Mating patterns and demography in the tristylous daffodil *Narcissus triandrus*

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Mating patterns in plant populations are influenced by interactions between reproductive traits and ecological conditions, both factors that are likely to vary geographically. *Narcissus triandrus*, a wide-ranging heterostylous herb, exhibits populations with either two (dimorphic) or three (trimorphic) style morphs and displays substantial geographical variation in demographic attributes and floral morphology. Here, we investigate this variation to determine if demography, morphology, and mating system differ between the two sexual systems. Our surveys in Portugal and NW Spain indicated that dimorphic populations were less dense, of smaller size, and had larger plants and flowers compared to trimorphic populations. Outcrossing rates estimated using allozyme markers revealed similar outcrossing rates in dimorphic and trimorphic populations (t_m dimorphic $= 0.759$; t_m trimorphic $= 0.710$). All populations experienced significant inbreeding in progeny (mean $F = 0.143$). In contrast, parental estimates of inbreeding were not significantly different from zero (mean $F = 0.062$), implying that few inbred offspring survive to reproductive maturity due to inbreeding depression. Although the majority of inbreeding results from selfing, significant levels of biparental inbreeding were also detected in eight of the nine populations (mean $s_s - s_m = 0.081$). Density was negatively associated with levels of selfing but positively associated with biparental inbreeding. Population size was positively associated with outcrossing but not biparental inbreeding. There were no consistent differences among the style morphs in outcrossing or biparental inbreeding indicating that the maintenance of trimorphism *vs* dimorphism is unlikely to be associated with inbreeding of maternal parents.
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Introduction

Mating patterns in flowering plants are products of complex interactions between reproductive traits and the ecology of populations (Lloyd, 1980; Holsinger, 1996). An important aspect of mating is the amount of self-fertilization experienced by individuals. Selfing has important genetic consequences, including its influence on the strength of inbreeding depression (Charlesworth and Charlesworth, 1987) and the partitioning of genetic variation within and among populations (Hamrick and Godt, 1989). In species that lack strong self-incompatibility, selfing may increase when insufficient quantities of outcrossed pollen are deposited on stigmas. In animal-pollinated plants, spatial isolation and small population size generally act to reduce the frequency of pollinator visitation (Sih and Baltus, 1987; Ågren, 1996). Thus, under such conditions selfing is predicted to increase (Moeller and Geber, 2005). Indeed, several studies have investigated the association between outcrossing rates and density or population size (vanTreuren *et al*, 1993; Raijmann *et al*, 1994; Routley *et al*, 1999; Lu, 2000; Barrett *et al*, 2004a; Zoro Bi *et al*, 2005). However, results from these studies have been mixed indicating that the

ecology of selfing rates warrants further investigation, particularly in wide-ranging species exposed to contrasting environmental conditions.

The demographic characteristics of populations, through their impact on pollinator behaviour, might also be expected to influence the intensity of biparental inbreeding. Observations of spatially restricted pollen and seed dispersal in plants have led to the prediction that populations will often be genetically subdivided at a local scale (Wright, 1943; Turner *et al*, 1982; Heywood, 1991; Kalisz *et al*, 2001). Pollinator foraging distances are generally negatively correlated with density, such that at high-density pollen dispersal distances are shorter than at low density (Levin and Kerster, 1969b; Kameyama *et al*, 2002). Thus, in dense populations, pollinator behaviour in combination with genetic subdivision is likely to result in increased biparental inbreeding with potential consequences for plant fitness.

Narcissus triandrus is a wide-ranging, bee-pollinated herb with populations that vary in demographic and morphological characters likely to influence mating patterns. This species possesses a tristylous sexual system and a particular focus of our study was to examine how mating patterns influence the maintenance of this genetic polymorphism. In tristylous populations there are three floral morphs that differ reciprocally in the placement of their anthers and stigmas (long-, mid- and short-styled, hereafter referred to as L-, M- and S-morphs). The style morphs in most tristylous species possess a heteromorphic incompatibility system that

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prevents selfing and intra-morph mating. The resulting disassortative mating generally gives rise to an isoplethic equilibrium of morph ratios (Fisher, 1941, 1944; Charlesworth, 1979; Heuch, 1979; Barrett, 1993). However, some tristylous species lack this type of incompatibility providing opportunities for selfing and intra-morph mating (Barrett and Cruzan, 1994). Increased selfing rates have been implicated in causing deviations from isoplethy and the breakdown of tristyly via the loss or fixation of the morph with the highest selfing rate (Charlesworth, 1979).

N. triandrus lacks heteromorphic incompatibility and instead possesses a late-acting self-incompatibility system that is often weakly expressed (Barrett *et al*, 1997; Sage *et al*, 1999). Population morph ratios usually deviate strongly from 1:1:1 (isoplethy) (Fernandes, 1965; Barrett *et al*, 1997). Based on large-scale surveys conducted in the Iberian Peninsula (Barrett *et al*, 2004b), three distinctive patterns characterize populations: (1) most populations are L-morph biased; (2) there is a negative association between the frequencies of the L- and M-morphs; and (3) some populations lack the M-morph and are thus dimorphic. Only dimorphic populations occur in the north-west part of the range whereas in central Portugal dimorphic and trimorphic populations are often found in close geographic proximity. Preliminary observations indicate that variation in the sexual system of *N. triandrus* is associated with geographical variation in floral morphology (see Barrett *et al*, 2004b) and possibly also demographic features of populations including their size and density. Thus, *N. triandrus* provides an opportunity to investigate the influence of demography and reproductive morphology on mating patterns with potential relevance to the maintenance of stylar trimorphism *vs* dimorphism.

Here, we address three specific questions concerning the relations between mating patterns, floral morphology and demography in *N. triandrus*. (1) Are there differences between dimorphic and trimorphic populations in demographic and morphological characteristics? (2) Do outcrossing rates and biparental inbreeding vary significantly among populations that differ in demography and sexual system? (3) Are there morph-specific differences in the rates of self-fertilization and biparental inbreeding? We discuss the implication of our results for the maintenance of sexual-system polymorphism in *N. triandrus* and factors responsible for the loss of the M-morph from trimorphic populations.

Materials and methods

Study species
N. triandrus L. (Amaryllidaceae) is a diminutive perennial geophyte, common in the central and northern parts of the Iberian Peninsula. Populations start flowering in late February at the southern margin of the range in Andalucía and continue until mid May at higher elevations in central and northern Spain and Portugal. Flowering plants produce a single stem with 1–9 (mean 1.6) pale yellow to cream pendulous flowers that last up to 14 days. Solitary bees, particularly *Anthophora pilipes* Fabr., are the primary pollinators, although in the cooler Atlantic zone in northern Spain and Portugal *Bombus* spp. visit flowers. Several intra-specific taxa have been described in *N. triandrus* (Blanchard, 1990) but here we

focus exclusively on the widespread *N. triandrus* var. *triandrus* as this taxon displays the greatest morph-frequency variation including all dimorphic populations reported for the species (see Barrett *et al*, 2004b).

Population sampling for morphological and demographic measurements
In 2003 and 2004, we sampled 76 populations (37 dimorphic and 39 trimorphic) in central Portugal and NW Spain recording latitude and longitude at each site. Localities for all populations are available from the first author on request. In each population, we estimated the style morph ratio (see Barrett *et al*, 2004b) and population size. Populations were identifiable as discrete colonies of plants separated from other populations usually by several kilometres. Two separate counts were made and the average was taken as the final estimate of population size. *N. triandrus* does not propagate clonally so the sampling of genets is unambiguous. In 58 populations (21 dimorphic and 37 trimorphic) plant height was measured from a random sample of individuals (mean = 30.71 individuals, range = 7−79 individuals) and in 37 populations (13 dimorphic and 24 trimorphic) we also measured perianth length and flower number (mean = 95.8 individuals, range = 14−224 individuals). We determined near neighbour distances in 22 of these populations (eight dimorphic and 14 trimorphic) by randomly selecting focal individuals and measuring the distance from these individuals to their three closest neighbours. We previously established that this measure of density is closely correlated with counts of the numbers of individuals per square meter (*unpublished data*). The number of focal individuals used for density measurements varied depending on population size as the number of focal individuals was limited in small populations (mean = 37.4, range = 13–46).

We used ANCOVA to determine the association between sexual system (trimorphic *vs* dimorphic), plant height, flower size and flower number (Glm procedure ver. 8.2 SAS Institute, 2001). The analysis was conducted using population means. We treated sexual system as a fixed effect and latitude as a covariate as both flower size and number increase with latitude (Barrett *et al*, 2004b). To test for significant differences in population size and near neighbour distance between dimorphic and trimorphic populations we used ANOVA (Glm procedure ver. 8.2, SAS Institute, 2001). When appropriate, we transformed data (eg log or square root) to meet the assumptions of statistical tests. In these cases least squared means and standard errors were back-transformed for presentation in figures.

Estimates of mating patterns
To estimate population mean and morph-specific maternal outcrossing rates, we collected seed families from a total of nine populations (five dimorphic, four trimorphic) during April and May 2004. Individuals were collected from populations with a minimum of 15 seed families per morph to achieve sufficient sample sizes for morph-specific estimates. Therefore, populations do not represent the entire range of morph frequencies or population sizes found in *N. triandrus*. The mean number of families per style morph sampled was 27.8 (range = 16–36). From each seed family, we assayed eight

to ten seeds. For one dimorphic population, the number of families for the S-morph with a sufficient number of seeds was very low, so we estimated the outcrossing rate of the L-morph only.

We performed horizontal starch gel electrophoresis following the methods outlined in Barrett *et al* (1997). We crushed seeds in a small amount of 0.02 M Na_2HPO_4 buffer (pH 7.4) containing DL-dithiothreitol (1 mg/ml). Chromatography paper wicks (Whatman 3 mm) were used to absorb the resulting homogenate. The wicks were then inserted into 12–14% starch gels. We stained gels for enzymes using the methods of Wendel and Weeden (1991). We initially screened 18 enzyme systems and from these three polymorphic loci were reliably resolved. A histidine-citrate buffer (pH 6.5) was used to resolve phosphoglucomutase (*PGM-1*) and phosphoglucose isomerase (*PGI-1*) and a lithium borate buffer (pH 8.3) was used to resolve aspartate aminotransferase (*AAT-2*). We detected three alleles at the *AAT-2* locus, four alleles at *PGM-1* and six alleles at the *PGI-1* locus.

We used Ritland's MLTR program for windows version 3.0, September 2004, available at http://genetics.forestryubc.ca/ritland/programs.html (see also Ritland, 1990b) to estimate multi- and single-locus maternal outcrossing rates as well as parental inbreeding coefficients. This program uses maximum likelihood procedures to infer the genotypes of the maternal parents, the allele frequencies of the pollen pool and the proportion of progeny that result from outcrossing (*t*). Parental inbreeding coefficients (*F*) are estimated from the inferred maternal genotype frequencies. The program assumes that allele frequencies do not differ significantly among morphs. If there are chance associations of the marker alleles with respect to the style morphs there will be a downward bias in *t* when a common pollen pool is used to estimate the outcrossing rate rather than separate pollen pools. As trial runs indicated that there were significant differences in allele frequencies among the morphs at some loci, outcrossing rates were estimated using separate pollen pools (Eckert and Barrett, 1994; Baker *et al*, 2000). We derived SE of the outcrossing rates from 1000 bootstraps using the seed family as the unit of resampling.

Significant differences in outcrossing rate between the style morphs in populations were assessed by pairwise comparisons of 1000 bootstrap estimates following the methods of Eckert and Barrett (1994). Pairwise differences between the 1000 bootstrap estimates for each morph were calculated as $t_i - t_j$ (where t_i and t_j represent the bootstrap estimates for the *i*th and *j*th morph). With this approach, morphs were considered to be significantly different if $100(1-\alpha_{pc}/2)$ percentage of the differences between paired bootstrap values were either all greater than zero or all less than zero, where α_{pc} represents the type-I error rate per contrast. Similarly, we calculated overall differences among morphs and between dimorphic and trimorphic populations by determining the average of the L-, M- and S-morph t_m for a given bootstrap. Morphs or populations were considered to be significantly different when, as above, $100(1-\alpha_{pc}/2)$ percentage of the differences between paired bootstraps were either all greater than zero or all less than zero. Type-I error rate was calculated as $\alpha_{pc} = 1 - (1-0.05)^{1/C}$ where C represents the number of multiple contrasts (Sidák, 1967).

We calculated estimates of biparental inbreeding $(s_s - s_m)$ where s_s is the single-locus selfing rate and s_m is the multi-locus selfing rate (Ritland, 2002). These estimates are useful in comparisons among populations of the same species when the same set of loci are used (Lu, 2000; Ritland, 2002). However, we note that experimental estimates of biparental inbreeding indicate that this method may underestimate the true level of biparental inbreeding experienced in populations (Griffin and Eckert, 2003). We measured inbreeding depression ($\delta = 1 - $fitness of selfed progeny/fitness of outcroseed progeny) for survival from seed to reproductive maturity from the multi-locus estimates of outcrossing rate (*t*) and parental inbreeding (*F*) using the equilibrium estimator of Ritland (1990a).

$$\delta = 1 - \left[\frac{2tF}{(1-t)(1-F)} \right]$$

To determine significant differences, we examined the distributions of bootstrap values for each parameter $(s_s - s_m, F, \delta)$ in an analogous way to the method described for outcrossing rates. Values of $s_s - s_m$ and F in which the confidence interval overlapped with 0 were considered not significantly different from 0. Similarly, values of δ where the confidence interval overlapped with 1 were not significantly different from 1.

We employed ANCOVA (JMP ver. 4.0 SAS Institute, 2000) to evaluate the extent to which variation in population size, near neighbour distance and sexual system were associated with variation in outcrossing rate. Sexual system was treated as a fixed effect and both population size and near neighbour distance were incorporated as covariates. Population size was log transformed. Any nonsignificant interactions of the covariates with sexual system were removed using backwards elimination. We weighted the outcrossing estimates by the inverse of their squared SE to take into account sampling errors associated with the estimates. Estimates of biparental inbreeding were analyzed in the same manner.

Results

Demographic and morphological characteristics of dimorphic and trimorphic populations

Dimorphic and trimorphic populations of *N. triandrus* differ significantly in demographic characteristics (Figure 1a and b). Mean population size was significantly smaller in dimorphic populations compared to trimorphic populations ($F_{1,74} = 16.49$, $P = 0.0001$). In addition, average distance to the nearest three neighbours was found to be significantly greater in dimorphic compared to trimorphic populations ($F_{1,20} = 4.46$, $P = 0.047$) as was the distance to the first, second and third nearest neighbours ($F_{1,20} = 10.25$, $P = 0.0045$; $F_{1,20} = 8.02$, $P = 0.0103$; $F_{1,20} = 12.16$, $P = 0.0023$).

In testing for associations between morphological characteristics and sexual system, ANCOVAs revealed no significant interaction between sexual system and latitude and were removed from the model (Table 1). Plants in dimorphic populations were found to be significantly taller than plants in trimorphic populations ($F_{1,55} = 15.85$, $P = 0.0002$; Figure 1c). Latitude also explained a significant proportion of the variation in plant
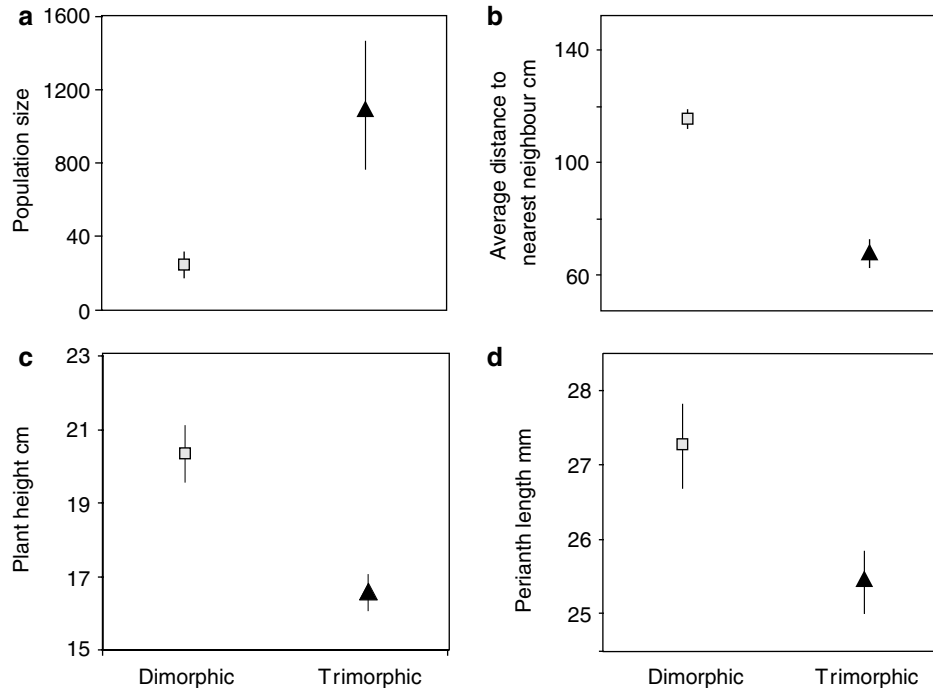
Figure 1 Demographic and morphological characteristics of dimorphic and trimorphic populations of *N. triandrus* var. *triandrus*. (**a**) The association between log population size and sexual system. (**b**) The association between the average distance of focal individuals to their three nearest neighbours and sexual system. (**c**) The association between the average plant height of populations and sexual system. (**d**) The association between the average perianth length of populations and sexual system. Means are shown ± 1 SE.

Table 1 ANCOVA of three morphological characters among dimorphic and trimorphic populations of *Narcissus triandrus*

Morphological trait	Source	F	P-value
Plant height	Sexual system	15.85 (1,55)	0.0002
	Latitude	4.09 (1,55)	0.048
Perianth length	Sexual system	19.28 (1,34)	0.041
	Latitude	1.19 (1,34)	0.283
Flower number	Sexual system	0.01 (1,34)	0.972
	Latitude	5.48 (1,34)	0.025

Sexual system is a fixed effect and latitude a covariate. Degrees of freedom are indicated in parentheses.

height ($F_{1,55} = 4.09$, $P = 0.048$). Perianth length was found to be significantly greater in dimorphic populations compared to trimorphic populations ($F_{1,34} = 19.28$, $P = 0.041$; Figure 1d). The covariate latitude did not explain a significant proportion of the variation in perianth length ($F_{1,34} = 1.19$, $P = 0.283$). Conversely, latitude was found to be significantly associated with flower number ($F_{1,34} = 5.48$, $P = 0.025$) but sexual system was not ($F_{1,34} = 0.01$, $P = 0.972$).

Mating patterns
Outcrossing rates: There was a marginal difference in outcrossing rates between the two sexual systems of *N. triandrus*. Dimorphic populations exhibited slightly higher outcrossing rates than trimorphic populations (t_m dimorphic $= 0.759 \pm 0.0272$, t_m trimorphic $= 0.710 \pm 0.0195$, proportion of $t_{di} - t_{tri} < 0 = 0.060$). However,

morph-specific estimates within dimorphic and trimorphic populations revealed no large or consistent differences among the style morphs (Figure 2a and b). Of the eight populations examined, in only one (population 222 – dimorphic) was there a significant difference between the style morphs with the L-morph exhibiting a significantly higher outcrossing rate than the S-morph (t_m long $= 0.919 \pm 0.068$, t_m short $= 0.536 \pm 0.0749$, proportion of $t_l - t_s < 0 = 0.000$). In dimorphic population 246 there was a trend towards significance although the direction of the effect was reversed with the L-morph with a lower outcrossing rate compared to the S-morph (t_m long $= 0.795 \pm 0.0518$, t_m short $= 0.930 \pm 0.0408$, proportion of $t_l - t_s > 0 = 0.024$). When morph-specific outcrossing rates among all dimorphic populations were compared, there was a significantly higher outcrossing rate in the L-morph compared to the S-morph (t_m long $= 0.801 \pm 0.0264$, t_m short $= 0.710 \pm 0.0342$, proportion of $t_l - t_s < 0 = 0.017$). However, a single population was responsible for this difference and when it was removed from the analysis this difference was lost (proportion of $t_l - t_s < 0 = 0.314$).

There were no significant differences in outcrossing rates among the style morphs in trimorphic populations after correcting for multiple comparisons (Figure 2b). However, there was a trend towards significance in two instances. In population 276 the M-morph had a higher outcrossing rate than the L-morph (t_m long $= 0.541 \pm 0.0647$, t_m mid $= 0.719 \pm 0.0809$, proportion of $t_l - t_m > 0 = 0.034$) and in population 205 the M-morph had a lower outcrossing rate than the L-morph (t_m long $= 0.914 \pm 0.0444$, t_m mid $= 0.747 \pm 0.0464$, proportion of $t_l - t_m < 0 = 0.012$). Interestingly, this occurred in populations with the highest (population 276 = 0.432)
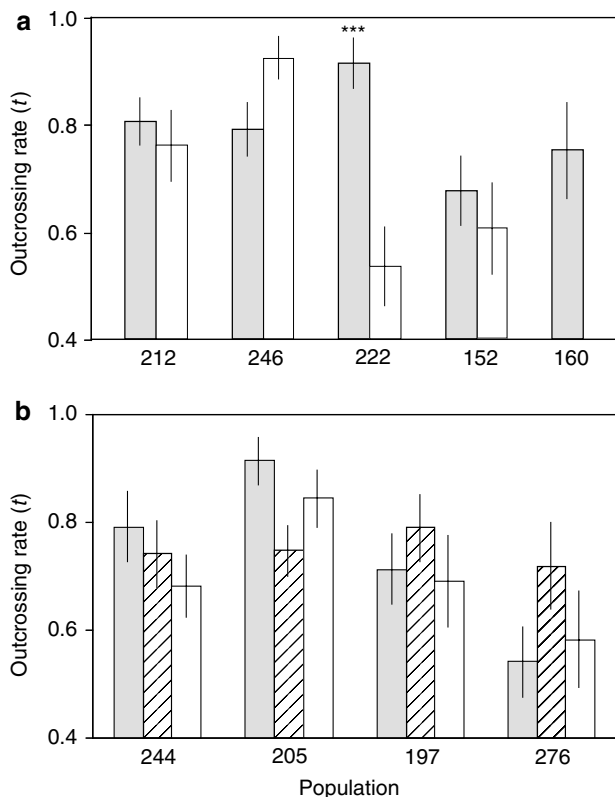
Figure 2 Estimated multi-locus outcrossing rates (t) in nine populations of *N. triandrus* var. *triandrus*. Morph-specific estimates were obtained using separate pollen pools in Ritland's (1990a) MLTR program and error bars are standard errors based on 1000 bootstrap estimates. (**a**) Outcrossing rates of the L- and S-morphs in four dimorphic populations (***$P<0.0001$). For one dimorphic population (population 160) an outcrossing rate was estimated for only the L-morph. (**b**) Outcrossing rates of the L-, M- and S-morphs in four trimorphic populations. L-morph = filled bars, M-morph = cross-hatched bars and S-morph = open bars.
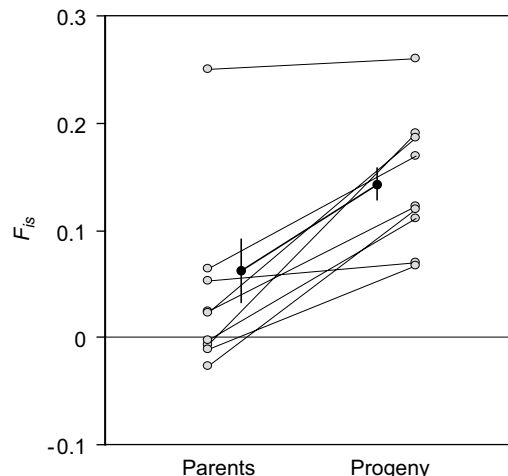

Figure 3 The progeny and parental inbreeding coefficients in nine populations of *N. triandrus* var. *triandrus*. Estimates were obtained using Ritland's MLTR (1990a) program. Progeny inbreeding coefficients were calculated from population selfing rates and the parental inbreeding coefficients (Ritland, 1990a). The overall average inbreeding coefficients and error bars are displayed for parents and progeny (black circles) along with individual population means for the nine populations (grey circles). Error bars are SE based on 1000 bootstrap estimates.

and lowest frequencies of the M-morph (population 205 = 0.174). When morph-specific differences among all trimorphic populations were compared no significant differences were evident (t_m long = 0.740 ± 0.0305, t_m mid = 0.749 ± 0.0319, t_m short = 0.670 ± 0.0363).

Biparental inbreeding: Population estimates of biparental inbreeding in *N. triandrus* were significantly different from 0 in all but one population (population 160 – dimorphic). Morph-specific estimates of biparental inbreeding revealed no significant differences among the style morphs within populations. Among all dimorphic populations there were no significant differences among morphs in biparental inbreeding (s_s-s_m long = 0.0865 ± 0.0129, s_s-s_m short = 0.0715 ± 0.0116). Similarly, in trimorphic populations there were also no significant differences among the morphs (s_s-s_m long = 0.108 ± 0.0121, s_s-s_m mid = 0.099 ± 0.0112, s_s-s_m short = 0.096 ± 0.0112). However, comparison of the two sexual systems revealed a significant difference in biparental inbreeding between dimorphic and trimorphic populations (s_s-s_m dimorphic = 0.102 ± 0.0068, s_s-s_m trimorphic = 0.0646 ± 0.0085, proportion of $(s_s-s_m)_{di}-(s_s-s_m)_{tri}<0 = 0.001$).

Inbreeding depression: Not unexpectedly, given the observed selfing and biparental inbreeding, there was evidence of significant inbreeding, in progeny (mean $F = 0.143$, range = 0.066-0.261). However, by contrast parental inbreeding estimates were not significantly different from zero in eight out of the nine populations investigated (mean $F = 0.062$, range = −0.027–0.252). This indicates that few inbred offspring survive to reproductive maturity due to strong inbreeding depression (Figure 3).

Demography and mating patterns: The effects of population size and density on outcrossing rate were first examined independently (Table 2). There was no relation between maternal outcrossing rates and population size in *N. triandrus* ($b \pm SE = -0.0079 \pm 0.0192$, $t_7 = -0.41$, $P = 0.6929$, $R^2 = 0.023$). However, ANCOVA revealed a significant interaction between average near neighbour distance and sexual system ($F_{1,5} = 6.801$, $P = 0.0478$). Therefore, outcrossing rates were regressed against average near neighbour distances separately for each sexual system. For dimorphic populations, there was a significant association between outcrossing rate and average near neighbour distance ($b \pm SE = -0.0025 \pm 0.0007$, $t_3 = -3.78$, $P = 0.0324$, $R^2 = 0.826$; Figure 4a). Trimorphic populations had a significant association between outcrossing rate and average near neighbour distance ($b \pm SE = -0.0073 \pm 0.0016$, $t_2 = -4.67$, $P = 0.0430$, $R^2 = 0.9158$). Similarly, univariate analysis revealed that population size was not correlated with population estimates of biparental inbreeding ($b \pm SE = -0.0085 \pm 0.0068$, $t_7 = 1.25$, $P = 0.2514$, $R^2 = 0.183$). However, average near neighbour distance was significantly associated with estimates of biparental inbreeding ($b \pm SE = -0.0005 \pm 0.0002$, $t_7 = -2.83$, $P = 0.0253$, $R^2 = 0.534$; Figure 4b).

Table 2 Correlations between outcrossing rates, biparental inbreeding and demographic characteristics among nine *Narcissus triandrus* populations

Mating system trait	Demographic trait	t	R^2	P-value
Outcrossing rate	Population size	−0.41 (7)	0.023	0.6929
Outcrossing rate (dimorphic populations)	Average distance to near neighbours	−3.78 (3)	0.826	0.0324
Outcrossing rate (trimorphic populations)	Average distance to near neighbours	−4.67 (2)	0.916	0.0430
Biparental inbreeding	Population size	1.25 (7)	0.183	0.2514
Biparental inbreeding	Average distance to near neighbours	−2.83 (7)	0.534	0.0235

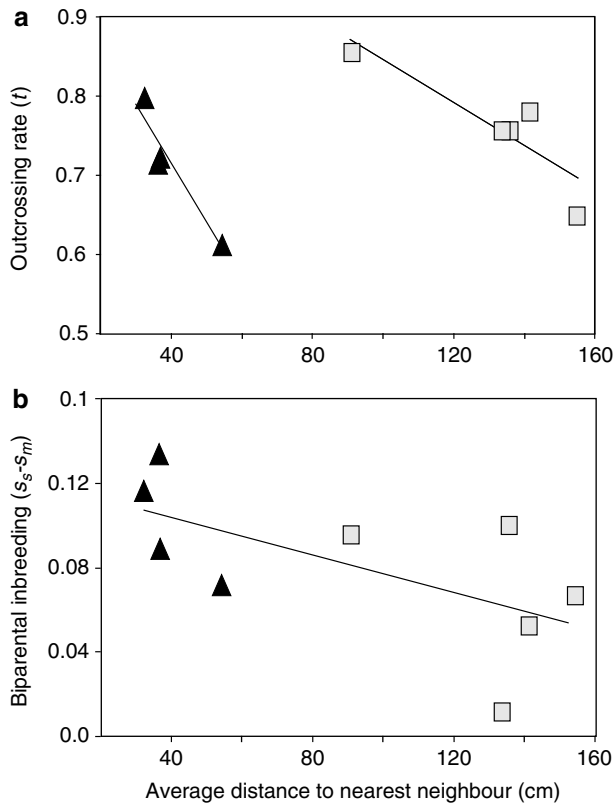Degrees of freedom are indicated in parentheses.

Figure 4 The correlation between inbreeding and density in nine populations of *N. triandrus* var. *triandrus*. (**a**) The association between outcrossing rate (*t*) and average near neighbour distance in dimorphic and trimorphic populations. (**b**) The association between biparental inbreeding ($s_s - s_m$) and average near neighbour distance. Triangles indicate trimorphic populations and squares dimorphic populations.

ANCOVA of outcrossing rate against density, population size and sexual system identified a significant effect of sexual system ($F_{1,5} = 52.32$, $P = 0.0008$). There was an inverse relationship between outcrossing rate and near neighbour distance (partial regression coefficient ± SE = −0.0007 ± 0.0003, $t_5 = -6.12$, $P = 0.0017$), but a positive relationship between outcrossing rate and population size (partial regression coefficient ± SE = 0.0377 ± 0.0121, $t_5 = -3.13$, $P = 0.0259$). All interactions were nonsignificant and removed from the model. For biparental inbreeding, multiple regression analysis revealed that biparental inbreeding was not associated with population size (partial regression coefficient ± SE = −0.0051 ± 0.0080, $t_6 = -0.64$, $P = 0.5449$) but was marginally nega-

tively associated with near neighbour distance (partial regression coefficient ± SE = −0.0007 ± 0.0003, $t_6 = -2.29$, $P = 0.0617$).

Discussion

With the advent of genetic markers, mating patterns are now routinely measured in plant populations and a considerable database now exists for outcrossing rate variation in flowering plants (Barrett and Eckert, 1990; Vogler and Kalisz, 2001). Most estimates of outcrossing have been obtained for self-compatible species, presumably because of the assumption that self-incompatible species experience limited variation in mating patterns. Previous work on *N. triandrus* indicates that the late-acting self-incompatibility system in this species is weakly expressed and significant levels of selfing can occur under field conditions (Barrett *et al*, 1997; Sage *et al*, 1999). A major goal of this study was, therefore, to investigate the role that demographic and morphological factors might play in influencing mating patterns and to evaluate the hypothesis that morph-specific differences in selfing could play a role in sexual-system evolution.

Demographic and morphological characteristics of dimorphic and trimorphic populations

We investigated demographic and morphological differences between dimorphic and trimorphic populations of *N. triandrus*. Our study revealed that populations with these contrasting sexual systems differ significantly in size, density, plant size and flower size (Figure 1). Dimorphic populations were smaller in size and on average had greater near neighbour distances compared to trimorphic populations. Dimorphic populations also have significantly larger plants that possess larger flowers than trimorphic populations, although a difference in flower number between the two sexual systems was not evident. In other plant species these demographic and floral characters are often associated with variation in mating patterns (see Table 1, Barrett and Eckert, 1990), we were therefore interested in determining to what extent this also occurs in *N. triandrus*.

Mating patterns and morph loss in *N. triandrus*

Our estimates of mating patterns in nine populations of *N. triandrus* var. *triandrus* indicated that this taxon is largely outcrossing. The average outcrossing rate was 0.74 (range = 0.61–0.86) with only a small (5%) mean difference between dimorphic and trimorphic populations. Significant amounts of selfing were, however, detected in all of the populations that we investigated.

Our earlier estimates of outcrossing rates in *N. triandrus* involved four trimorphic populations of var. *cernuus* from southern and central Spain (mean $t_m = 0.77$, range $= 0.68–0.87$, Barrett *et al*, 1997). Estimates from these two studies are therefore quite similar. Populations of var. *cernuus* are exclusively trimorphic whereas populations of var. *triandrus* are either dimorphic or trimorphic. Hence, there is no obvious indication from these population-level estimates that outcrossing-rate variation plays a significant role in the maintenance or loss of stylar trimorphism.

Morph-specific differences in selfing rate within tristylous populations can be an important factor in the evolution of morph ratios and in the destabilization of tristyly (Charlesworth, 1979; Weller 1992; Barrett, 1993). For example, Charlesworth's (1979) model concerning the breakdown of tristyly predicts that the M-morph is likely to experience higher selfing rates and indeed there is empirical evidence that supports this idea (Kohn and Barrett, 1992). If inbreeding depression is low, higher selfing in the M-morph can lead to its fixation in populations or, conversely, if inbreeding depression is severe the M-morph can decrease in frequency and be lost from populations (Charlesworth, 1979). Morphological and theoretical evidence indicate that contemporary dimorphic populations of *N. triandrus* are likely to be derived from trimorphic populations through loss of the M-morph (Barrett *et al*, 2004b). Therefore, consistently higher selfing rates in the M-morph of *N. triandrus* var. *triandrus* could provide a mechanism responsible for the origin of dimorphic populations.

Our results provided no clear support for the hypothesis that differences in the levels of self-fertilization among the morphs are responsible for biased morph ratios and the morph loss that characterizes populations of *N. triandrus*. Our genetic estimates of inbreeding depression were uniformly high in all but one population. Consequently, differences in selfing rates among the morphs could potentially lead to the decline and loss of the most highly selfing morph through viability and fertility selection. However, there were no consistent differences in outcrossing rate among the morphs that can easily explain either morph-ratio bias or morph loss from populations. Although there was a general lack of consistency in levels of outcrossing among morphs, several populations exhibited morph-specific differences in selfing, although in only one population were these differences statistically significant (Figure 2). This could indicate that the necessary conditions for morph loss occur only rarely. For example, in population 205 the selfing rate of the M-morph was 16% higher than the L-morph and in this population the M-morph was at a low frequency (0.17). It is probable that the circumstances that lead to morph loss in *N. triandrus* var. *triandrus* are locally determined and that in this particular population continued selfing of the M-morph could conceivably result in its loss from the population.

Although the evidence that self-fertilization is involved in the loss of the M-morph from populations of *N. triandrus* is weak at best, self-pollination in *Narcissus* species has other detrimental consequences in addition to inbreeding depression. *N. triandrus* possesses an ovarian self-incompatibility system in which self pollen tube growth causes the arrest of developing ovules prior to fertilization, a phenomenon known as ovule discount-ing (Barrett *et al*, 1996). Pollination studies have revealed that prior or simultaneous self-pollination results in reduced seed set (Sage *et al*, 1999). Future work on morph-specific estimates of self-pollination and ovule discounting are required to evaluate whether differences in levels of self-pollination among the style morphs may be contributing to the origin of dimorphic populations from trimorphic populations.

Mating and population structure

Single-locus estimates of outcrossing rate were significantly lower than multi-locus estimates in eight out of the nine populations of *N. triandrus*. This provides evidence for biparental inbreeding probably as a result of spatial genetic subdivision within populations. Seeds of this species have no obvious dispersal mechanisms and simply fall in the vicinity of maternal parents when capsules dehisce. Owing to the diminutive stature of the plants this likely results in relatively restricted seed dispersal. Spatial structure is expected to develop in populations with limited seed dispersal and local pollinator foraging. These features would enhance opportunities for mating among relatives. Although biparental inbreeding appears to be a pervasive feature of *N. triandrus* populations we found no evidence that the style morphs differ in this component of inbreeding, or that it is involved with transitions between trimorphism to dimorphism.

We detected a positive association between plant density and biparental inbreeding in populations of *N. triandrus* (Figure 4b). In dense populations, pollinator behaviour in conjunction with restricted seed dispersal can often result in local biparental inbreeding. Positive associations between biparental inbreeding and plant density have been reported in the self-incompatible *Phlox drummondii* (Watkins and Levin, 1990) and bird-pollinated *Banksia cuneata* (Coates and Sokolowski, 1992). Our analysis of biparental inbreeding also revealed an association with sexual system. Dimorphic populations despite being smaller in size engaged in significantly less biparental inbreeding than trimorphic populations. The lower biparental inbreeding levels may reflect greater seed dispersal distances caused by the greater height of dimorphic plants compared to trimorphic plants (Figure 1c). However, this pattern could also reflect the observed differences in density of dimorphic and trimorphic populations (Figure 1b). Pollinator foraging in low density populations has been shown to result in fewer visits to near neighbours thus reducing the incidence of mating among relatives (Levin and Kerster, 1969a, b; Kameyama *et al*, 2002; Degen *et al*, 2004). The proximate ecological mechanisms that cause differences in density between trimorphic and dimorphic populations are unknown but may be associated with extent of suitable habitat for seedling establishment.

Associations between mating patterns and population size have been demonstrated in several plant species (Raijmann *et al*, 1994; Routley *et al*, 1999), although this pattern does not always occur (vanTreuren *et al*, 1993; Barrett *et al*, 2004a; Zoro Bi *et al*, 2005). In *Salvia pratensis* population density, rather than population size was inversely related to selfing rate (vanTreuren *et al*, 1993). Our data revealed an association between near neighbour distances and outcrossing rate, as well as between

population size and outcrossing rate, when the variation in density was controlled for statistically. Although trimorphic populations had higher plant densities and larger population sizes than dimorphic populations, selfing rates were marginally higher in trimorphic populations. This suggests that factors other than density and population size are affecting the mating system of dimorphic and trimorphic populations. These could include flower size variation (Rick et al, 1978) and differences in the types of pollinators visiting populations of the two sexual systems.

Our estimates of inbreeding in the progeny of N. triandrus indicate a significant deficit of heterozygotes. However, the analysis of parental genotypes revealed that they are largely the products of outcrossing. This indicates that selfed offspring of N. triandrus do not survive to reproductive maturity as a result of strong inbreeding depression. Indeed, in eight of the nine populations measurements of inbreeding depression were not significantly different from one. Genetic estimates of inbreeding depression in other plant species with mixed mating also indicate very low survival of selfed offspring in comparison with outcrossed offspring (Eckert and Barrett, 1994; Routley et al, 1999; Dorken et al, 2002; Barrett et al, 2004a; Medrano et al, 2005). Given the genetic costs of selfing in N. triandrus it seems unlikely that partial selfing is commonly adaptive in this species. Why leaky self-incompatibility is maintained in N. triandrus is therefore unclear.

Acknowledgements

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