

## Seasonal variation in the mating system of a selfing annual with large floral displays

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• **Background and Aims** Flowering plants display considerable variation in mating system, specifically the relative frequency of cross- and self-fertilization. The majority of estimates of outcrossing rate do not account for temporal variation, particularly during the flowering season. Here, we investigated seasonal variation in mating and fertility in *Incarvillea sinensis* (Bignoniaceae), an annual with showy, insect-pollinated, ‘one-day’ flowers capable of delayed selfing. We examined the influence of several biotic and abiotic environmental factors on day-to-day variation in fruit set, seed set and patterns of mating.

• **Methods** We recorded daily flower number and pollinator abundance in nine 3 × 3-m patches in a population at Mu Us Sand land, Inner Mongolia, China. From marked flowers we collected data on daily fruit and seed set and estimated outcrossing rate and biparental inbreeding using six microsatellite loci and 172 open-pollinated families throughout the flowering period.

• **Key Results** Flower density increased significantly over most of the 50-d flowering season, but was associated with a decline in levels of pollinator service by bees, particularly on windy days. Fruit and seed set declined over time, especially during the latter third of the flowering period. Multilocus estimates of outcrossing rate were obtained using two methods (the programs MLTR and BORICE) and both indicated high selfing rates of ~80 %. There was evidence for a significant increase in levels of selfing as the flowering season progressed and pollinator visitation declined. Biparental inbreeding also declined significantly as the flowering season progressed.

• **Conclusions** Temporal variation in outcrossing rates may be a common feature of the mating biology of annual, insect-pollinated plants of harsh environments but our study is the first to examine seasonal mating-system dynamics in this context. Despite having large flowers and showy floral displays, *I. sinensis* attracted relatively few pollinators. Delayed selfing by corolla dragging largely explains the occurrence of mixed mating in *I. sinensis*, and this mode of self-fertilization probably functions to promote reproductive assurance when pollinator service is limited by windy environmental conditions and competition from co-occurring flowering plants.

**Key words:** corolla dragging, delayed selfing, *Incarvillea*, mixed mating, pollinator service, reproductive assurance, sand desert.

### INTRODUCTION

Mating patterns vary considerably within and among species of flowering plants, particularly those that are self-compatible. Various reproductive, environmental and demographic factors influence outcrossing rates and determining their relative importance is a key issue in plant evolutionary ecology (Lloyd, 1979; Barrett and Husband, 1990; Eckert *et al.*, 2010; Karron *et al.*, 2012). The increased use of genetic markers has enabled quantitative measurements of mating patterns and these have provided numerous insights into the ecological and genetic correlates of outcrossing rate (Schemske and Lande, 1985; Barrett and Eckert, 1990; Goodwillie *et al.*, 2005). Although mating patterns are now routinely measured, the vast majority involve a single mean estimate of outcrossing based on a sample of open-pollinated seed families collected from a population at one time. There have been relatively few studies of temporal

variation in mating patterns and virtually all report year-to-year variation (e.g. Cheliak *et al.*, 1985; Barrett *et al.*, 1993; Eckert *et al.*, 2009; Coates *et al.*, 2013). Very little is known about seasonal variation in plant mating systems and how environmental factors may influence changes in outcrossing rate. For animal-pollinated species with extended blooming periods and mixed mating, a single sample of open-pollinated families is unlikely to capture the true extent of variation in patterns of mating.

Among the diverse factors influencing mating patterns in self-compatible plants, environmental conditions affecting the quantity and quality of pollinator service are of particular importance. Infrequent pollinator visitation to flowers owing to unfavourable environmental conditions (Schemske *et al.*, 1978; Fan *et al.*, 2012), competition from co-occurring species (Levin, 1972; Bell *et al.*, 2005) or low pollinator densities in mass flowering populations (Barrett, 1980; Delmas *et al.*, 2015) are among the many ecological factors that can cause pollen

limitation of seed set (Ashman *et al.*, 2004; Knight *et al.*, 2005). Many animal-pollinated species possess mechanisms of autonomous self-pollination that relieve pollen limitation of seed set caused by unreliable pollinator service (Lloyd, 1979; Schoen and Brown, 1991). However, among the various modes of autonomous self-pollination (Lloyd, 1979; Barrett and Harder, 1996), only delayed selfing is considered adaptive because it does not generally result in significant costs from pollen or seed discounting (Lloyd, 1992). Delayed self-pollination therefore has the potential to provide reproductive assurance if plants receive insufficient outcross pollen to fertilize available ovules (Kalisz *et al.*, 2004; Eckert *et al.*, 2006; Fenster and Martén-Rodríguez, 2007). Indeed, delayed selfing is probably an important reproductive factor contributing to the significant proportion (approx. 42 %) of flowering plant species that possess mixed mating systems (Goodwillie *et al.*, 2005). The extent of delayed selfing can be investigated by examining the temporal dynamics of mating patterns in relation to pollinator visitation. Mating in plant populations is often context dependent, depending on a variety of biotic and abiotic factors influencing pollinator service within and between seasons.

*Incarvillea* (Bignoniaceae) comprises 18 species of temperate herbs largely endemic to the Himalaya–Hengduan mountain regions of China (Zhang and Santisuk, 2003; Chen *et al.*, 2005). Members of the genus possess large showy pink, red or yellow flowers and several are used in ornamental horticulture. Flowers of *Incarvillea* exhibit several specialized floral structures, including a bilobed sensitive stigma (Ai *et al.*, 2013), as occurs in other members of the Bignoniaceae (Milet-Pinheiro *et al.*, 2009), and conspicuous anther appendages that function as a pollen-dispensing mechanism (Cutting, 1921; Han *et al.*, 2008; Verma *et al.*, 2008). Most species are bee-pollinated, alpine perennials and are presumably outcrossing, at least to some degree. *Incarvillea mairei* is the only species for which the mating system has been quantified and multilocus outcrossing rates ranged from 0.97 to 0.99 in three self-compatible populations (Ai *et al.*, 2013). Thus, at least in this species the specialized floral morphology is associated with high outcrossing rates. However, apparently specialized pollination mechanisms can also be associated with delayed selfing, particularly when pollinator service is unreliable (Fenster and Martén-Rodríguez, 2007), and observations of pollinator visitation to *Incarvillea* spp. indicate that populations are often pollen-limited (Qu *et al.*, 2007; Verma *et al.*, 2008; Ai *et al.*, 2013). Under these circumstances floral adaptations promoting outcrossing may be compromised and mating patterns have the potential to include a significant selfing component.

*Incarvillea sinensis* is a widely distributed species ranging from south-west to north-east China and to the Russian Far East. It occurs over a broad range of ecological conditions and includes perennial populations and the only known annual populations in the genus (Chen *et al.*, 2005, 2012). A previous study of an annual population of *I. sinensis* var. *sinensis* (hereafter *I. sinensis*) from a sand desert in Inner Mongolia characterized by harsh conditions including strong winds, documented a novel mechanism of delayed selfing, involving wind-induced corolla dragging, and a pollen–ovule ratio indicative of obligate autogamy (Qu *et al.*, 2007). However, the population also possessed showy pink flowers, bilobed stigmas and anther appendages, similar to outcrossing *Incarvillea mairei* (Ai *et al.*, 2013),

and the sole pollinators at the study site (*Anthophora borealis* and *Bombus sporadicus*) were observed visiting flowers, suggesting some degree of outcrossing. *Incarvillea sinensis* blooms for an extended period and possesses ‘one-day’ flowers (unlike other *Incarvillea* species) enabling the study of day-to-day variation in the pollination environment on reproduction. These features motivated us to investigate whether seasonal variation in environmental conditions throughout the blooming period of *I. sinensis* may provide an explanation for the co-occurrence of both outcrossing and selfing floral adaptations in this species.

Here, we investigate further the sand desert population of *I. sinensis* studied by Qu *et al.* (2007) by examining the dynamics of mating and fertility during the blooming period in relation to flower abundance, pollinator visitation and wind speed. Our study addressed the following specific questions: (1) Does the mating system of *I. sinensis* vary during the blooming period as a result of the influence of biotic (pollinators) and abiotic (wind) factors? We predicted that if pollinator service was unreliable, the population would exhibit a mixed mating system, with increased selfing associated with reduced pollinator activity. (2) Is there evidence for temporal variation during the flowering season in components of female fertility (i.e. fruit and seed set)? We predicted that maternal reproductive success would be associated with changes in flowering density and pollinator abundance. Our study is the first to investigate the seasonal dynamics of mating patterns in relation to pollinator visitation in a plant population and illustrates how ‘point estimates’ of mating patterns in self-compatible species may mask considerably variability.

## MATERIALS AND METHODS

### *Study species and site*

*Incarvillea sinensis* Lamarck var. *sinensis* produces 5–50 rose-red, funnel-form perfect flowers on racemose inflorescences (Fig. 1). Most individuals have only a single flower open per day and these last for 9 h, opening in the early morning and closing in early afternoon (0230–1400 h); at our study area populations bloom from early July to late August. The large (2.5–4.0 cm) insect-pollinated flowers are showy and possess sensitive stigmas composed of two round lobes (Yang *et al.*, 2004; Yang, 2005; Fig. 1A). The four stamens in each flower are didynamous and epipetalous, with anthers pressed closely against the style. Pistils of *I. sinensis* produce on average 165–81 ovules (range 44–263). Experimental studies indicate that *I. sinensis* is highly self-compatible, non-dichogamous, and at the end of anthesis the abscising corolla is moved away from the ovary base by wind resulting in contact between the anthers and stigmas, a mechanism of delayed self-pollination known as ‘corolla dragging’ (Qu *et al.*, 2007).

Our study was conducted at Mu Us Sand land (39°02′N, 109°51′E), a semi-arid desert in the south-east of the Ordos Plateau in Inner Mongolia, China. This area has constant winds that blow for more than 200 d each year. The population we examined was large (approx. 3500 plants) and composed of dense flowering patches at peak blooming (Fig. 1C). At our study site there were several other sympatric flowering species, particularly *Hedysarum leave* (Fabaceae) and *Leonurus artemisia* (Lamiaceae), which flower from mid-July to September and



FIG. 1. The showy floral displays of *Incarvillea sinensis* at the study site in Inner Mongolia, China. (A) Flower illustrating the bilobed stigma (arrow) and four epipetalous didynamous stamens. (B) Flower being visited by *Anthophora borealis*. Note the pollen grains on the stigma. (C) Showy floral display at peak flowering in the population investigated in this study.

share pollinators with *I. sinensis*. Our observations confirmed earlier reports that the only pollinators at the study site were *Anthophora borealis* and *Bombus sporadicus*. Further details of the study site and floral biology of the population are presented in [Qu et al. \(2007\)](#).

#### *Flowering phenology, pollinator observations and wind speed*

We selected nine  $3 \times 3$ -m patches approx. 20 m apart using line transects positioned throughout the population to

investigate changes in flower density during the blooming period from July to August 2012, and for pollinator observations and the sampling of open-pollinated fruits for mating system analysis (see below). [Table 1](#) provides information on the sample sizes for the number of individuals, flowers and progeny, and the mean and median number of progeny sampled per maternal individual from each patch. We conducted a daily census of the number of flowers and pollinators visiting each patch for 50 and 44 d, respectively, during the flowering period. Pollinator observations were conducted for 20 min per patch

from 0700 to 1200 h, with a different order of patches each day. To assess the importance of wind speed on pollinator activity and wind-dragged delayed selfing, we obtained data on the mean maximum wind speed per hour between 1000 and 1400 h each day from Ordos Sand land Ecological Research Station, Chinese Academy of Sciences. The location of this research station is about 100 m from our study site. The mean maximum wind speed per day is defined as the mean values of maximum wind speed per hour. Wind speeds were recorded for all but 2 d from Day 16 to 44 of the blooming period. Prior to this period data were not available.

#### Seasonal variation in fertility and mating system

**Sampling.** From early July to mid-August (44 d), we randomly marked approx. 30 open flowers each day among the sample of plants (one flower per individual) and later collected a total of 1051 mature capsules from 172 maternal plants among the nine patches. Supporting Information Table S1 provides information on the sample sizes each day during the flowering season. Given the large sampling effort required in this study, more than one capsule per plant was sampled from a majority of plants in the study to maintain daily sample sizes. Table S2 provides information on the number of fruits sampled per plant during the entire season. We used this information to investigate daily variation in per cent fruit set and seed set during the flowering season. Seed set was estimated by counts of the number of mature seeds, aborted seeds and unfertilized ovules per capsule and was obtained from the mean seed set values from the approx. 30 flowers marked each day. In this species aborted seed are easily distinguished from mature seed, as they are shrivelled and about one-tenth the size of a viable seed. We sowed 10–20 seeds from each capsule separately in germination trays in a glasshouse at the Institute of Botany, Chinese Academy of Sciences, and after 2 weeks we randomly chose two seedlings per capsule ( $n = 2041$  seedlings from 172 maternal parents) for analysis of mating patterns.

**DNA extraction and genotyping.** We extracted genomic DNA from the leaves of all maternal plants and seedlings using the DP305 Plant Genomic DNA Extraction Kit (Tiangen, Beijing, China). We amplified the DNA of adults and seedlings with six microsatellite loci: 01, 03, 05, 08, 821 and 831 (Yu *et al.*, 2011) and the forward primer for each locus was labelled with TAMRA (01, 03), HEX (05, 08) and FAM (821, 831). We performed PCR in a total volume of 20  $\mu$ L, containing 5.0 ng of template DNA, 2  $\mu$ L 10  $\times$  PCR buffer, 10 mM dNTPs mixture, 0.25  $\mu$ M of each primer and 1 U of *Taq* DNA polymerase. The PCR profile consisted of an initial denaturation step of 10 min at 94  $^{\circ}$ C followed by 35 cycles of 45 s at 94  $^{\circ}$ C, 30 s at an annealing temperature of 53–58  $^{\circ}$ C and 45 s at 72  $^{\circ}$ C, and a final extension step of 10 min at 72  $^{\circ}$ C. We ran the PCR products on an ABI 3730 automatic sequencer (Applied Biosystems, Foster City, CA, USA) and genotypes were analysed using Genemapper software version 4.0 and results were checked three times. We tested for the presence of null alleles using MICRO-CHECKER (van Oosterhout *et al.*, 2004).

**Mating system estimation.** We calculated summary statistics for the six microsatellite loci: the number of alleles per locus ( $N_a$ ),

expected heterozygosity ( $H_e$ ), observed heterozygosity ( $H_o$ ) and inbreeding coefficients ( $F_{is}$ ) per locus based on maternal individuals, using GENALEX version 6.501 (Peakall & Smouse, 2006). We estimated outcrossing rates using two different methods: MLTR version 3.4 (Ritland, 2002) and the more recent program BORICE (Koelling *et al.*, 2012), and compared the results. The maximum-likelihood computer program MLTR calculates the multilocus ( $t_m$ ) and single-locus ( $t_s$ ) outcrossing rates using the Newton–Raphson iteration based on the mixed mating model. The difference between the two parameters ( $t_m - t_s$ ) provides an estimate of biparental inbreeding. We estimated all mating system parameters at the population level, and standard errors and 95 % confidence intervals were obtained from 1000 bootstrap replicates. The program BORICE (Koelling *et al.*, 2012) provides more accurate estimates of outcrossing rate when family sizes are small and maternal genotypes are unknown, although in our case maternal genotypes were known. For BORICE, we used a chain of 100 000 steps with a burn in for the first 10 000 steps. The outcrossing rate tuning parameter, allele frequency tuning parameter and initial population outcrossing rate were set as 0.05, 0.1 and 0.5, respectively.

**Statistical analyses.** We used independent sample *t*-tests to determine whether there were significant differences in estimates of mean maximum wind speed between days with and without pollinator visitation to flowers. We examined the relationship between sampling day in the flowering period and pollinator number, multilocus outcrossing rate and biparental inbreeding using linear regression. We also examined the relationship between sampling day and daily flower number, fruit set and seed set with second-degree polynomials. To investigate the extent to which non-independence of samples collected from the same individual may have influenced temporal variation, we analysed seed category data (outcrossing or selfing, recorded as 1 or 0) using a generalized linear mixed model (GLMM, with binomial errors for the response variable), where time (day) was included as a continuous explanatory variable, and maternal plant ID as a random factor. We used independent sample *t*-tests to determine whether there were significant differences in estimates of outcrossing rate from MLTR and BORICE. For the analysis of outcrossing rate and biparental inbreeding we accounted for the sampling error associated with each daily estimate by weighting the estimate by the inverse of its squared standard error (see Neter *et al.*, 1990; Barrett *et al.*, 1994). Analyses were performed with SPSS version 20 (IBM Statistics), JMP 7 (SAS, 2007) and package lme4 v.1.1-8 (<https://cran.r-project.org/web/packages/lme4/index.html>) in *R* (CRAN; <http://www.r-project.org/>).

## RESULTS

### *Flower density, pollinator visitation and wind speed during the flowering season*

During the 50-d flowering season of *I. sinensis*, we recorded a total of 5124 flowers among the nine patches. The number of flowers per day ranged from 11 to 323 (mean  $\pm$  s.d. = 119.16  $\pm$  61.23) and increased continuously as the flowering season progressed but with a steep decline at the end of the

TABLE 1. Details of sample sizes for the nine patches of *Incarvillea sinensis* investigated in this study

Patch	No. of individuals	No. of flowers	No. of maternal individuals sampled*	Progeny sampled	Mean no. of progeny per maternal individual	Median no. of progeny per maternal individual
1	15	369	12	238	19.83	14
2	56	670	33	326	9.88	8
3	8	261	8	198	24.75	22
4	16	613	15	220	14.67	14
5	21	649	19	260	13.68	12
6	27	369	19	195	10.26	9.5
7	44	569	36	241	6.69	6
8	62	732	6	8	1.33	6
9	33	1025	24	355	14.79	14
Total	282	5257	172	2041	115.90	91.5
Mean	31.33	584.11	19.11	226.78	23.18	11.44
Median	27.00	613.00	19.00	238.00	13.68	12

\* The number of maternal individuals sampled is less than the number of marked individuals because of damage to plants in some plots, for example plot 8.

season (Fig. 2A:  $F_{2,40} = 17.42$ ;  $P < 0.0001$ , Flowers =  $21.39 + 11.29 \times \text{Day} - 0.22 \times \text{Day}^2$ ;  $r^2 = 0.47$ ). Our daily censuses of insects visiting flowers recorded a total of 73 pollinators during the entire flowering season with the vast majority of visits by *Bombus sporadicus* and *Anthophora borealis* (Fig. 1B). We observed frequent contact between these bees and the sexual organs of *I. sinensis* and the occurrence of pollen on their bodies, thus confirming the earlier findings of Qu *et al.* (2007) that these two species of bees are effective pollinators. The number of pollinators observed per day was generally low, ranging from 0 to 7 (mean  $\pm$  s.d. =  $1.74 \pm 1.88$ ) and on 10 of the 44 days we observed no activity, mostly as a result of wind. Mean maximum wind speed was higher on days with no pollinator activity ( $8.00 \pm 3.00 \text{ m s}^{-1}$ ) than on days in which pollinators were observed visiting flowers ( $6.12 \pm 1.68 \text{ m s}^{-1}$ ); however, there was no significant difference between these values ( $P = 0.196$ ). Overall pollinator numbers decreased significantly from the beginning to the latter part of the flowering season (Fig. 2B:  $F_{1,35} = 4.68$ ;  $P = 0.038$ , Pollinators =  $3.03 - 0.05 \times \text{Day}$ ,  $r^2 = 0.12$ ). There was no evidence for a change in the relative abundance of the two pollinator species during the flowering season (Fig. S1). Estimates of mean maximum wind speed per day in the area close to where our study population is located were mostly higher than  $2 \text{ m s}^{-1}$  between 1000 and 1400 h from Day 16 of the flowering period onwards (Fig. 2C). High wind speeds  $> 2 \text{ m s}^{-1}$  are sufficient to result in delayed selfing by corolla dragging (Qu *et al.* 2007).

#### Fruit and seed set during the flowering season

Daily fruit set ranged from 28.0 to 98.3 % (mean  $\pm$  s.d. =  $83.9 \pm 15.4$  %) with a steep decline evident during the last third of the flowering period [Fig. 3A:  $F_{2,35} = 48.08$ ;  $P < 0.001$ , predictive equation for % fruit set  $d^{-1} = 109.24 - 0.81 \times \text{Day} - 0.05 \times (\text{Day} - 21.37)^2$ ,  $r^2 = 0.73$ ]. Per cent seed set was consistently high (82.3–95.9 %, mean  $\pm$  s.d. =  $92.2 \pm 3.6$  %) but also decreased as the season progressed [Fig. 3B:  $F_{2,35} = 7.66$ ;  $P = 0.002$ , predictive equation for % seed set  $d^{-1} = 95.80 - 0.077 \times \text{Day} - 0.012 \times (\text{Day} - 21.37)^2$ ,  $r^2 = 0.30$ ].

#### Genetic diversity, outcrossing rate and biparental inbreeding

The six microsatellite loci that we investigated in *I. sinensis* yielded 41 alleles with an effective number of 3–10 alleles per locus. The expected heterozygosity ( $H_e$ ) and the observed heterozygosity ( $H_o$ ) per locus ranged from 0.29 to 0.83 and from 0.06 to 0.23, respectively. The inbreeding coefficients ( $F_{is}$ ) varied from 0.71 to 0.81 per locus (Table 2). Using MICRO-CHECKER we detected more homozygosity than expected under Hardy–Weinberg equilibrium at all six loci ( $P > 0.05$ ), as expected given the high selfing rates that we measured in the population (see below).

Based on the results from MLTR, the multilocus outcrossing rate ( $t_m$ ) per day ranged from 0.05 to 0.50 (mean  $\pm$  s.d. =  $0.23 \pm 0.01$ ) and exhibited a gradual but significant decline during the flowering period (Fig. 4A:  $F_{1,36} = 14.51$ ;  $P = 0.0005$ ; predictive equation for daily estimate of outcrossing rate =  $0.297 - 0.0037 \times \text{Day}$ ,  $r^2 = 0.29$ ). Both the intercept and the slope were significant (intercept  $t = 11.74$ ,  $P < 0.000$ ; slope  $t = -43.81$ ,  $P < 0.0005$ ). Using BORICE, the posterior distributions for outcrossing rate ( $t_{max}$ ) per day ranged from 0.04 to 0.50 (mean  $\pm$  s.d. =  $0.23 \pm 0.09$ ) and showed a similar trend to those obtained for  $t_m$  using MLTR (Fig. 4B:  $F_{1,36} = 24.49$ ;  $P < 0.001$ ; predictive equation for daily estimate of outcrossing rate =  $0.327 - 0.0045 \times \text{Day}$ ,  $r^2 = 0.40$ ). There was no significant difference in the mean daily outcrossing rate obtained from MLTR and BORICE ( $t = 0.381$ , d.f. = 74,  $P = 0.705$ ). When we controlled for the non-independence of samples (81.98 % of plants in the total sample), by taking into account maternal plant identity, the probability of outcrossing decreased over time, although the influence was only marginally significant (GLMM,  $Z = -1.744$ ,  $P = 0.081$ ).

Daily estimates of biparental inbreeding ranged from  $-0.03$  to 0.14 (mean  $\pm$  s.d. =  $0.06 \pm 0.01$ ) and also showed a significant downward trend with lower levels of biparental inbreeding at the end of the season (Fig. 5:  $F_{1,36} = 16.39$ ,  $P = 0.0003$ , predictive equation for daily estimate of biparental inbreeding =  $0.038 - 0.0005 \times \text{Day}$ ,  $r^2 = 0.29$ ). Both the intercept and the slope were significant (intercept  $t = 11.30$ ,  $P < 0.0001$ ; slope  $t = -4.05$ ,  $P = 0.0003$ ).

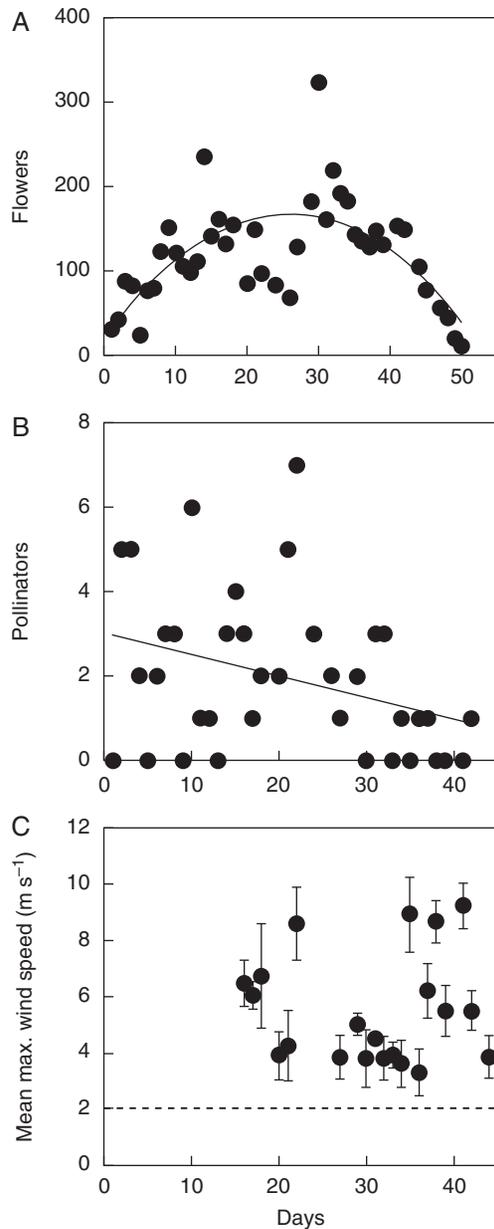


FIG. 2. Daily variation in (A) the number of flowers, (B) the number of pollinators and (C) mean maximum wind speed ( $\text{m s}^{-1}$ ) and standard deviation between 1000 and 1400h during part of the flowering season of *Incarvillea sinensis* at the population investigated in Inner Mongolia, China. See Methods for details of the measurement of these variables. The dotted line in C indicates the wind speed above which corolla dragging can cause delayed self-pollination. No measurements of wind speed were available prior to Day 16 of the flowering season.

## DISCUSSION

Estimates of mating and fertility are most commonly obtained from flower samples exposed to a brief period of the entire pollination environment experienced by plants. The estimates may therefore not capture functionally significant variation in pollination conditions, particularly for self-compatible species occupying temporally heterogeneous environments with uncertain

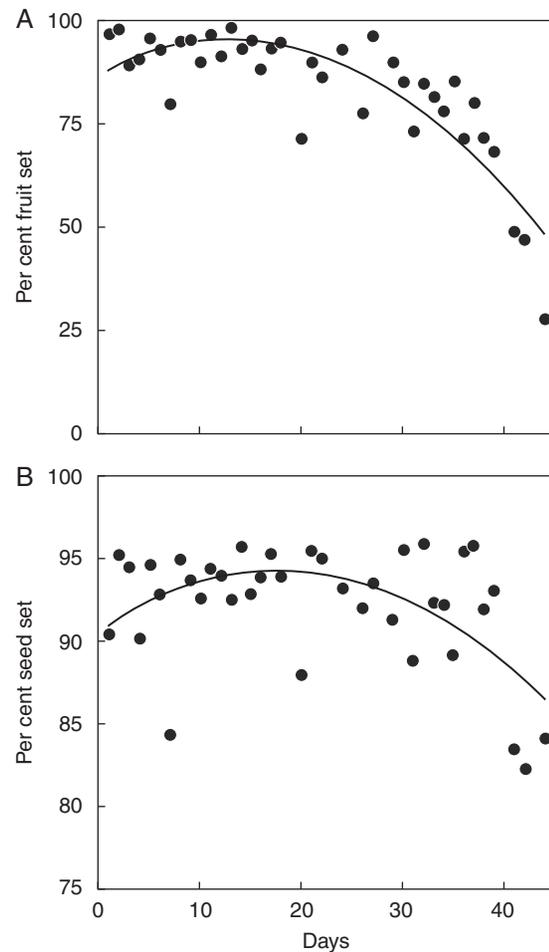


FIG. 3. Daily variation in (A) per cent fruit set and (B) per cent seed set during the flowering season of *Incarvillea sinensis* at the population investigated in Inner Mongolia, China. See Methods for details of the measurement of these variables and Results for statistical details of the fitted curves.

pollinator service. Our investigation of *I. sinensis* revealed striking day-to-day variation in mating and fertility with overall trends of declining fertility (Fig. 3A, B) and outcrossing (Fig. 4A, B) in the second half of the flowering season, indicating deteriorating conditions for cross-pollination. Our discussion focuses on the factors that might explain these patterns of variation and we consider how features of the floral biology of *I. sinensis* may function to compensate for insufficient pollinator visitation to maintain high fertility and outcrossing.

### *Ecology, mechanism and adaptive significance of delayed self-fertilization*

*Incarvillea sinensis* is exceptional for the genus in having both perennial and annual populations. Although knowledge of the mating systems of *Incarvillea* species is limited, our study suggests that in common with many other herbaceous groups, the evolution of the annual from the perennial life history is associated with a transition to increased selfing (Stebbins, 1974; Barrett *et al.*, 1996). Pollen limitation of maternal fertility is

TABLE 2. The number of observed alleles ( $N_a$ ), observed and expected heterozygosity ( $H_o$  and  $H_e$ ) and inbreeding coefficient ( $F_{is}$ ) for each of the six microsatellite loci sampled from 172 individuals of *Incarvillea sinensis* in the study population

Locus	$N_a$	$H_o$	$H_e$	$F_{is}$
01	10	0.17	0.83	0.80
05	8	0.20	0.82	0.75
821	7	0.16	0.81	0.81
03	8	0.23	0.80	0.71
08	5	0.16	0.72	0.78
831	3	0.06	0.29	0.80
Mean (s.d.)	6.83 (1.01)	0.16 (0.02)	0.71 (0.09)	0.78 (0.02)

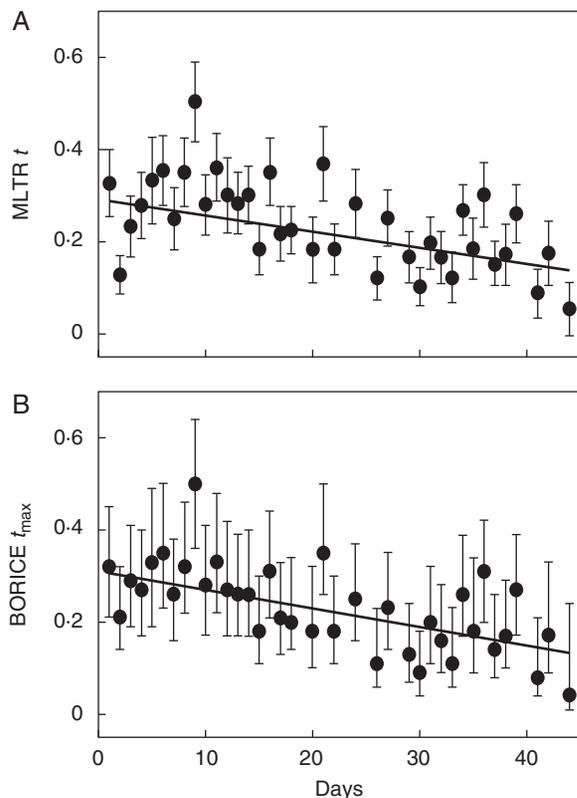


FIG. 4. Daily variation in (A) the mean multilocus outcrossing rate ( $t_m$ ) and standard error from MLTR; (B) the maximum posterior outcrossing rate ( $t_{max}$ ) and the credibility intervals (2.5 and 97.5 %) from BORICE, during the flowering season of *Incarvillea sinensis* at the population investigated in Inner Mongolia, China. See Methods for details of measurements of these variables and Results for statistical details of the fitted lines.

reported in several perennial *Incarvillea* species (Verma et al., 2008; Ai et al., 2013). However, these species do not appear to exhibit mechanisms of delayed selfing. In contrast, the annual population of *I. sinensis* we investigated exhibits pollen limitation of fruit set, has a specialized mechanism of delayed selfing and exhibits high selfing rates (Qu et al., 2007; Fig. 4 of this study). This combination of features seems likely to be causally associated with the harsh conditions that characterize the sand deserts in which the population occurs. At the study site, the growing season is short, high wind speeds are common and the

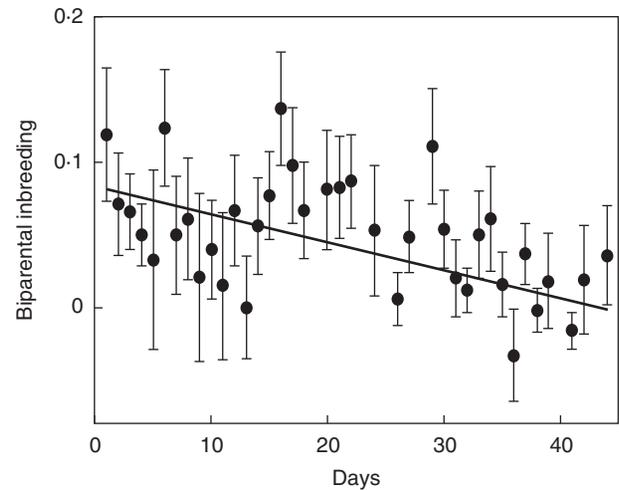


FIG. 5. Mean biparental inbreeding and standard error during the flowering season of *Incarvillea sinensis* at the population investigated in Inner Mongolia, China. See Methods for details of measurements and Results for statistical details of the fitted line.

pollination environment is highly variable with very low or no pollinator activity on most days (Fig. 2B, C; Fig. S1). Under these conditions delayed selfing probably functions to provide reproductive assurance.

The mechanism of delayed selfing in *I. sinensis* differs from most species that exhibit this mode of self-pollination. Only flowers that are not visited by a pollinator and in which the stigma lobes remain open can self-pollinate. Measurements of mean maximum wind speed for part of the 2012 flowering season (Fig. 2C), and those of Qu et al. (2007; see their fig. 2A) for the entire season of 2006, indicate that values in our study population were consistently above the threshold required for corolla dragging. The process of wind-driven corolla dragging cannot cause self-pollination in a flower previously visited by a pollinator because the sensitive stigma remains closed after cross-pollination, preventing self-pollen deposition on stigmas (Qu et al., 2007). The only other case of delayed self-pollination resulting from corolla dragging that we are aware of is reported in *Mimulus guttatus* (Dole, 1990, 1992), although wind does not appear to be required and the role of corolla dragging as a source of selfing has been questioned (Dudash and Ritland, 1991).

Most self-pollination in *I. sinensis* occurs after opportunities for animal-mediated cross-pollination have occurred, suggesting that the mechanism of delayed selfing is an adaptive response to unsatisfactory pollinator service. Experimental manipulations are required to provide convincing evidence that modes of selfing provide reproductive assurance (Schoen and Lloyd, 1992; Eckert et al., 2006). Qu et al. (2007) compared fruit set in open-pollinated versus supplemental hand-pollinated flowers of *I. sinensis* at our study site and found evidence for pollen limitation. Moreover, they also found that emasculated flowers set significantly less fruit than either open-pollinated flowers or those in which pollinators were excluded and were allowed to self-pollinate autonomously. Their results therefore support the hypothesis that delayed selfing in *I. sinensis* is adaptive and provides reproductive assurance.

Both components of female fertility that we estimated in our study population showed a steep decline during the latter part of the flowering season (Fig. 3A, B). Several factors seem likely to be involved. The abundance of *Bombus sporadicus* and *Anthophora borealis* declined during the flowering season (Fig. 2B; Fig. S1), but this is unlikely to be the result of higher wind speeds, as there is no indication that they increased in intensity (Fig. 2C). A more significant factor was probably competition from co-flowering *Hedysarum leave* and *Leonurus artemisia*. Both species began flowering mid-way through the blooming period of *I. sinensis* and we observed preferential visitation by bees to these two co-flowering species. An earlier study at our study site by Qu (2007) reported higher sugar concentration in the nectar of *H. leave* compared with *I. sinensis* and this may help to explain the preference for this species by bees. Competition for pollinators is often invoked as a mechanism causing reduced fertility in co-flowering species that share pollinators (reviewed by Mitchell et al., 2009), but few studies (but see Bell et al., 2005) have demonstrated that this directly influences the fertility and mating of species.

A combination of high wind speeds and competition for pollinators causing unsatisfactory pollinator service probably contributed to some of the decline in fruit and seed set of *I. sinensis* during the latter part of the flowering season; however, these are probably not the only factors involved. Indeed, if flowers were not visited by pollinators, we might have expected delayed selfing to maintain levels of fertility similar to values recorded earlier in the season. Significantly, in field experiments on *I. sinensis* by Qu et al. (2007), flowers in their pollinator exclusion treatment set levels of seed set from delayed self-pollination equivalent to those from hand self-pollination, cross-pollination and open pollination. Their results indicate that delayed selfing is an effective means of maximizing seed set. Hence, it seems unlikely that the end-of-season decline in fertility resulted entirely from pollen limitation.

Several other factors probably played a role in causing the decline in fertility during the flowering season. Lower temperatures later in the season could potentially slow pollen tube growth, resulting in a reduced frequency of ovule fertilization and/or a reduced maturation of fruits. Other causes of the seasonal drop in fertility may have involved the internal resource economy of *I. sinensis*. Architectural influences on resource gradients and 'position effects' could have influenced the probability of fruit maturation and seed set in flowers produced later in the flowering season (reviewed by Diggle, 2003). It is commonly observed that flowers produced later in the season produce fewer fruits and seeds and declines in fertility may often result from resource limitation (e.g. Dudash, 1993; Aizen, 2001). Such a pattern is not unexpected for a species such as *I. sinensis* that grows in a harsh desert environment and in which fertility is maintained at a high level for most of the flowering season. Lastly, inbreeding depression may possibly also contribute to the seasonal decline in fertility as a result of increased selfing rates and the abortion of developing embryos.

#### *Incarvillea* exhibits a mixed mating system

At our study site, we estimated the multilocus outcrossing rate on 38 days encompassing the entire flowering season of

*I. sinensis*. This represents the most fine-scale temporal examination of mating patterns in a flowering plant. Regardless of which of the two methods we used to estimate outcrossing rates, our analysis revealed striking day-to-day variation in the frequency of outcrossing ranging from days in which flowers were highly selfing to those in which seed families were equally divided between selfed and outcrossed progeny. The population we investigated possesses a mixed mating system but with the majority of progeny (approx. 80 %) resulting from self-fertilization. Thus, *I. sinensis* can be added to the growing number of self-compatible, animal-pollinated plant species in which mixed mating is reported (Goodwillie et al., 2005). The number of unvisited flowers remaining in the population once pollinators ceased their activities around noon appears to largely determine the degree of mixed mating on a given day. Although we cannot rule out some degree of pollinator-mediated selfing during bee visits to flowers, it is probable that the delayed selfing of unvisited flowers is the principal cause of mixed mating. Geitonogamous pollination is infrequent at our site because individuals of *I. sinensis* usually open only one flower per day.

The elevated rate of selfing we recorded throughout much of the season was unexpected given the floral biology of the species and the high flowering densities in our study population (Fig. 1C). Possession of showy zygomorphic flowers, sensitive stigmas and anther appendages that function in cross-pollen dispersal are common traits in outcrossing species of Bignoniaceae. However, several examples have been recently reported in which species possessing specialized traits presumed to be associated with outcrossing have on investigation exhibited moderate to high selfing rates (e.g. Anderson et al., 2005; Herlihy and Eckert, 2005; Zhang et al., 2005; reviewed by Fenster and Martén-Rodríguez, 2007). In some cases high selfing rates are associated with mechanisms of autonomous self-pollination, as in our study. The long-term maintenance of both sets of floral traits in *I. sinensis* will depend on the costs and benefits of selfing versus outcrossing and the history of chronic pollen limitation in populations. Future investigation of the relative fitness of selfed and outcrossed progeny in *I. sinensis* would be useful to examine whether evolutionary progression to higher selfing rates, smaller flowers and the selfing syndrome might potentially occur, as is evident in many other taxa with annual species (Stebbins, 1974; Sicard and Lenhard, 2011; Wright et al., 2013; Barrett et al., 2014). Alternatively, stable mixed mating might be a common feature of desert populations of *I. sinensis* and may be maintained by a balance of selective forces involving temporal variation in outcross pollen limitation and reproductive assurance.

Outcrossing rates exhibited striking day-to-day variation but a general trend of increased selfing towards the end of the blooming period (Fig. 4A, B). The most likely explanation for this pattern is that the decline in pollinator abundance during the season resulted in a larger number of flowers that were not visited by bees. Unvisited flowers practised delayed self-pollination, thus elevating selfing rates. Decreased pollinator abundance towards the end of the flowering period may have also caused the lower levels of biparental inbreeding evident during this time (Fig. 5), because this mating parameter requires pollinator-mediated cross-pollination between related individuals (Ritland, 2002; Griffin and Eckert, 2003). However,

measured rates of outcrossing and biparental inbreeding were not correlated in our study (Spearman rank correlation:  $r = 0.201$ ,  $P = 0.23$ ). Moreover, in the moderate to highly selfing *Aquilegia canadensis*, Herlihy and Eckert (2004) found that in large dense populations similar to the one we investigated, higher outcrossing was associated with reductions in levels of biparental inbreeding, a pattern opposite to the weak trend that was evident in our data.

In conclusion, our study involved a detailed analysis of the seasonal dynamics of mating and fertility in an annual population of *I. sinensis*. Because this species has a broad geographical range occupying diverse habitats distinct from the sand deserts of Inner Mongolia, it will be important to examine whether the main findings of our work are general. Of particular interest would be an investigation of the floral biology and mating systems of perennial populations of *I. sinensis* to determine if they are more highly outcrossing, as might be predicted based on the general association between longevity and outcrossing rate (Stebbins, 1950; Barrett *et al.*, 1996; Morgan, 2001; Scofield and Schultz, 2006). Relatively few angiosperm species consist of both annual and perennial populations and therefore *I. sinensis* could provide a future model system for investigating the correlated evolution of life history, floral biology and mating system.

#### SUPPLEMENTARY DATA

Supplementary data are available online at [www.aob.oxfordjournals.org](http://www.aob.oxfordjournals.org) and consist of the following. Table S1: Daily sample sizes for fruit and seed used for mating system estimates among patches of *Incarvillea sinensis* in our study population. Table S2: The number of fruits sampled per plant among the nine patches of *Incarvillea sinensis* in our study population. Figure S1: The numbers of individuals of the two pollinator species visiting the population daily throughout the season.

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