–stigma separations in *N. longispathus* are comparable to those reported for *Datura stramonium* (Motten and Stone, 2000) and *Aquilegia caerulea* (Brunet and Eckert, 1998), and broader than those of several other species with herkogamy variation (e.g. *Minulus ringens*, Karron et al., 1997; *Ipomoea purpurea*, Epperson and Clegg, 1987; *Nicotiana rustica*, Breese, 1959; *Turnera ulmifolia*, Shore and Barrett, 1990). The occurrence of this range of herkogamy raises the question of what selective forces maintain the variation in populations of *N. longispathus*. Clearly, understanding the reproductive consequences of herkogamy variation in terms of pollination and mating is critical for addressing this issue.

Previous studies that have investigated how anther–stigma separation influences outcrossing rate have generally found a monotonically increasing relationship between the two variables (see references in the Introduction). Logically, it would have been expected that the closer the anthers and stigmas are located in a flower the more likely autonomous and/or pollinator facilitated self-pollination would occur. The present results for *N. longispathus* depart from earlier investigations and this simple functional expectation. Outcrossing rates did not increase monotonically with intermediate distances separating anthers and stigmas. Nevertheless, tests of these relationships did not reach statistical significance if more-stringent significance levels were used to account for the unplanned nature of comparisons. Low statistical power is a well-known limitation inherent in investigations comparing mating-system parameters among experimental treatments, populations, or groups of plants (Leclerc-Potvin...
of Narcissus longispathus, indicating that few selfed offspring reach reproductive maturity (Barrett et al., 2004). Assuming that inbreeding depression is uniformly strong and unrelated to individual differences in anther–stigma separation (Carr et al., 1997; Chang and Rausher, 1999; but see Stone and Motten, 2002; Takebayashi and Delph, 2000), variation in outcrossing rates should translate into differential survival prospects for the resulting progeny and, therefore, to natural selection on anther–stigma separation. The shape of the selection function (e.g. directional, stabilizing) would then be expected to mimic the shape of the relationship linking outcrossing rate and anther–stigma distance. Monotonic increases in outcrossing rate with increasing anther–stigma separation of the sort found in most species will thus likely translate into directional selection favouring increased herkogamy. In contrast, results of this study point to the possibility of pollinator-mediated stabilizing selection on anther–stigma separation in the Cuevas Bermejas population. This scenario would imply the existence of some outcrossing disadvantage when anther–stigma separation increases beyond a certain optimum level, a pattern that does not seem to have been previously documented for other species. On the other hand, however, the hypothesis of pollinator-mediated stabilizing selection on anther–stigma separation is difficult to reconcile with the broad intrapopulation variability in herkogamy observed. Corroborating the generality of the results of this study will thus require further work.

Stability of mixed mating and inbreeding equilibrium

Narcissus longispathus has a mixed mating system with populations consistently exhibiting moderate levels of selfing (Barrett et al., 2004). Remarkably, the mean multilocus outcrossing rate for the Cuevas Bermejas population reported in this study ($t_m = 0.598$) is virtually identical to the estimate obtained by Barrett et al. (2004) for seed progenies collected in the same population 13 years earlier in 1990 ($t_m = 0.595$). The estimates of the inbreeding coefficient of parent plants in this population were also similar and not significantly different from zero (1990, $f = 0.031$; 2003, $f = -0.098$). These values are considerably lower than the expected level of inbreeding in a population at equilibrium with the observed outcrossing rate, $f_{exp} = 0.251$ [computed as $f_{exp} = (1 - t_m)/(1 + t_m)$; e.g. Spiess, 1989]. This indicates a consistently greater heterozygosity of adult plants relative to seeds in the two study years. Although these comparisons involve data for only 2 years, they suggest that the mating system and inbreeding parameters remained close to invariant over more than one decade in the Cuevas Bermejas population. As a consequence of similarities in $t_m$ and $f$,
inbreeding depression estimates over the whole life cycle (6) obtained using Ritland's equilibrium estimator (Ritland, 1990b) were also similar and close to unity in the two study years. Collectively, these results would support the view that the Cuevas Bermejas N. longispathus population is in inbreeding equilibrium, and that selection against selfed progeny is consistently strong in the population.

Similarly strong selection against selfed progeny in other animal-pollinated species with mixed mating systems has recently been reported: e.g. Decodon verticillatus (Eckert and Barrett, 1994), Shorea leprosula (Lee et al., 2000), Sagittaria latifolia (Dorken et al., 2002), Aquilegia canadensis (Herlihy and Eckert, 2002), Daphne laureola (Medrano et al., 2004). The high inbreeding depression estimates for the whole life cycle detected in these studies imply that few, if any, selfed progeny survive long enough to enter the adult reproductive stage and therefore self seeds make little or no genetic contribution to the next generation (reviewed by Barrett, 2003). However, these marker-based approaches for measuring inbreeding depression using the Ritland estimator (Ritland, 1990b) are based on the critical but usually untested assumption that populations are at inbreeding equilibrium. The present studies of the Cuevas Bermejas population of N. longispathus indicate that, at least for this population, this assumption is reasonable.

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LITERATURE CITED


Darwin C. 1877. The different forms of flowers on plants of the same species. London: John Murray.


