plant biology

Plant Biology ISSN 1435-8603

RESEARCH PAPER

Mating system in Mexican populations of the annual herb *Solanum rostratum* Dunal (Solanaceae)

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Keywords

Annual herb; enantiostyly; heteranthery; microsatellites; mixed mating; outcrossing rate; selfing.

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Editor

J. Arroyo

Received: 19 February 2012; Accepted: 5 November 2012

doi:10.1111/j.1438-8677.2012.00715.x

ABSTRACT

Traditionally, annual colonising species are expected to have high rates of self-fertilisation, although recent theoretical and empirical studies have shown that cross-fertilisation can be selected for under heterogeneous pollination environments. *Solanum rostratum* is a self-compatible annual herb that colonises disturbed habitats. Despite the lack of physiological mechanisms to prevent self-fertilisation, pollen transfer between individuals is expected to be favoured because of its complex floral morphology. In previous studies of *S. rostratum* it has been shown that anther dimorphism within flowers results in precise pollen placement on the pollinator's body, and the presence of mirror-image floral morphs within plants promotes outcrossing in experimental arrays. However, the mating system of natural populations of *S. rostratum* has never been assessed, and thus whether it is predominantly selfing or outcrossing remains unknown. We hypothesise that floral and inflorescence morphology of *S. rostratum* should facilitate cross-fertilisation, making it a predominantly outcrossing despite its lack of a self-incompatibility system. To test this hypothesis, we estimated outcrossing rates by genotyping 700 individuals at 13 microsatellite loci, sampled from four populations across a 690-km transect in the species' native range. We found that populations had mean outcrossing rates of 0.70 ± 0.03, with multiple sires contributing to paternity of each progeny array (average effective number of sires = 8.97 ± 0.57). This indicates that natural populations *S. rostratum* have relatively high levels of outcrossing, probably facilitated by its floral and inflorescence morphology. We speculate that partial selfing in this species may be an unavoidable consequence of displaying multiple flowers at the same time (geitonogamy), as well as the result of self-pollen transfer by illegitimate visitors.

INTRODUCTION

Populations of colonising species or those occurring at the margins of a species distribution often grow under conditions where mate and pollinator availability are limited or unpredictable (Baker 1974; Knight *et al.* 2005). Because self-pollinating individuals can set seed under pollen limited conditions (Eckert *et al.* 2006), it is thought that natural selection should favour an association between colonising ability and self-fertilisation (Baker 1955; Busch 2011). However, selection for characters that facilitate self-fertilisation, including the breakdown of self-incompatibility (Baker 1967) and reduced herkogamy (Motten & Stone 2000), can be weakened and even opposed by a multitude of biological phenomena. These phenomena include inbreeding depression (Charlesworth & Charlesworth 1987), spatially or temporally variable pollination environments (Massol & Cheptou 2011), life-history characteristics such as seed banks, perenniality or clonal reproduction (Baker 1967; Pannell & Barrett 1998; Vallejo-Marin & O'Brien 2007), as well as details of the dispersal process such as multiple introductions, and simultaneous arrival of many founders (Busch 2011). Establishing the mating system of colonising species is therefore an important task to better understand the conditions in which self- or cross-fertilisation, or a mixed mating strategy, are favoured in natural populations.

Selection for increased self-fertilisation in colonising plants is expected to be particularly high for annual species, because their lack of a perennial life history or clonal reproduction prevents them from buffering the costs of reproductive failure in any one reproductive season (Pannell & Barrett 1998; Vallejo-Marin *et al.* 2010a; Barrett 2011). In fact there are multiple examples of low outcrossing rates in annual species that colonise ephemeral or disturbed habitats, including *Medicago truncatula* Gaertn. ($t < 0.01$), an opportunistic herb widespread around the Mediterranean basin (Siol *et al.* 2008), *Solanum ptycanthum* Dunal, a weed of agricultural fields ($t < 0.17$) (Hermanutz 1991), and *Datura stramonium* L., an aggressive coloniser of disturbed habitats ($t = 0.02$–0.08) (Motten & Antonovics 1992). In general, weedy, short-lived colonising plants are often characterised by low levels of outcrossing (outcrossing rate, $t < 0.2$; Schemske & Lande 1985), although many exceptions occur (*e.g.* Burdon *et al.* 1988; Abbott & Forbes 1993; reviewed in Barrett 2011).

The possibility that outcrossing can be selectively favoured in short-lived species has recently been supported in theoretical models investigating co-evolution between mating system and dispersal in a metapopulation (*e.g.* Massol & Cheptou 2011).

For example, if there is heterogeneity in the pollination environment among sub-populations, either at spatial or temporal scales, selection can favour the evolution of outcrossing associated with dispersal (Cheptou & Massol 2009; Massol & Cheptou 2011). This counterintuitive result arises because heterogeneity in the pollination environment creates interpatch variation in fitness, which in turn selects for increased dispersal in outcrossers (Cheptou 2012). High outcrossing rates (t = 0.77–0.99) have indeed been detected using genetic markers in natural populations of some annual colonising species (Sun & Ritland 1998; Cheptou *et al.* 2002; Friedman & Barrett 2008). In these cases, outcrossing is enforced by physiological mechanisms to reduce self-fertilisation, such as partial or complete self-incompatibility. The maintenance of outcrossing in some of these species (*e.g. Ambrosia artemisiifolia*) might be facilitated by the presence of long-lived seed banks, large population sizes or wind-pollination (Pannell & Barrett 1998; Friedman & Barrett 2008).

Here, we investigate the mating system of *Solanum rostratum* Dunal (Solanaceae), an annual, self-compatible, herbaceous plant native to Mexico and possibly the USA (Whalen 1979). Populations of *S. rostratum* occur in disturbed habitats such as roadsides and abandoned fields and pastures in North America. As an annual herb of disturbed and ephemeral habitats, *S. rostratum* is expected to grow in conditions associated with limited availability of mates and pollinators (pollen limited). Nevertheless, the floral and inflorescence characteristics present in this species (*e.g.* strong herkogamy and enantiostyly; Todd 1882; Bowers 1975) are expected to promote pollen transfer between individuals and should allow the maintenance of high rates of cross-fertilisation. Specifically, each of the nectarless flowers of *S. rostratum* is heterantherous, with two morphologically distinct sets of anthers that serve different functions ('feeding' and 'pollinating' anthers; Bowers 1975; Vallejo-Marin *et al.* 2009, 2010b). The four yellow 'feeding' anthers are located at the centre of the flower and serve to attract and reward pollen-collecting small to large bees that resort to vibrations to extract pollen from the poricidal anthers (buzz pollination; Vallejo-Marin *et al.* 2009). The single 'pollinating' anther, usually tinged brown or red and deflected to either the right- or left-hand side of the flower opposite to the style, contributes disproportionately to ovule fertilisation (Vallejo-Marin *et al.* 2009). The spatial separation of the pollinating anther and the stigma (herkogamy) on opposite sides of a flower, and the deposition of pollen in specific areas of the pollinator's body could contribute to the reduction of self-pollination within a flower. In fact, preliminary observations suggest a low capacity for autonomous seed set (M. Vallejo-Marín and L. Solís-Montero, unpublished).

In addition, *S. rostratum* is monomorphically enantiostylous, *i.e.* each plant bears two distinct floral morphs that are mirror-image flowers, with the style and one anther deflected to either the right- or left-hand side of the plane of symmetry. Plants produce equal proportions of left- and right-styled flowers alternating at each node (Jesson & Barrett 2005). Experimental manipulations in artificial arrays of *S. rostratum* in Canada have demonstrated that this type of enantiostyly promotes outcrossing by reducing pollen transfer within flowers of the same morph within a plant (Jesson & Barrett 2005). Nevertheless, the mating system (*i.e.* the relative contribution of self-fertilisation and outcrossing to offspring production) has never been estimated for this species in natural populations where pollinator assemblages can be much more diverse and spatially heterogeneous (L. Solís-Montero, unpublished).

We hypothesise that the floral and inflorescence morphology of the annual *S. rostratum* facilitates a predominantly outcrossing mating system in natural populations exposed to diverse pollination environments, despite a lack of physiological mechanisms (*e.g.* self-incompatibility) preventing self-fertilisation. Taking advantage of recently developed microsatellite markers for *S. rostratum* (Vallejo-Marín *et al.* 2011), we addressed the following specific objectives: (i) estimation of outcrossing rates in natural populations of *S. rostratum* and (ii) determination of other mating parameters, including the contribution of mating between relatives to selfing rates (biparental inbreeding), and the average number of plants contributing to paternity of individual families. Our study provides a first step in understanding reproductive dynamics in natural populations of this annual species.

MATERIAL AND METHODS

Sampling design

To estimate mating parameters in *S. rostratum*, we sampled four Mexican populations (Table 1) in localities that represented the habitats where this species is commonly found (roadsides, and open or abandoned fields). These four populations were chosen to cover a large area at the core of the native distribution of this species (Whalen 1979). The distance between sampled populations ranged from 40 km (DOL and SLG) to 690 km (TEM and VDU). Between 20 October 2010

Table 1. Localities and sample sizes of the Mexican populations of *Solanum rostratum* used to study genetic diversity and mating system parameters.

Code	Population	Latitude (N)	Longitude (W)	Elevation (m)	Population size	Maternal families sampled	Genotyped offspring (mean; median; range per family)	Germination proportion (SE)
DOL	Dolores, Guanajuato	21.161°	100.886°	1913	150	19	179 (9.42; 10; 2–12)	0.723 (0.06)
SLG	San Luis La Paz, Guanajuato	21.309°	100.514°	2050	50	19	156 (8.21; 8; 5–12)	0.956 (0.02)
TEM	Teotihuacan, Estado de Mexico	19.683°	98.858°	2277	2000	19	187 (9.84; 10; 2–12)	0.763 (0.05)
VDU	Vicente Guerrero, Durango	23.744°	103.996°	1926	150	20	178 (8.90; 10; 3–12)	0.738 (0.06)
Total						77	700	

Population sizes are only approximate values estimated by one observer. Germination proportion represents the average across families in each population for 1,216 total seeds planted in the four populations.

and 17 January 2011 we collected seeds from up to 20 randomly chosen individuals per population, trying to sample four to five mature fruits per plant, although some individuals did not produce many fruits, and thus fewer fruits (and seeds) were collected. We randomly sampled individuals regardless of fruit production to avoid biasing our results to only plants with high fruit set. To facilitate drying of the material and to prevent fungal attack, we placed fruits in paper bags and kept them under dry conditions at room temperature or briefly placed them in a drying oven at 40 °C. We then extracted seeds from the fruits, and transported them to the University of Stirling, where they were kept at 5 °C in airtight containers.

To obtain material for genetic analysis, we collected leaf tissue from young seedlings (2–3 weeks post-germination), as a preliminary attempt to extract DNA directly from seeds yielded poor quality material that gave unreliable results. To maximise germination, we pre-treated seeds with a 1000 ppm solution of gibberellic acid for 24 h before planting. Between five and 20 seeds (depending on seed availability; see above) per family were planted in plastic trays containing fine soil, and placed in a heated glasshouse at the University of Stirling under 16 h supplemented light per day. Germination began 5–10 days after planting, and germination success at the time of tissue collection was high (79.5 ± 2.6%, family mean ± SE, n = 1,216 seeds). Up to 12 seedlings per family were randomly chosen for tissue collection; for families with few planted seeds we necessarily sampled fewer seedlings. In total we sampled between 156 and 187 seedlings per population for a total of 700 offspring belonging to 77 maternal families (average number of offspring per family = 9.09; median = 10, range = 2–12; Table 1).

DNA extraction and genotyping

We employed a modified CTAB protocol (Doyle & Doyle 1990) to extract DNA from leaves dried in silica gel, and quantified DNA yield using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA). We genotyped each individual at 13 microsatellite loci following the protocols described in Vallejo-Marín *et al.* (2011). These 13 microsatellite loci can be amplified in a single multiplex reaction, and include both di- and tri-nucleotide repeats. Each multiplex reaction contained 1 × Qiagen Type-it Microsatellite PCR kit (Qiagen, Crawley, West Sussex, UK), various concentrations of each of the 13 fluorescent forward primers labelled with one of 6-FAM (Eurofins MWG Operon, Ebersberg, Germany), VIC, PET or NED (Applied Biosystems, Foster City, CA, USA) dyes, reverse primers and 5–50 ng of template. We performed PCR cycles in a Veriti thermocycler (Applied Biosystems), and cycles consisted of a denaturing step of 5 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, 58 °C for 180 s, and 72 °C for 30 s, and a final elongation step of 30 min at 60 °C. We checked PCR products with a 3% agarose 1 × TBE electrophoresis, and sent them to DNA Sequencing and Services (Dundee, UK) for fragment analysis in an ABI 3730xl capillary sequencer with a GeneScan 500 LIZ internal size standard (Applied Biosystems).

Genetic and statistical analysis

We analysed fluorescence profiles using STRand (Toonen & Hughes 2001) and exported the data to MsatAllele (Alberto 2009) in R version 2.13.1 (R Core Development Team 2012) to assign peaks to a suitable allele bin range. We repeated the genotyping of a random sample of 11% of the individuals in three populations, and used Pedant (Johnson & Haydon 2007) to calculate per-allele genotyping errors. Pedant allows separately estimating stochastic errors introduced by allelic dropout (E1, where one allele of a heterozygote randomly fails to amplify) and false alleles (E2, where a true allele is mis-genotyped due to PCR artefacts or human errors when recording data; Johnson & Haydon 2007). We used the full data set, minus repeat genotypes, to calculate the frequency of null alleles per locus per population using an individual inbreeding model that simultaneously estimates null allele frequency and inbreeding coefficients, as implemented in the program INEst (Chybicki & Burczyk 2009). This approach avoids the potential upward bias in null allele frequency estimation that can arise when a population experiences some inbreeding. We used a Gibbs sampler with 10,000 iterations to calculate mean and SE for the estimated frequency of null alleles ($null_{IIM} \pm SE$), and assigned statistical significance at $P < 0.01$ if the value $null_{IIM} - 2.33*SE$ did not overlap zero. Because this test does not apply a correction for multiple comparisons, it represents a conservative detection level for the presence of null alleles.

For each population–locus combination, we calculated the number of alleles (N_a), average expected heterozygosity (H_e) and observed heterozygosity (H_o) using the program GENALEX 6.4 (Peakall & Smouse 2006). To calculate polymorphic information content (PIC) and exclusion probabilities per population–locus combination and across loci in each population we used the program CERVUS (Slate *et al.* 2004). We used FSTAT 2.9.3.2. (Goudet 1995) to estimate the inbreeding coefficient (F_{is}) of the progeny arrays, calculated separately for each population. We tested statistical significance of the inbreeding coefficients using 52,000 randomisations, and adjusted significance thresholds for multiple tests using a Bonferroni correction.

Mating system analysis

We used the genotypes of the progeny arrays to estimate mating system parameters using the computer program MLTR 3.2 (Ritland 2002). We performed calculations separately for each population, excluding loci with significant frequencies of null alleles (Table S1). However, including all 13 loci in the analysis had very little effect in the estimated outcrossing rates (data not shown), indicating that these results are robust to the inclusion of loci with null alleles. We calculated multilocus and single locus outcrossing rates (t_m and t_s, respectively). The difference $t_m - t_s$ can be used to estimate biparental inbreeding, the increase in homozygosity resulting from mating between relatives (Ritland 2002). Under biparental inbreeding, the magnitude of the difference $t_m - t_s$ should be positive, as single locus estimates of outcrossing rates will include apparent selfing due to mating between relatives. We also calculated two parameters of the correlated matings model: (i) r_t, the correlation of outcrossing among two members of a family, which is also the normalised variance in outcrossing rates among families (Ritland 2002), and (ii) r_p the correlation of paternity, which measures the probability that two siblings are outcrossed full-sibs (Ritland 1989). The correlation of paternity can also be estimated for single or multiple loci (r_{ps} and r_{pm}, respectively), and the difference $r_{ps} - r_{pm}$ can be employed to indicate

whether outcrossed matings within a progeny array occur between related males. For instance, a positive $r_{ps} - r_{pm}$ may occur when population substructure results in genetic similarity among male parents (Ritland 2002).

Mating system parameters were estimated using the expectation maximisation method, which is recommended for data sets with missing data (Ritland 2002), and allowing for the presence of undetected null alleles. Standard errors of the estimates were approximated as the standard deviation of 1000 bootstrap replicates, resampling maternal families. To assign statistical significance, we set a nominal level of $\alpha = 0.05$, and adjusted it for multiple comparisons using a Bonferroni correction ($\alpha_C = 0.05 / 4$ populations $= 0.0125$). The null hypothesis was rejected when a fraction $1 - \alpha_C$ of the bootstrap estimates exceeded the test value; the test value for the null hypothesis was zero for all mating parameters (*e.g.* $t_m = 0$; or $t_m - t_s = 0$; Barrett *et al.* 2004). Furthermore, to determine the potential biases on outcrossing rate estimation resulting from including maternal families with few genotyped offspring, we repeated the analyses excluding families with less than five offspring genotyped (8/77 families). However, the estimated mating parameters were very similar (*e.g.* difference in population outcrossing rates between the full and reduced data sets $\Delta t_m = -0.042$, 0.010, 0.009 and 0.018, for populations DOL, SLG, TEM and VDU, respectively; population average of reduced data set $t_m = 0.70 \pm 0.02$); below we present results of the analysis of the full data set only.

RESULTS

Genetic diversity

Table 2 shows a summary of the genetic diversity measured in the four populations of *S. rostratum*. All 13 microsatellite loci were polymorphic in each of the four populations, and possessed a low to moderate number of alleles per locus (3–14 across all populations) with intermediate polymorphic information content (mean per population = 0.39–0.51; Table 2). Expected average heterozygosity across loci ranged between 0.48 in population SLG to 0.57 in VDU (Table 2), with within-population values ranging from 0.019 (locus Sr9, SLG) to 0.84 (Sr5, TEM) (Table S1).

We detected a deficiency of heterozygotes across loci within populations, as indicated by the positive and highly statistically significant values of the inbreeding coefficient, F_{is} (Table 2). The individual inbreeding model detected the presence of null alleles in some loci with noticeably high inbreeding coefficients: Sr5 (DOL), Sr6 (DOL, SLG), Sr12 (TEM, VDU) and Sr22 (SLG) (Table S1). Combined exclusion probabilities with the 13 loci were high (Table 2). The analysis of repeat genotypes showed allelic dropout estimates (E1) undistinguishable from zero in 12 of 13 loci, and relatively low for the remaining one (point estimate 0.01 for Sr33; Table S2); the error rate arising from false alleles (E2) was estimated as zero for all 13 loci. The full results of the genetic diversity for each population–locus combination are presented in Table S1.

Outcrossing rate and mating system parameters

The populations of *S. rostratum* studied here showed intermediate to high outcrossing rates (Table 3). The average multilocus outcrossing rate across populations was 0.70 ± 0.03, ranging from 0.65 ± 0.07 (mean $\pm$ SE) in DOL to 0.77 ± 0.05 in the northernmost population of VDU. The 95% CI of the outcrossing rates for each population are shown in Fig. 1, and suggest that variation among the studied populations is modest. We found evidence for a statistically significant difference in the outcrossing rates calculated with single or multiple loci, indicating the presence of biparental inbreeding in two of the four populations. However, the contribution of mating between relatives to apparent selfing was small (Table 3).

The analysis of correlated mating patterns showed a low but significant correlation of outcrossing rates among siblings (r_t) in all populations (Table 3). This correlation was on average 13% of its maximum value of 1.0, indicating that outcrossing/selfing events occur with relative independence among maternal siblings. Similarly, the correlation of outcross paternity (r_{pm}) was low but significant in all populations (0.11 ± 0.01), suggesting that, on average, approximately one in ten maternal sibs are expected to be fathered by the same plant. The comparison between single and multilocus estimates of correlated paternity was not statistically different from zero, which indicates that correlated paternity does not occur *via* related male parents.

Table 2. Summary of genetic diversity, polymorphic information content and probabilities of exclusion in four populations of *Solanum rostratum* obtained by high-throughput genotyping at 13 microsatellite loci.

Population	N_{ind}	P	N_a (range)	PIC	PE_{sp}	PE_P	PE_C	PE_I	PE_{SI}	H_e (SE)	H_o (SE)	F_{is}
DOL	179	13	4.92 (2–12)	0.454	0.9283	0.9933	0.9997	>0.9999	0.9994	0.505 (0.061)	0.358 (0.042)	0.288**
SLG	156	13	4.54 (2–9)	0.437	0.9305	0.9935	0.9998	>0.9999	0.9992	0.481 (0.070)	0.342 (0.056)	0.286**
TEM	187	13	4.23 (2–8)	0.390	0.8682	0.9842	0.9992	>0.9999	0.9981	0.429 (0.062)	0.319 (0.050)	0.256**
VDU	178	13	5.00 (2–10)	0.507	0.9450	0.9959	0.9999	>0.9999	0.9998	0.566 (0.039)	0.451 (0.039)	0.203**
Species level	700	13	6.69 (3–14)	0.5315	0.9679	0.9983	>0.9999	>0.9999	0.9998	0.579 (0.050)	0.368 (0.037)	0.256**

The full results for each population-locus combination are given in Table S1. N_{ind} = Number of individuals genotyped; P = number of polymorphic loci; N_a = average number of alleles per locus; H_e = unbiased heterozygosity and H_0 = observed heterozygosity were calculated using GENALEX (Peakall & Smouse 2006). Inbreeding coefficients (F_{is}) and associated P-values were determined using 52,000 permutations and adjusting significant thresholds using a Bonferroni correction in FSTAT (Goudet 1995). PIC = Polymorphic information content and various exclusion probabilities were computed using CERVUS (Slate *et al.* 2004); PE_{sp} = combined probability of exclusion of a single parent; PE_P = combined exclusion for paternity (mother known); PE_C = combined exclusion probability of a parent pair; PE_j = combined exclusion probability of identity; PE_{si} = combined exclusion probability of identity among outcrossed sibs. Species level values were calculated on a combined data set of all populations. **P-value < 0.001.

Table 3. Mating system parameters in four Mexican populations of *Solanum rostratum*.

Population	N_{ind}	# Loci included	Loci excluded	t_m	t_s	$t_m - t_s$	r_t	r_{pm}	r_{ps}	$r_{ps} - r_{pm}$
DOL	179	11	Sr5, Sr6	0.646** (0.066)	0.596** (0.064)	0.050* (0.030)	0.160** (0.058)	0.100** (0.026)	0.100** (0.019)	0^{NS} (0.017)
SLG	156	11	Sr6, Sr22	0.683** (0.051)	0.611** (0.048)	0.072* (0.028)	0.076** (0.051)	0.131** (0.036)	0.107** (0.020)	-0.025^{NS} (0.025)
TEM	187	12	Srl2	0.696** (0.028)	0.662** (0.059)	0.034^{NS} (0.024)	0.180** (0.092)	0.110** (0.022)	0.087** (0.009)	-0.023^{NS} (0.018)
VDU	178	12	Sr12	0.773** (0.048)	0.704** (0.053)	0.068* (0.025)	0.119** (0.048)	0.109** (0.034)	0.096** (0.024)	-0.013^{NS} (0.022)

For each population, the microsatellite loci with significant frequencies of null alleles were excluded (Table SI). The following mating parameters were calculated in MLTR (Ritland 2002), using expectation-maximisation, and setting pollen and ovule frequencies to be equal: multilocus outcrossing rate (t_m), single-locus outcrossing rate (4), correlation of outcrossing rate (r_t), correlation of paternity within sibships for single and multilocus cases (r_{ps} and r_{pm}, respectively), biparental inbreeding ($t_m - t_s$, and extent of outcrossed paternity by related male parents ($r_{ps} - r_{pm}$,). SE shown in parentheses and calculated from 1000 bootstrap replicates by resampling maternal families. *P*-values were obtained from the bootstrap sample as described in the Material and Methods. The null hypothesis for significance testing was in all cases that the mating ; parameter was zero; *e.g* $t_m = 0$ Or $t_m - t_s = 0$. *$P < 0.05$, **$P < 0.01$, NS $P > 0.05$. Bold-face values indicate significance after Bonferroni correction for multiple comparisons among the four populations.

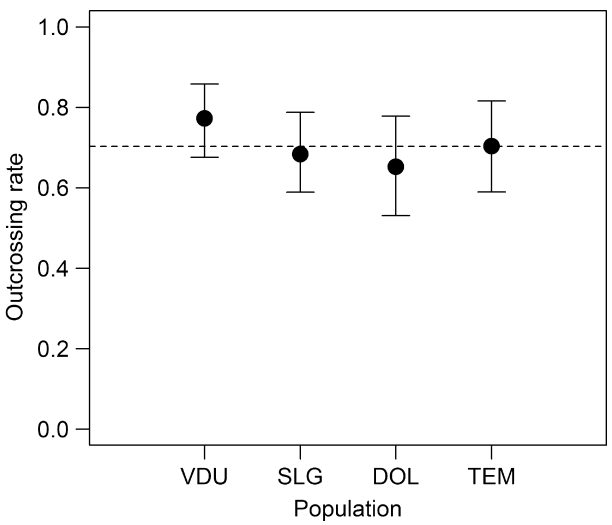

Fig. 1. Multilocus outcrossing rates (t_m) and associated 95% confidence intervals in four Mexican populations of *Solanum rostratum*. The dashed line shows the average outcrossing rate across populations ($\overline{t_m} = 0.70 \pm 0.03$). Population names as in Table 1.

DISCUSSION

We found that *S. rostratum* can be described as mixed-mating ($0.20 \leq t \geq 0.80$; Schemske & Lande 1985), although the range of outcrossing rates observed (t_m = 0.65–0.77) falls towards the high end of the mixed mating spectrum. The high outcrossing and multiple paternity within progeny arrays observed here also suggest a reliance on pollen transfer between plants for the reproduction of this species. We hypothesise that a predominantly outcrossing system in this annual, self-compatible species is probably facilitated by its floral morphology.

Factors influencing outcrossing rate in *S. rostratum*

Outcrossing in *S. rostratum* could be facilitated by the strong herkogamy of its flowers, which should reduce within-flower selfing (Webb & Lloyd 1986). In a preliminary field experiment, in which we excluded pollinators using mesh bags, we found that excluding pollinators successfully prevented fruit and seed formation (L. Solís-Montero, unpublished), suggesting that the realised capacity for autonomous self-fertilisation in *S. rostratum* is very low. During the legitimate visits of medium to large bees, heteranthery results in the deposition of pollen in specific locations on the pollinator's body (*e.g.* the pollinating anther deposits pollen opposite to the stigma; Bowers 1975; Jesson & Barrett 2005; Vallejo-Marin *et al.* 2009). Because pollen deposition and pick-up occur on opposite sides of the pollinator's body (Bowers 1975; Jesson & Barrett 2005), floral morphology should thus reduce pollinator-assisted selfing within flowers (Lloyd & Schoen 1992). However, as buzz pollination can produce small pollen clouds around the pollinator's body, within-flower selfing may still arise during visitation. In particular, self-pollination may occur when smaller bees and other opportunistic insects illegitimately manipulate the flower (with or without buzzing; Renner 1989; M. Vallejo-Marín and L. Solís-Montero, personal observations).

Theory predicts that the probability of geitonogamous selfing by legitimate pollinators should be reduced by monomorphic enantiostyly (Jesson *et al.* 2003; but see Dulberger 1981; Fenster 1995), and experimental arrays of *S. rostratum* have confirmed this expectation (Jesson & Barrett 2005). Nevertheless, geitonogamy can still occur in large individuals of *S. rostratum,* which simultaneously present left- and right-styled flowers in the same plant or inflorescence (Fenster 1995; Jesson *et al.* 2003). Our field surveys have found that individual plants in fact may display numerous open flowers at the same time, with large individuals displaying up to 98 flowers open concurrently (VDU population, unpublished results). These observations indicate that geitonogamous selfing is indeed a very likely possibility in natural populations of *S. rostratum*. In summary, floral morphology – herkogamy, deposition of pollen in different areas of the pollinator's body though heteranthery – and inflorescence architecture (alternating floral morphs along an inflorescence) of *S. rostratum* are both expected to reduce self-fertilisation, while the residual selfing observed may partly reflect the action of illegitimate visitors and the imperfect avoidance of geitonogamous selfing arising as a consequence of producing large floral displays.

Potential effects of post-pollination processes on observed outcrossing rates in *S. rostratum*

The actual value of outcrossing rate measured here represents an upper estimate of the primary outcrossing rate because it does not account for differential post-zygotic mortality between syngamy and germination of selfed and outcrossed offspring (Charlesworth & Charlesworth 1987). To date, no evidence is available to suggest differential mortality of selfed and outcrossed embryos in *S. rostratum*, as hand-pollinations reveal no difference in either fruit set or the number of seeds produced per fruit between cross- and self-fertilisation treatments (Bowers 1975). In our study, seed germination was high across populations, and the outcrossing rate was not correlated with germination proportion. However, Jesson & Barrett (2005) estimated outcrossing rates with allozymes at both the seed and seedling stages in *S. rostratum*, and found that outcrossing rates in experimental arrays in Canada were higher when measured in seedlings than when measured in seeds ($t = 0.81 \pm 0.09$ *versus* $t = 0.71 \pm 0.08$, respectively). Based on these results, we expect that if a bias is introduced into our data set by inbreeding depression acting between germination and seedling establishment, its magnitude on the average outcrossing rate per population should be moderate and would not qualitatively affect our conclusions. Further work, however, is required to determine the amount of inbreeding depression in *S. rostratum*.

Patterns of correlated paternity

The estimation of correlated paternity provides insight on the dynamics of outcross paternity in natural populations of *S. rostratum*. For the four populations studied here, the effective number of sires per progeny array was 8.97 ± 0.57 ($N_{ep} = 1/\overline{r_{pm}}$) (Hardy *et al.* 2004). It is important to note that the estimated patterns of correlated paternity were done in a pooled sample of offspring of three to five fruits per plant, and provides only a snapshot of the patterns of correlated paternity in each population. Analysing more fruits per individual is likely to increase the number of sires per progeny array, although the variation in sire number among different fruits will depend on whether flowers open at different times of the flowering season and experience different pollination environments. Nevertheless, the average number of sires for *S. rostratum* falls on the upper side of the range of values available for other herbaceous species, in which usually only a subset of the total fruit production is analysed (Hardy *et al.* 2004). Number of sires per progeny array in these species ranges from 3.24 ± 0.56 in 13 populations of the self-incompatible daisy *Rutidosis leptorrhynchoides* F. Muell. (Young & Brown 1999) to 8.39 ± 2.11 in eight populations of the yellow starthistle *Centaurea solstitialis* L. (Sun & Ritland 1998). As the number of sires is influenced by whether plants receive deposits of pollen from the same or different individuals during a single visit and by whether mating occurs with a limited number of near–neighbours (Sun & Ritland 1998), the relatively low levels of correlated paternity detected here for *S. rostratum* may indicate that outcross pollination involves pollen from multiple and often unrelated individuals (as shown by the non-significant $r_{ps} - r_{pm}$).

Selection for cross-fertilisation in *S. rostratum*

Our study suggests that natural populations of *S. rostratum* are characterised by relatively high outcrossing rates, and we have argued that mechanistically, cross-fertilisation may be facilitated by floral and inflorescence characteristics. However, it remains to be established whether the theoretical conditions expected to result in selection for outcrossing or mixed mating in colonising species are met in *S. rostratum*. For example, pollinator observations carried out in Central Mexico indicate large variation in the identity and number of pollinators in different populations (M. Vallejo-Marín and L. Solís-Montero, unpublished). In addition, preliminary studies of pollen supplementation in the field, suggest that there is heterogeneity in the extent to which seed set in different populations is pollen limited (L. Solís-Montero, unpublished). These preliminary results are consistent with heterogeneity in pollination environments and fitness across patches, which is one of the prerequisites necessary for the correlated evolution of outcrossing and dispersal predicted by some theoretical models (*e.g.* Massol & Cheptou 2011). Unfortunately, it is currently not known whether *S. rostratum* also presents other features facilitating the maintenance of outcrossing in colonising metapopulations, such as seed banks (Pannell & Barrett 1998). Further studies on the heterogeneity in reproductive success among populations, the presence of seed banks and the level of inbreeding depression under field conditions will help determining whether the theoretical conditions favouring outcrossing in colonising populations are met in this species.

CONCLUSIONS

Our study characterises the mating patterns of natural populations of the annual herb *S. rostratum* using a set of newly developed microsatellite markers that allow high-throughput genotyping. We show that outcrossing rates are relatively high in this annual, self-compatible species. We speculate that high outcrossing rates in natural populations of *S. rostratum* growing in periodically disturbed environments (*e.g.* roadsides and fallow fields) are facilitated by a combination of herkogamy, precise pollen placement due to heteranthery and enantiostyly. Although future experimental work is required to test these hypotheses, *S. rostratum* illustrates the potential for floral morphology to contribute to the maintenance of outcrossing in short-lived species growing in human-altered environments.

ACKNOWLEDGEMENTS

We thank J. Fornoni, R. Perez, N. Suarez, C. Castillo and C. Dominguez for support during fieldwork; L. Mitchell for assistance in the glasshouse; O. Lepais for advice in genetic analysis; and S. Barrett, the associate editor J. Arroyo, and three anonymous reviewers for insightful comments on an earlier version of the manuscript. This work was partially funded by a Horizon PhD studentship from the University of Stirling to LSM, a summer internship grant from Stirling's Biological and Environmental Sciences Department to DS, and a Royal Society of London Research Grant (RG2010R1) and a Scottish Plant Health Licence (PH/38/2010) to MVM.

SUPPORTING INFORMATION

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

Table S1. Characteristics of microsatellite loci for *Solanum rostratum* presented for each population–locus combination.

Table S2. Genotyping error rates for the 13 microsatellite loci.

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