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Do annual and perennial populations of an insect-pollinated plant species differ in mating system?

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• **Background and Aims** Theory predicts that outcrossing should be more prevalent among perennials than annuals, a pattern confirmed by comparative evidence from diverse angiosperm families. However, intraspecific comparisons between annual and perennial populations are few because such variation is uncommon among flowering plants. Here, we test the hypothesis that perennial populations outcross more than annual populations by investigating *Incarvillea sinensis*, a wide-ranging insect-pollinated herb native to China. The occurrence of both allopatric and sympatric populations allows us to examine the stability of mating system differences between life histories under varying ecological conditions.
• **Methods** We estimated outcrossing rates and biparental inbreeding in 16 allopatric and five sympatric populations in which both life histories coexisted using 20 microsatellite loci. In each population we measured height, branch number, corolla size, tube length and herkogamy for ~30 individuals. In a sympatric population, we recorded daily flower number, pollinator visitation and the fruit and seed set of annual and perennial plants.
• **Key Results** As predicted, outcrossing rates (t) were considerably higher in perennial (mean = 0.76) than annual (mean = 0.09) populations. This difference in mating system was also maintained at sympatric sites where plants grew intermixed. In both allopatric and sympatric populations the degree of herkogamy was consistently larger in outcrossing than selfing plants. Perennials were more branched, with more and larger flowers than in annuals. In a sympatric population, annuals had a significantly higher fruit and seed set than perennials.
• **Conclusions** Genetically based differences in herkogamy between annuals and perennials appear to play a key role in governing outcrossing rates in populations, regardless of variation in local ecological conditions. The maintenance of mating system and life history trait differentiation between perennial and annual populations of *I. sinensis* probably results from correlated evolution in response to local environmental conditions.

Key words: Floral biology, herkogamy, life history evolution, mating system, outcrossing rates, pollination.

INTRODUCTION

Mating systems of flowering plants range from obligate outcrossing, through mixed mating, to predominant selfing. This diversity in mating patterns is distributed non-randomly with respect to habitat characteristics, plant life histories and phylogeny. Henslow (1879) first pointed out that self-fertilizing species tended to be annual, whereas perennials were more likely to be outcrossing, thus establishing a potential functional link between plant mating and life history. This association has subsequently been strengthened by comparative evidence from numerous angiosperm families (Stebbins, 1950; Baker, 1959; Lloyd, 1980; Barrett and Eckert, 1990; Duminil *et al.*, 2009; Friedman, 2020). More recent explicit phylogenetic analyses have generally supported these associations between mating patterns and life history; species with greater longevity are more likely to exhibit higher outcrossing rates than shorter-lived species (Barrett *et al.*, 1996; Bena *et al.*, 1998; Munoz *et al.*, 2016). An understanding of the floral and ecological determinants of mating system variation

is necessary to elucidate how and why mating systems evolve and are maintained (Barrett and Harder, 2017).

Several hypotheses have been advanced to explain associations between mating systems and plant life histories. For example, unfavourable environmental conditions during reproduction will have a greater influence on fitness in annuals than perennials, with selection for mechanisms promoting autonomous selfing providing reproductive assurance in annuals (Stebbins, 1974; Lloyd, 1980; Eckert *et al.*, 2006). Many annuals occupy unpredictable, often seasonally arid environments, with brief periods for growth and reproduction (Evans *et al.*, 2005; Datson *et al.*, 2008). Under these conditions, low population density and/or unreliable pollinator service may cause selection for selfing to alleviate pollen limitation and reproductive failure (Munoz *et al.*, 2016). In contrast, perennials more often occur in saturated competitive environments in which the higher offspring quality that generally arises from outcrossing is beneficial. Knowledge of the particular habitat conditions to which species are adapted is important for understanding the evolution and maintenance of both mating system

and life history differentiation. However, it is difficult to determine the order of establishment of mating system and life history, or whether these traits arise jointly through correlated selective forces (*sensu* 'selective covariance'; Armbruster and Schwaegerle, 1996), without explicit phylogenetic analyses.

Fitness differences between selfed and outcrossed offspring play a key role in the evolution and maintenance of plant mating systems (Lloyd, 1979; Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987). Several theories involving inbreeding depression help to explain the association between mating system and life history. For example, inbreeding depression compounded multiplicatively over the lifetime of perennials has been considered a major factor maintaining outcrossing (Morgan *et al.*, 1997). Also, it has been suggested that because perennials are usually larger than annuals, their growth involves a higher number of mitotic divisions, thus elevating mutational load and causing more severe inbreeding depression (Morgan, 2001; Scofield and Schulz, 2006). Zhang (2000) proposed that, provided inbreeding depression is less than one-half, selfing should increase the optimal allocation to reproduction at the expense of survival and drive the evolution of annuality. These hypotheses help to explain the finding of an increasing severity of inbreeding depression with longevity (Husband and Schemske, 1996; Duminil *et al.*, 2009; Angeloni *et al.*, 2011). Lesaffre and Billiard (2019) modelled the joint evolution of lifespan and self-fertilization. They found that if inbreeding depression affected adult survival, selfing promoted the evolution of shorter lifespans, with the range of conditions under which selfing can evolve rapidly diminishing with increased longevity.

Most investigations of the relations between mating system and life history have been based on the comparative analysis of interspecific variation through large-scale surveys of diverse angiosperm taxa (Barrett and Eckert, 1990; Barrett *et al.*, 1996; Duminil *et al.*, 2009). Although this approach has provided important insights, it does not account for other confounding variables associated with the phylogeny, ecology, life history and reproductive traits of species that may distinguish annuals and perennials and potentially influence mating patterns (Friedman, 2020). Few angiosperm species are reported in which both annual and perennial populations occur [exceptions include *Eschscholzia californica* (Cook, 1962), *Mimulus guttatus* (Friedman and Rubin, 2015), *Oenothera deltoides* (Evans *et al.*, 2005) and *Zostera marina* (Reynolds *et al.*, 2017)], perhaps because mating system and life history differentiation contribute to ecological isolation and speciation. To our knowledge, only a single intraspecific study has quantified mating patterns in annual and perennial populations. Barbier (1989) estimated outcrossing rates from allozyme data for one annual and two perennial populations of the wild rice *Oryza rufipogon*. The annual population was highly selfing and the two perennial populations were mixed mating.

Incarvillea sinensis (Bignoniaceae) is a showy insect-pollinated herb in a genus comprising 16 species (Grierson, 1961; Wang *et al.*, 1990). Significantly, this species includes perennial populations and the only known annual populations in *Incarvillea* (Grierson, 1961; Chen *et al.*, 2005, 2012). At present it is not known whether annual and perennial populations of *I. sinensis* represent different biological species. Grierson (1961) classified perennial populations as *I. sinensis* subsp. *variabilis* and annual populations as *I. sinensis* subsp. *sinensis*. Interestingly, perennial populations have two distinct flower colours, red and pale yellow, whereas annual populations have only red flowers. In *Flora Reipublicae Popularis Sinicae*, populations with red flowers are classified as *I. sinensis* var. *sinensis* and populations with yellow flowers as *I. sinensis* var. *przewalskii* (Wang *et al.*, 1990). A recent molecular phylogeographic investigation of *I. sinensis* based on samples from 47 localities throughout the range identified two divergent chloroplast DNA lineages corresponding to the southern perennial populations and northern annual populations (Chen *et al.*, 2012). Thus, although annual and perennial populations of *I. sinensis* are currently treated taxonomically as a single species it is possible that some degree of reproductive isolation exists between them. Regardless of their species or intraspecific taxonomic status, the closely related annual and perennial lineages of *I. sinensis* serve as a valuable model system for investigating predicted associations between life history and mating system, and this was the primary goal of our study.

Here, we test the hypothesis that perennial populations of *I. sinensis* should outcross more than annual populations using a broad geographical sampling of populations in China, including an area of geographical overlap between the life histories (Fig. 1). Our sample included 16 populations of a single life history and five populations in which both annual and perennial plants grew intermixed. Hereafter, we refer to populations in these two situations as allopatric and sympatric populations, respectively. In each population we also measured a range of vegetative and reproductive traits in an effort to determine whether annual and perennial plants possessed features commonly associated with mating system differences in plants. For example, there is abundant evidence that selfing populations usually have reduced floral displays, smaller flowers and much reduced herkogamy (stigma–anther separation) compared with outcrossing populations (Lloyd, 1965; Ornduff, 1969; Stebbins, 1974; Morgan and Barrett, 1989).

Our approach was to first compare traits in allopatric populations and then determine whether any differences that distinguished the life histories were also maintained at sympatric sites. The coexistence of both annual and perennial life histories at several locations provided us with an opportunity for more fine-scale comparisons not confounded with potential ecological differences between allopatric sites. At one sympatric site we compared the flowering phenology, pollinator visitation and maternal fertility to evaluate components of reproductive success of plants exhibiting perennial and annual life histories. Our study addressed the following specific questions. (1) Do perennial and annual populations differ in outcrossing rates? We predicted that outcrossing would be greater among perennial than annual populations. (2) Do the two life histories differ in vegetative and floral traits? Because annual plants reproduce within a single season and may benefit from more rapid growth and a capacity for reproductive assurance through autonomous selfing, we predicted that plants in annual populations would exhibit a shorter flowering season, smaller flowers of reduced longevity and less stigma–anther separation in comparison with perennial populations. (3) Are there differences between perennial and annual plants in flowering phenology, pollinator visitation and levels of fruit and seed set when they co-occur in
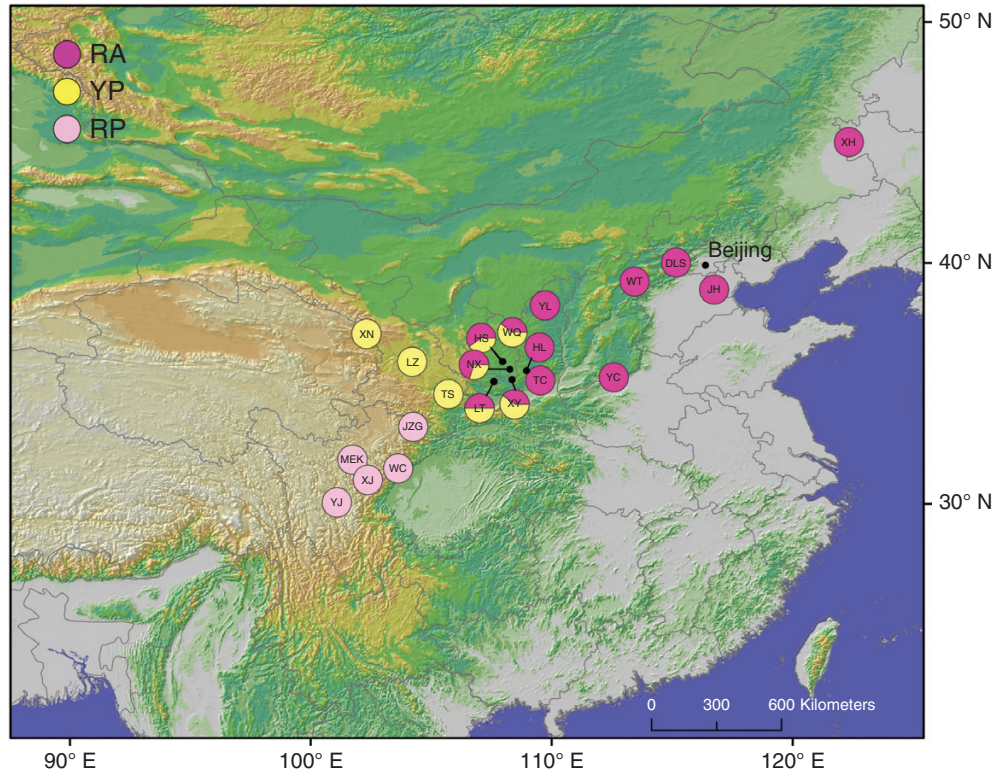
FIG. 1. The geographical distribution of *I. sinensis* populations in China sampled for this study. Populations are coloured according to life history. Dark pink circles represent annual populations (RA), yellow circles are perennial populations with yellow flowers (YP), and light pink circles are perennial populations with red flowers (RP). Circles with dark pink and yellow represent sympatric populations.

sympatry? As well as providing overall information on the reproductive success of the contrasting life histories in sympatry, information on the patterns of flowering and the types and abundance of pollinators visiting flowers could provide insight into whether or not there are opportunities for gene exchange between annual and perennial plants.

MATERIALS AND METHODS

Study system

Incarvillea sinensis ranges from south-west to north-east China and to the Russian Far East, occurring over a broad range of ecological conditions, particularly open areas including sand deserts, fields, mountain and hill slopes, riversides and disturbed sites. Plants in perennial populations exhibit woody, branched roots and are distributed in Sichuan, Gansu and Qinghai Provinces of China. Annual populations possess a single main root and are distributed from eastern Gansu Province to north-eastern China and the Russian Far East.

Sampling and field collections

During June to September of 2016–19 we collected plants and maternal seed families from populations at 21 locations in the Chinese provinces of Sichuan, Gansu, Qinghai, Shenxi, Shanxi, Hebei and Jilin. This involved 14 sites (13 allopatric, one sympatric) in 2016, five sites (three allopatric, two sympatric) in 2018 and two sympatric sites in 2019. The collections were from eight annual populations with red flowers, eight perennial populations (three with yellow flowers and five with red flowers), and five sympatric populations in which red-flowered annual plants and yellow-flowered perennial plants coexisted at the same site. The locations of populations are mapped in Fig. 1 and detailed locality information, sample sizes and population genetic data from the populations are presented in Supplementary Data Table S1.

Mating system estimation

Sampling. At allopatric sites we randomly sampled 8–16 leaves and maternal seed families from plants in the population for estimates of mating system (Supplementary Data Table S1). For each plant, we selected two mature capsules (11 seeds per capsule) from different stages in the flowering phenology (early and late flowers). We chose mature capsules at different developmental stages because our previous work on this species revealed significant seasonal variation in selfing rate (Yin *et al.*, 2016). At the five sympatric sites, we sampled 19–32 maternal seed families per population evenly distributed between the two life histories (Supplementary Data Table S1).

DNA extraction and genotyping. We extracted genomic DNA from silica-dried leaves of maternal plants and 20–22 seeds per family using the D2485-02 HP Plant DNA Kit (Omega, Norcross, GA, USA). Microsatellite markers in annual and

perennial plants were differentiated and exhibited low transferability, so we resolved seven specific microsatellite loci (01, 03, 08, mI16, mw2, mw3, mw4) in annual populations and 11 specific microsatellite loci (mI2, mI4, mI6, mI14, mw5, mw6, mw7, mw8, mw9, mw10, mw11) in perennial populations and two loci shared between them (mI9 and mw1). Among these microsatellite loci, 01, 03 and 08 were previously described by Yu *et al.* (2011) and the remainder were screened from transcriptome data (Supplementary Data Table S2). The forward primer for each locus was labelled with FAM or HEX. We performed PCR in a total volume of 20 µL, containing 5.0 ng of template DNA, 2 µL of 10 × PCR buffer, 10 mM dNTP mixture, 0.25 µM of each primer and 1 U of *Taq* DNA polymerase. The PCR profile consisted of an initial denaturation step of 10 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at an annealing temperature of 53–58 °C and 45 s at 72 °C, and a final extension step of 10 min at 72 °C. We ran the PCR products on an ABI 3730 automatic sequencer (Applied Biosystems, Foster City, CA, USA), identified genotypes using GeneMapper software version 4.0 (Applied Biosystems) and manually checked our results three times. We also tested for the presence of null alleles using Micro-Checker (van Oosterhout *et al.*, 2004).

Mating system and population genetic estimates. We calculated summary statistics using GenAlEx version 6.501 (Peakall and Smouse, 2006) for nine or 13 microsatellite loci in each population: 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. Maximum likelihood estimates of mating system parameters were calculated using MLTR v. 3.4 (Ritland, 2002). Our previous mating system estimates of *I. sinensis* (Yin *et al.*, 2016) showed close agreement between this method and an alternative program, BORICE (Koelling *et al.*, 2012), so we only present results for MLTR in the main text of our article but results from BORICE are presented in the supplementary information (Supplementary Data Note S1, Fig. S1). We used the Newton–Raphson (NR) method with genotype data of maternal plants and seeds to calculate the following mating system parameters: the single-locus outcrossing rate (t_s), the multilocus outcrossing rate (t_m) and a measure of biparental inbreeding ($t_m - t_s$). We estimated all mating system parameters at the population level for allopatric populations and for samples of plants for each life history in sympatric populations. Standard errors and 95 % confidence intervals were obtained from 1000 bootstrap replicates.

Variation in vegetative and floral traits among allopatric and sympatric populations

We measured a range of vegetative and floral traits in populations sampled for mating system estimates. Supplementary Data Table S3 provides information on the number of individuals and flowers in each population. Plant height and branch number were measured in seven perennial populations (7–24 individuals per population, median = 21), seven annual populations (9–30 individuals per population, median = 30) and the four sympatric populations (17–60 individuals per population, median = 40). We measured four floral traits commonly associated with mating system variation: corolla length, corolla width, corolla tube length, and distance between the stigma and lower anthers (herkogamy) in seven perennial populations (14–48 flowers per population, median = 30.5), except population XJ (eight flowers); seven annual populations (43–76 flowers per population, median = 55), except XH (eight flowers) and JH (four flowers); and four sympatric populations (27–82 flowers per population, median = 53.5) during peak flowering. We made all measurements, except height and branch number, using digital callipers in the field on fresh material. In sympatric population WQ, we sampled two mature buds per individual from 15 annual and 16 perennial plants and put them in centrifuge tubes with 1–1.5 mL of ethanol. Finally, all ovules were counted by eye, and all pollen grains were placed in a liquid suspension from which 10 µL was removed using a pipette, and the grains were then counted under an optical light microscope (×40) in the laboratory.

Flowering phenology, pollinator observations and fertility at a sympatric site

In sympatric population WQ, we set up 25 plots (2 m × 2 m) throughout the population to investigate flowering phenology during the blooming period from June to September in 2017. We recorded daily flower number in each plot for 114 d during the flowering period. In two plots, we observed insects visiting flowers of annual and perennial plants and recorded visitation times nearly every day from 0800 to 0900 h (24 July to 30 August 2017). Visitors that could not be identified in the field were caught and later identified by an expert entomologist (Jia-Xing Huang, Institute of Apiculture, Chinese Academy of Agricultural Sciences).

To compare the reproductive success of life histories we randomly chose 31 annual and 24 perennial plants and measured fruit set (fraction of flowers setting fruit) at the end of the flowering period. We also collected three mature capsules per individual from 25 annual and 25 perennial plants and measured capsule length, capsule dry weight and seed number.

Statistical analyses

Statistical analyses involved two basic approaches. One approach compared vegetative and floral traits for annual and perennial plants at four sympatric sites, with life history and population as crossed fixed effects, in which the effects of population were crossed with life history. The second approach involved observations from all populations and considered year (2016–19), life history (annual and perennial) and flower colour (red and yellow) nested within life history as fixed effects. Tests of the latter nested effect compared only perennial populations, as all annual populations had red flowers. For related analyses of dependent variables that were measured for individual plants, we treated population nested within combinations of life history and flower colour as a random effect. Analyses of fixed effects also accounted for lack of independence associated with the spatial proximity of the sampled populations. Specifically, we used the spatial covariance among populations based on their latitude and

longitude to adjust the denominator degrees of freedom for *F*-tests based on Kenward and Roger (2009) (Supplementary Data Note S2).

Analyses involved general linear mixed models for all plant traits except branch number, and generalized linear (mixed) models for branch number and population mating characteristics (outcrossing rate and biparental inbreeding). We conducted all analyses using *R* version 3.5.3 (CRAN; http://www.r-project.org/). General linear models were fitted using package lme4 (Bates *et al.*, 2015). The generalized linear models of mating patterns considered the β distribution and logistic link function, as implemented in package betareg (Cribari-Neto and Zeileis, 2010). The generalized linear mixed model used for branch number considered the negative binomial distribution and ln link function, as implemented in package MASS (Venables and Ripley, 2002). All tests of fixed effects assessed partial effects (i.e. holding the effects of other independent variables constant) using the Anova function in package car (Fox and Weisberg, 2019). We tested random effects using package lme4 (Bates *et al.*, 2015).

RESULTS

Genetic diversity, outcrossing rates and biparental inbreeding

Allelic diversity at each microsatellite loci was limited in all populations, with allele number ranging from 1.00 to 2.89 and from 1.18 to 3.15 in annual and perennial populations, respectively. The expected heterozygosity (H_e) in annual and perennial populations ranged from 0.00 to 0.36 and from 0.05 to 0.41, respectively, and the observed heterozygosity (H_o) ranged from 0.00 to 0.11 and from 0.04 to 0.34, respectively. The inbreeding coefficient (F_{is}) in annual populations and perennial populations varied from 0.51 to 1.00 and from −0.13 to 0.23, respectively (Supplementary Data Table S1).

Multilocus maternal outcrossing rate (t_m) differed considerably between annual and perennial populations (Fig. 2). As predicted, perennial populations were largely outcrossing (mean = 0.76, range 0.53–0.90), whereas annual populations

were mostly self-fertilizing (mean = 0.09, range 0.00–0.21; *F*-test, *F* = 300.50, d.f. = 1, *P* < 0.001). Biparental inbreeding also differed statistically between annual (mean = 0.03, range 0.00–0.08) and perennial populations (mean = 0.09, range −0.07 to 0.19; *F*-test, *F* = 10.08, d.f. = 1; *P* < 0.001).

For the five sympatric sites, population genetic and mating system estimates closely paralleled the findings from allopatric populations. Values for the number of alleles per locus, observed heterozygosity and expected heterozygosity for perennial plants were significantly higher than for annual plants (Supplementary Data Table S1). Similarly, the outcrossing rate of perennial plants was substantially higher for annual plants, with values similar to those measured for the two life histories in allopatric populations (Fig. 2).

Life history and floral trait variation among populations

Plant height in *I. sinensis* varied considerably among annual (mean ± s.e. = 76.27 ± 5.21 cm; range 46.00–106.56 cm) and perennial (mean ± s.e. = 72.13 ± 7.58 cm; range 33.48–116.10 cm) populations, but did not differ between life histories (Wald χ^2 test, *P* > 0.05; Fig. 3A, Supplementary Data Table S4), years (Wald χ^2 test, *P* > 0.05; Supplementary Data Table S4) or flower colours within life history (Wald χ^2 test, *P* > 0.05; Supplementary Data Table S4). Most annual plants had a single main stem with branches (mean ± s.e. = 6.78 ± 0.78), but perennial plants had several stems each with branches (mean ± s.e. = 16.87 ± 3.55). The generalized linear mixed models analysis identified significant differences in branch number associated with life history (Wald χ^2 test, *P* < 0.001; Fig. 3B, Supplementary Data Table S4) and for the interaction between year and life history (Wald χ^2 test, *P* < 0.001, Supplementary Data Table S4). Perennial populations produced more branches than annual populations in 2018 (Tukey test, *z* = −7.44, *P* < 0.001), but not in 2016 (Tukey test, *z* = −0.64, *P* > 0.05).

As predicted, plants in perennial populations exhibited larger flowers with greater stigma–anther separation than annual populations (Fig. 4, Supplementary Data Table S3). Corolla
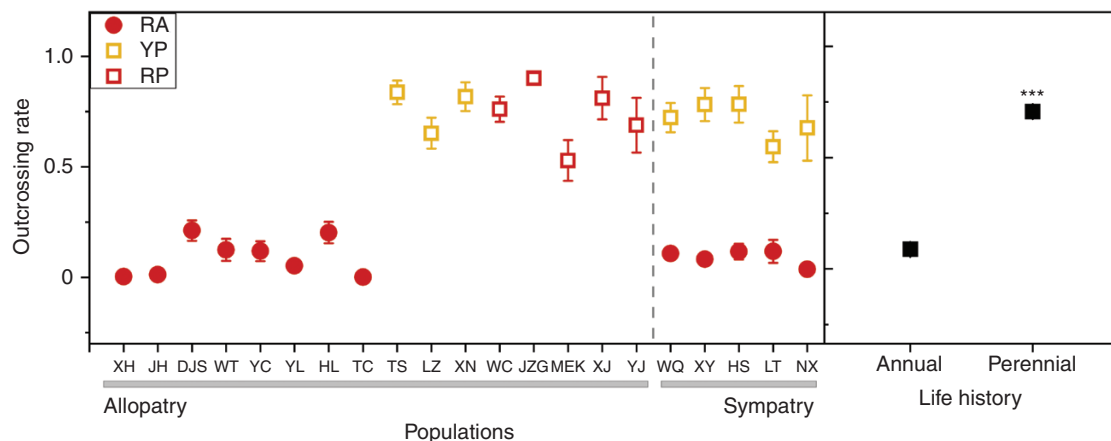

FIG. 2. Variation in mean population-level maternal outcrossing rates (t_m) of *I. sinensis* among allopatric annual and perennial populations, and at sympatric sites where the two life histories grow intermixed, with grand means for each life history. RA, annual populations with red flowers; YP, perennial populations with yellow flowers; RP, perennial populations with red flowers. ******P* < 0.001. Error bars are standard errors.
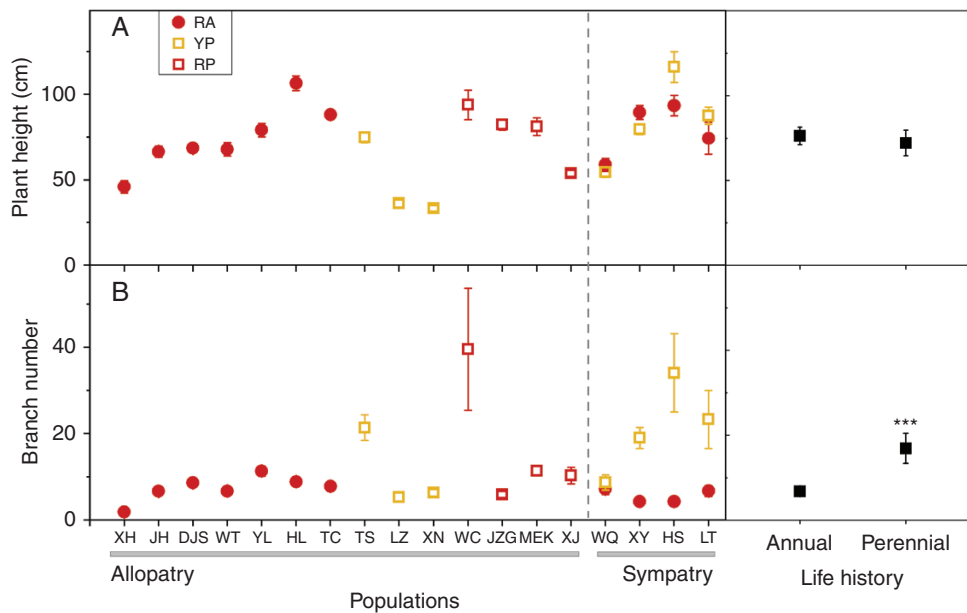
FIG. 3. Variation in vegetative traits of *I. sinensis* among allopatric annual and perennial populations, and in sympatric sites where the two life histories grow intermixed, with grand means for traits of each life history. (A) Plant height (cm). (B) Branch number. RA, annual populations with red flowers; YP, perennial populations with yellow flowers; RP, perennial populations with red flowers. ***$P < 0.001$. Error bars are standard errors.

length and width were longer and wider in perennial (length, mean ± s.e. = 30.65 ± 0.74 mm; width, 31.71 ± 0.94 mm) than annual populations (length, mean ± s.e. = 26.08 ± 0.65 mm, Wald χ^2 test, $P < 0.001$; width, 28.36 ± 0.64 mm, Wald χ^2 test, $P < 0.01$; Fig. 4A, B, Supplementary Data Table S4). Flowers from perennial populations also had longer corolla tubes (mean ± s.e. = 32.07 ± 0.77 mm) than those from annual populations (mean ± s.e. = 26.60 ± 0.74 mm, Wald χ^2 test, $P < 0.001$; Fig. 4C, Supplementary Data Table S4). Stigma–anther separation was significantly larger in perennial (mean ± s.e. = 9.60 ± 0.45 mm) than annual populations (mean ± s.e. = 5.69 ± 0.29 mm, Wald χ^2 test, $P < 0.001$; Fig. 4D, Supplementary Data Table S4) and the variation in herkogamy was positively associated with outcrossing rate (Fig. 5). None of the analyses differed between years (Wald χ^2 test, $P > 0.05$) or between flower colour for any of the four floral traits (Wald χ^2 test, $P > 0.05$).

Parallel trait differences between life histories were also evident for sites where annual and perennial plants grew intermixed, although not for all traits. For plant height, there was no difference between annual and perennial individuals in sympatric populations (Tukey test, $F = 1.87$, $P > 0.05$; Supplementary Data Table S3). For branch number, perennial plants had significantly more branches than annual plants (Tukey test, $F = 81.93$, $P < 0.001$; Supplementary Data Table S3), but there was only a marginally significant difference in population WQ (Tukey test, $z = -2.01$, $P = 0.04$). Corolla length and width, tube length and stigma–anther distance were all much larger in perennial than annual plants in four sympatric populations (Tukey test, $F = 163.50, 128.17, 350.02, 611.85$, all $P < 0.001$; Fig. 4, Supplementary Data Table S3). At sympatric site WQ, ovule number per flower was similar between annual (mean ± s.e. = 162.13 ± 8.91) and perennial (mean ± s.e. = 121.44 ± 8.09; $t = 3.39$, d.f. = 29, $P < 0.001$)

plants, but perennial plants produced more pollen grains per flower (mean ± s.e. = 31 996.53 ± 2439.76) than annual plants (mean ± s.e. = 12 899.31 ± 4822.97; $t = -6.72$, d.f. = 29, $P < 0.001$; Fig. 6A, B).

Floral display, insect visitors and maternal fertility in sympatry

At WQ, the floral longevity of individual flowers in annual and perennial plants differed; flowers of annuals lasted for 8–12 h, opening in the early morning and closing in the early afternoon (0230–1400 h), whereas flowers of perennials opened in the morning around 0400–0500 h and lasted for 2–3 d. Annual plants bloomed from late June to early September (~77 d), whereas perennials bloomed for a longer period, from early June to late September (~114 d; Fig. 7A). Flower number per individual per day was significantly higher in perennial plants (mean ± s.e. = 2.94 ± 0.17) than in annual plants (mean ± s.e. = 0.84 ± 0.15, t-test, $t = -9.02$, d.f. = 131, $P < 0.001$; Fig. 7A).

At WQ, we recorded nine and eight species of insect visiting the flowers of annual and perennial plants, respectively. All visitors touched the sexual organs and were therefore considered pollinators. The most abundant pollinator of annual plants was the hoverfly *Episyrphus balteatus*, whereas the bumble bee *Bombus patagiatus* was the most frequent pollinator of perennial plants (Fig. 7C). Significantly, both pollinators were observed visiting flowers of annual and perennial plants (Fig. 8). There was no overall difference in visitation frequency (insect visits per hour) between annual (mean ± s.e. = 6.58 ± 0.75) and perennial (mean ± s.e. = 6.26 ± 0.65, $t = 0.328$, d.f. = 104, $P > 0.05$) plants; however, visitation to flowers of annual plants was significantly lower during the first third of the flowering season compared with perennial plants, whereas daily visitation
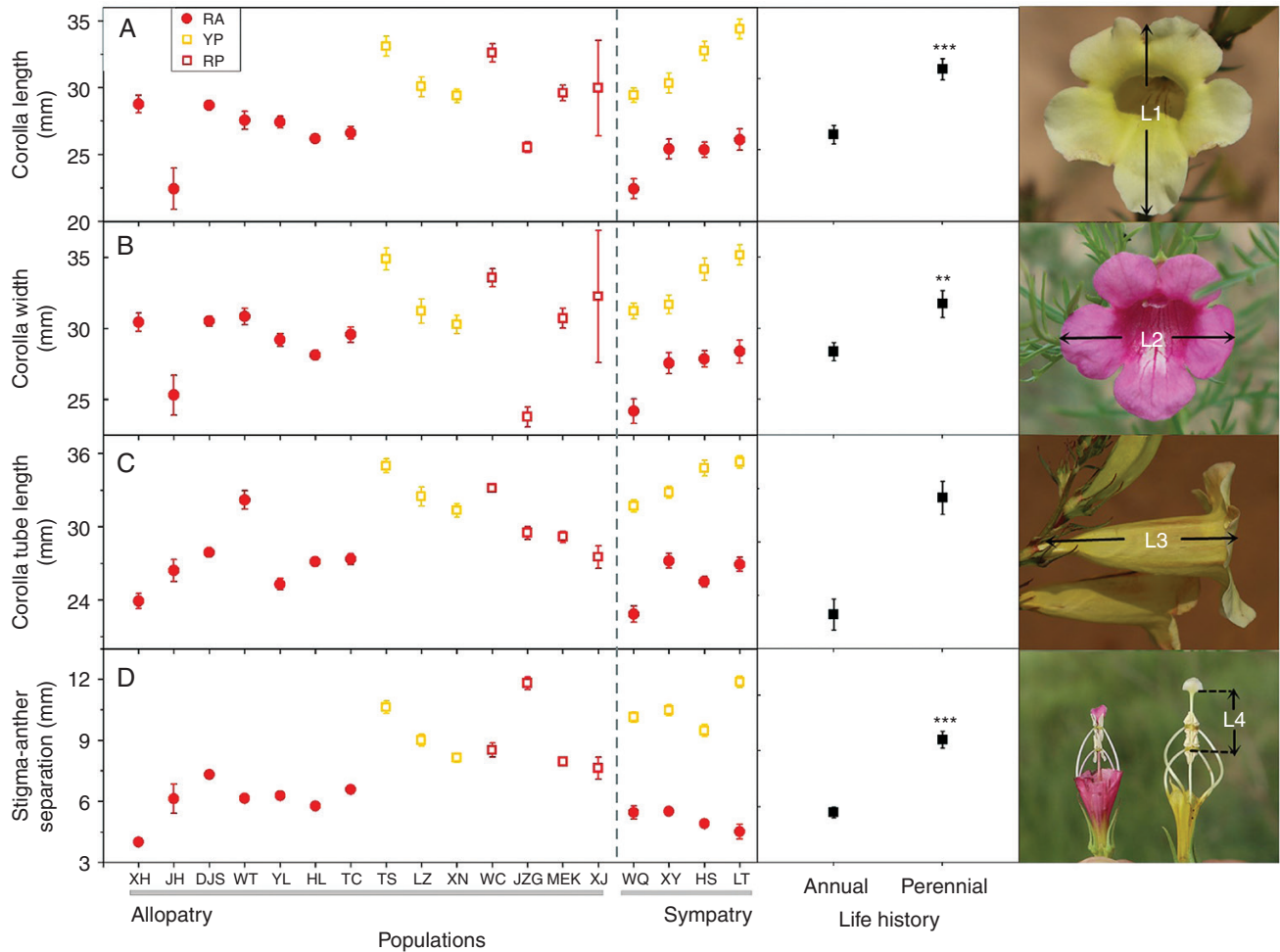
FIG. 4. Variation in floral traits of *I. sinensis* among allopatric annual and perennial populations, and in sympatric sites where the two life histories grow intermixed, with grand means for traits of each life history. (A) Corolla length (mm). (B) Corolla width (mm). (C) Corolla tube length (mm). (D) Stigma–anther separation (mm). RA, annual populations with red flowers; YP, perennial populations with yellow flowers; RP, perennial populations with red flowers. **$P < 0.01$, ***$P < 0.001$. Error bars are standard errors. L1–4 indicate the measurements of traits shown in (A)–(D).
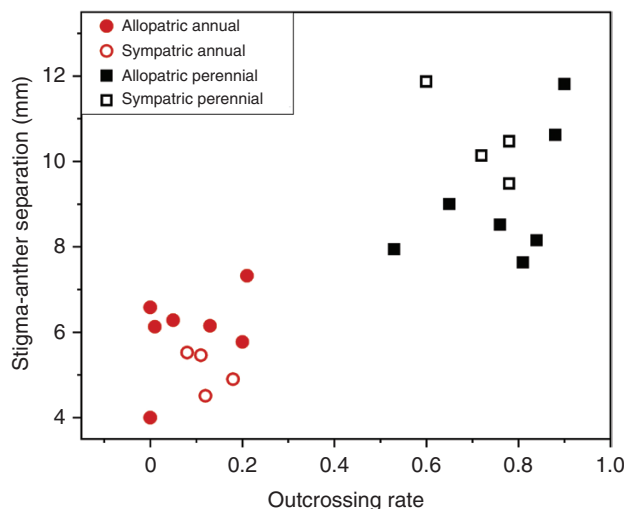

FIG. 5. Relation between mean stigma–anther separation (herkogamy) and outcrossing rate in allopatric and sympatric populations of *I. sinensis*.

was more often higher to flowers of annual plants during the middle of the flowering season (Fig. 7B).

At WQ, fruit set was significantly lower in perennial (mean ± s.d. = 22.49 ± 2.16 %) than annual plants (mean ± s.d. = 65.08 ± 18.07 %; $t = 10.93$, d.f. = 50, $P < 0.001$; Fig. 6C). Annual plants exhibited significantly longer (mean ± s.e. = 67.04 ± 1.01 mm) and heavier (mean ± s.e. = 0.28 ± 0.01 g) capsules than perennial plants (mean ± s.e. = 48.07 ± 0.82 mm; $t = 14.35$, d.f. = 74, $P < 0.001$; mean ± s.e. = 0.10 ± 0.00g; $t = 18.44$, d.f. = 74, $P < 0.001$; Fig. 6E, F). Finally, annual plants produced significantly more seeds per capsule (mean ± s.e. = 203.90 ± 4.35) than perennial plants (mean ± s.e. = 98.13 ± 3.25; $t = 19.50$, d.f. = 139, $P < 0.001$; Fig. 6D).

DISCUSSION

As predicted from theory and supported by comparative data, outcrossing rates were significantly higher in perennial than annual populations of *I. sinensis*. Remarkably, there was no
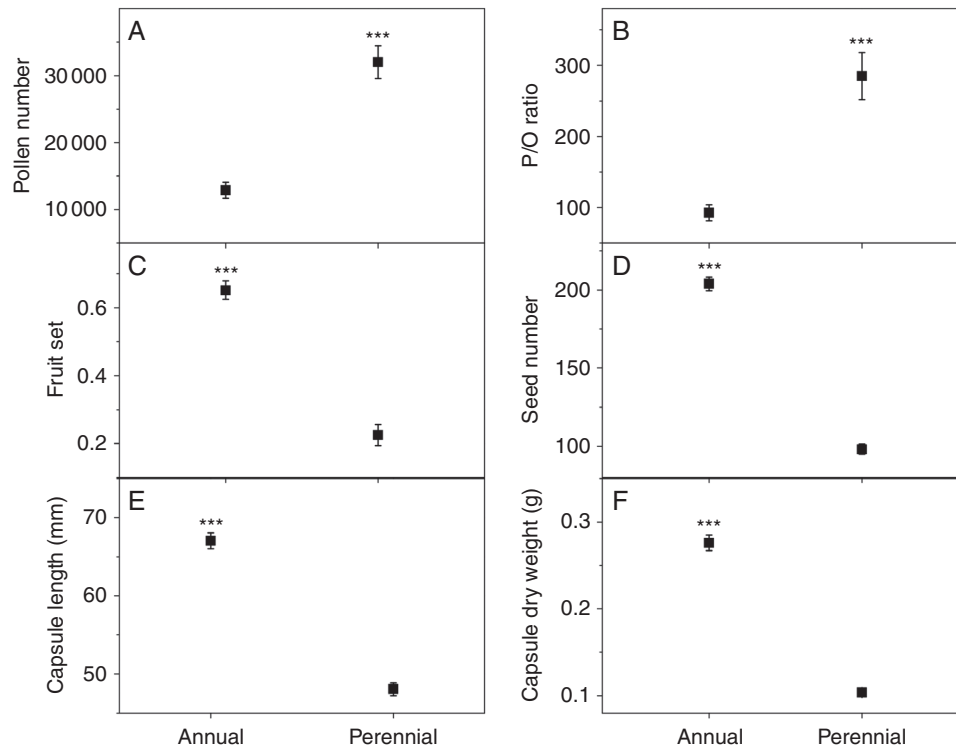
Fig. 6. Measures of maternal fertility, gamete production and pollen/ovule ratios in the sympatric population WQ of *I. sinensis* with annual and perennial plants in Shenxi Province, China. (A) Pollen number per flower. (B) Pollen/ovule (P/O) ratio. (C) Fruit set per individual. (D) Seed number per capsule. (E) Capsule length (mm). (F) capsule dry weight (g). ***$P < 0.001$. Error bars are standard errors.

overlap in the values between the two life histories for plants at both allopatric and sympatric sites (Fig. 2). Although the differences in outcrossing rate between allopatric populations were confounded by geography and probably habitat conditions, the differences were maintained when the two life histories grew intermixed in sympatry (Fig. 2). This suggests that the maintenance of mating system differentiation in allopatry is unlikely to be caused by ecological or demographic differences associated with the habitats that populations occupy. Rather, our finding that annual plants had smaller flowers with reduced herkogamy compared with perennial plants (Figs 4 and 5) strongly suggests that genetically based differences in the floral biology of annual and perennial plants largely accounted for the differences in mating patterns. Below, we address questions concerning the ecological factors responsible for the origin and maintenance of life history and mating system differentiation in *I. sinensis* and the floral mechanisms influencing mating.

Evolution and maintenance of annual life history and self-fertilization

Incarvillea sinensis is exceptional for the genus in having both perennial and annual populations, with annuality probably derived (Chen *et al.*, 2005). The evolutionary transition from the perennial to the annual habit is widespread among herbaceous angiosperms and often associated with radiations into seasonally arid environments with unpredictable climatic conditions (Stebbins, 1974; Barrett *et al.*, 1996; Fiz *et al.*, 2002; Evans *et al.*, 2005; Datson *et al.*, 2008; Cruz-Mazo *et al.*, 2009;

Friedman and Rubin, 2015), although transitions in the opposite direction occasionally occur (Barrett *et al.*, 1996; Bena *et al.*, 1998; Baldwin, 2007; Tank and Olmstead, 2008). Given the predominance of perenniality in *Incarvillea* and the geographically peripheral distribution of annual populations compared with the genus as a whole, this seems unlikely.

Incarvillea species possess large showy pink, red or yellow flowers that possess several specialized floral structures, including a bilobed sensitive stigma (Ai *et al.*, 2013) and conspicuous anther appendages that function as a pollen-dispensing mechanism (Cutting, 1921; Han *et al.*, 2008; Verma *et al.*, 2008). Knowledge of the mating systems of other *Incarvillea* species is limited, but the specialized floral morphology and showy flowers likely function to promote outcrossing, at least to some degree. *Incarvillea mairei* is the only other species in the genus for which mating has been quantified and in three self-compatible populations outcrossing rates ranged from 0.97 to 0.99 (Ai *et al.*, 2013). Given the floral biology of *Incarvillea* species, it seems reasonable to hypothesize that outcrossing or mixed mating are ancestral states in the genus and that high selfing rates are a derived condition.

Without explicit phylogenetic analysis of *Incarvillea* it is not possible to determine how often the association between annuality and selfing has originated and in what sequence. However, regardless of the number of origins, what is significant about our findings is that the mating system and life history association is maintained over a broad geographical range encompassing striking environmental variation. We were unable to find a single case in which either annual populations were even moderately outcrossing or perennial populations had
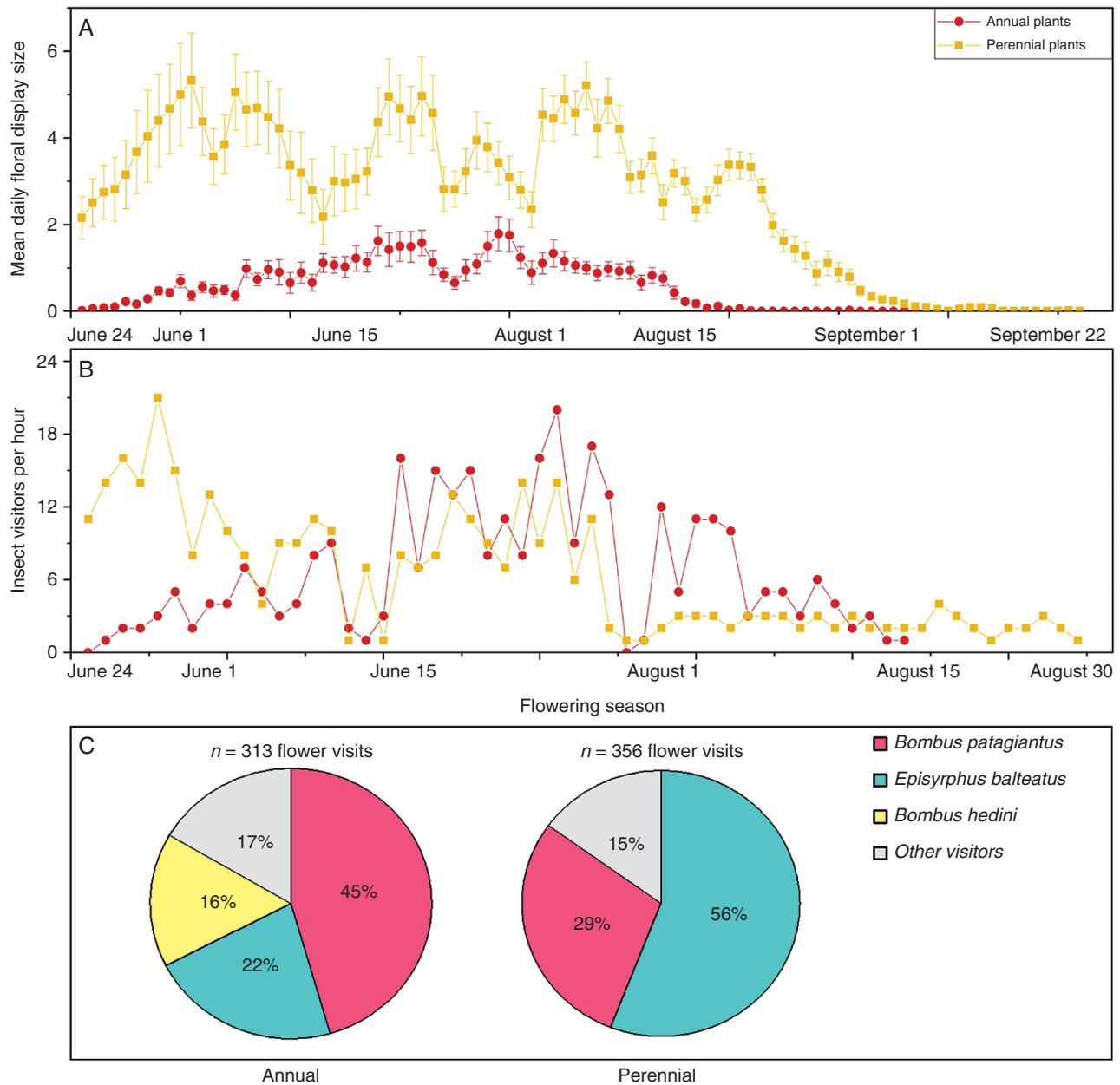
FIG. 7. Flowering phenology of mean daily floral display and insect visitation to flowers of annual and perennial plants of *I. sinensis* growing in sympatry at site WQ, Shenxi Province, China. (A) Daily variation in average number of open flowers per plant. (B) Daily variation in insect visits per flower throughout the flowering season. (C) Percentage frequencies of the most common insect visitors.

high selfing rates. Mating systems in angiosperms are highly variable with considerable interpopulation variation governed by ecological factors (reviewed in Barrett and Eckert, 1990; Whitehead *et al.*, 2018). Moreover, herkogamy, probably the most important quantitative floral trait governing mating (Barrett and Shore, 1987; van Kleunen and Ritland, 2004; Takebayashi *et al.*, 2006; Opedal, 2018), is also often highly variable both within and between populations and is recognized as being particularly evolutionarily labile (Shore and Barrett, 1990; Herlihy and Eckert, 2007; Duncan and Rausher, 2013; Opedal *et al.*, 2017). Indeed, it has been suggested that herkogamy is among the first traits to evolve in response to changes in environmental conditions, often rapidly (Mitchell

and Ashman, 2008; Roels and Kelly, 2011). The stability of the association between life history, degree of herkogamy and mating patterns in *I. sinensis* is therefore particularly striking and strongly suggestive of an adaptive basis.

Most *Incarvillea* species occur in the Himalayas and south-western China and have more restricted distributions than *I. sinensis*, which is the only species extending to north-eastern China and the Russian Far East (Chen *et al.*, 2012). Comparative analyses of the biogeography of mating systems indicate that selfing species often occur at higher latitudes (Moeller *et al.*, 2017) with larger geographical ranges (Grossenbacher *et al.*, 2015) than outcrossing sister species. Populations at the leading edge of an expanding range may suffer from a shortage of both

FIG. 8. Commonest insect visitors to flowers of annual and perennial plants of *I. sinensis* growing in sympatry at site WQ, Shenxi Province, China.

compatible mates and effective pollinators driving mating system shifts (Levin, 2012; Hargreaves *et al.*, 2014). Therefore, in *I. sinensis* the occurrence of selfing populations at higher latitudes may have resulted from selection for selfing and the annual habit during northward range expansion, perhaps associated with postglacial migration. Ancestral perennial populations in the Himalayas and south-western China likely migrated northwards (Chen *et al.*, 2012), encountering more seasonally arid environments. This change in ecology may have demanded the evolution of an annual life history and higher rates of selfing. Climate data from locations from which our populations were sampled indicate that annual populations are distributed over a region that experiences less total rainfall than areas in which perennial populations occur. Moreover, the coefficient of variation in daily rainfall was significantly higher in annual localities compared with perennial localities, suggesting that their environments experience greater unpredictability in local climate (Supplementary Data Fig. S2 and Note S3).

Evidence that annual populations of *I. sinensis* are likely adapted to unpredictable growing conditions comes from an earlier study of temporal variation in the mating system of an annual population in a sand desert in Inner Mongolia (Yin *et al.*, 2016). Pollinator activity was low in the population, fluctuating daily owing to variable wind speeds, and also showed a striking reduction in visitation as the season progressed. Selfing rates averaged 80 % but fluctuated throughout the season with a marked seasonal decline. The population exhibited pollen limitation of fruit set and, like other annual populations of *I. sinensis*, has a specialized mechanism of delayed selfing (Qu *et al.*, 2007). Comparisons of pollinator visitation between allopatric annual and perennial populations of *I. sinensis* would be valuable to determine whether the pollination environment of annual populations is generally more unpredictable than that of perennial populations.

Reproductive trait differentiation between annual and perennial populations

Flowers of perennial plants in both allopatry and sympatry were significantly larger than flowers of annual plants. This difference was evident regardless of whether perennial populations had yellow or red flowers. Larger-flowered perennial populations had much higher outcrossing rates than smaller-flowered annual populations (Fig. 5). Numerous studies in other herbaceous groups have observed this association (e.g. Lloyd, 1965; Ornduff, 1969; Cruden and Lyon, 1985; Morgan and Barrett, 1989) and reduced allocation to attractive structures, including smaller flower size, is predicted by sex allocation theory (Charlesworth and Charlesworth, 1987; Lloyd, 1987).

Goodwillie and colleagues (2010) reported that outcrossing rate varied positively with floral display size in a comparative analysis of 154 angiosperm species. Nevertheless, our measurements of the levels of pollination visitation to annual and perennial plants in sympatric population WQ revealed no difference in the frequency of visitation or diversity of pollinators visiting flowers, although pollinator quality (e.g. bumble bees as main visitors of perennials and hoverflies as primary visitors of annuals) may be important in influencing seed quantity and quality. The higher selfing rates and fruit set of annuals compared with perennials probably resulted from greater facilitated and autonomous selfing owing to the reduced herkogamy compared with perennials. The higher per flower fruit and seed set of annuals may occur because of greater resource investment in fruits and seeds compared with perennial plants, which probably invest more in vegetative growth and branching.

Unresolved questions

An important question that was not the focus of this study concerns whether annual and perennial populations of *I. sinensis* are different biological species. The earlier phylogeographic work on *I. sinensis* by Chen *et al.* (2012) reported a significant degree of genetic differentiation between southern perennial populations and northern annual populations, a finding consistent with the microsatellite divergence revealed by our studies. Chen *et al.* (2012) estimated divergence between the northern and southern clades at 4.4 MYA in the early Pliocene and proposed that it was initiated by mountain uplift of the Qinghai–Tibet Plateau followed by decreased effective gene flow through topographical isolation and divergent adaptation to the distinct habitats characterizing the southern and northern parts of the range. Our investigation suggests that the proposed adaptations probably involved correlated shifts in life history, floral traits and mating system and that these have played a role in promoting reproductive isolation between perennial and annual lineages of *I. sinensis*.

We investigated five sympatric sites in which annual and perennial plants retained the characteristics they exhibited in allopatry, despite extensive overlap in flowering time and several shared pollinators. Our preliminary studies have revealed that controlled crosses between annual and perennial plants result in high fruit and seed set and we have also observed a low frequency of hybrids in sympatric populations (F. Y. Wang *et al.*, unpubl. res.). Future work is required to determine the fertility of hybrids and potential barriers to gene exchange. Regardless of whether or not the two lineages of *I. sinensis* represent distinct biological species, they provide a valuable system for investigating the correlated evolution and maintenance of mating system and life history differentiation. Our findings highlight that evolutionary responses to ecological conditions may involve coordinated changes to all aspects of the mating biology of plants in concert with life history and floral evolution.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Table S1: location, life history, observed alleles, observed and expected heterozygosity, inbreeding coefficient, multilocus outcrossing rate and single-locus outcrossing rate of annual and perennial populations of *Incarvillea sinensis* investigated in this study. Table S2: microsatellite loci analysed, PCR conditions and motif and allele size ranges in *Incarvillea sinensis*. Table S3: vegetative and floral traits of annual and perennial populations of *Incarvillea sinensis* investigated in this study. Table S4: statistical results comparing vegetative and floral traits for annual and perennial plants, with year, life history and flower colour nested within life history as a fixed effect. Note S1: estimates of outcrossing rate in populations of *Incarvillea sinensis* using BORICE. Note S2: statistical methods and results on spatial autocorrelation of maternal outcrossing rate variation in populations of *Incarvillea sinensis*. Note S3: detailed information on climate data for localities of *Incarvillea sinensis*. Figure S1: variation in mean population-level maternal outcrossing rates of *Incarvillea sinensis* using BORICE. Figure S2: annual daily precipitation varies across the latitudinal range of sampled allopatric populations of *Incarvillea sinensis*.

DATA ACCESSIBILITY

Sample locations, morphology and microsatellite data: Dryad doi: 10.5061/dryad0.63xsj3tzx.

LITERATURE CITED

Ai H, Zhou W, Xu K, Wang H, Li D. 2013. The reproductive strategy of a pollinator-limited Himalayan plant, *Incarvillea mairei* (Bignoniaceae). *BMC Plant Biology* **13**: 195.

Angeloni F, Ouborg NJ, Leimu R. 2011. Meta-analysis on the association of population size and life history with inbreeding depression in plants. *Biological Conservation* **144**: 35–43.

Armbruster WS, Schwaegerle KE. 1996. Causes of covariation of phenotypic traits among populations. *Journal of Evolutionary Biology* **9**: 261–276.

Baker HG. 1959. Reproductive methods as factors in speciation in flowering plants. *Cold Spring Harbor Symposia on Quantitative Biology* **24**: 177–191.

Baldwin BG. 2007. Adaptive radiation of shrubby tarweeds (*Deinandra*) in the California Islands parallels diversification of the Hawaiian silversword alliance (Compositae-Madiinae). *American Journal of Botany* **94**: 237–248.

Barbier P. 1989. Genetic variation and ecotypic differentiation in the wild rice species *Oryza rufipogon*. II. Influence of the mating system and life-history traits on the genetic structure of populations. *Japanese Journal of Genetics* **64**: 273–285.

Barrett SCH, Eckert CG. 1990. Variation and evolution of mating systems in seed plants. In: Kawano S, ed. *Biological approaches and evolutionary trends in plants*. London: Academic Press, 229–254.

Barrett SCH, Harder LD. 2017. The ecology of mating and its evolutionary consequences in seed plants. *Annual Review of Ecology, Evolution, and Systematics* **48**: 135–157.

Barrett SCH, Shore JS. 1987. Variation and evolution of breeding systems in the *Turnera ulmifolia* L. complex (Turneraceae). *Evolution* **41**: 340–354.

Barrett SCH, Harder LD, Worley AC. 1996. The comparative biology of pollination and mating in flowering plants. *Philosophical Transactions of the Royal Society of London Series B. Biological Sciences* **351**: 1271–1280.

Bates D, Mächler M, Bolker B, Walker S. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* **67**: 1–48.

Bena G, Lejeune B, Prosperi JM, Olivieri I. 1998. Molecular phylogenetic approach for studying life-history evolution: the ambiguous example of the genus *Medicago* L. *Proceedings. Biological Sciences* **265**: 1141–1151.

Charlesworth D, Charlesworth B. 1987. Inbreeding depression and its evolutionary consequences. *Annual Review of Ecology and Systematics* **18**: 237–268.

Chen ST, Guan KY, Zhou ZK, Olmstead R, Cronk Q. 2005. Molecular phylogeny of *Incarvillea* (Bignoniaceae) based on ITS and *trnL-F* sequences. *American Journal of Botany* **92**: 625–633.

Chen ST, Xing YW, Su T, Zhou ZK, Dilcher EDL, Soltis DE. 2012. Phylogeographic analysis reveals significant spatial genetic structure of *Incarvillea sinensis* as a product of mountain building. *BMC Plant Biology* **12**: 58.

Cook SA. 1962. Genetic system, variation, and adaptation in *Eschscholzia californica*. *Evolution* **16**: 278–299.

Cribari-Neto F, Zeileis A. 2010. Beta regression in R. *Journal of Statistical Software* **34**: 1–24.

Cruden RW, Lyon DL. 1985. Patterns of biomass allocation to male and female functions in plants with different mating systems. *Oecologia* **66**: 299–306.

Cruz-Mazo G, Buide ML, Samuel R, Narbona E. 2009. Molecular phylogeny of *Scorzoneroides* (Asteraceae): evolution of heterocarpy and annual habit in unpredictable environments. *Molecular Phylogenetics and Evolution* **53**: 835–847.

Cutting EM. 1921. On the pollination mechanism of *Incarvillea delavayi* Franch. *Annals of Botany* **35**: 63–71.

Datson PM, Murray BG, Steiner KE. 2008. Climate and the evolution of annual/perennial life-histories in *Nemesia* (Scrophulariaceae). *Plant Systematics and Evolution* **270**: 39–57.

Duminil J, Hardy OJ, Petit RJ. 2009. Plant traits correlated with generation time directly affect inbreeding depression and mating system and indirectly genetic structure. *BMC Evolutionary Biology* **9**: 177.

Duncan TM, Rausher MD. 2013. Evolution of the selfing syndrome in *Ipomoea*. *Frontiers in Plant Science* **4**: 301.

Eckert CG, Samis KE, Dart S. 2006. Reproductive assurance and the evolution of uniparental reproduction in flowering plants. In: Harder LD, Barrett SCH, eds. *Ecology and evolution of flowers*. Oxford: Oxford University Press, 183–203.

Evans ME, Hearn DJ, Hahn WJ, Spangle JM, Venable DL. 2005. Climate and life history evolution in evening primroses (*Oenothera*, Onagraceae): a phylogenetic comparative analysis. *Evolution* **59**: 1914–1927.

Fiz O, Valcárcel V, Vargas P. 2002. Phylogenetic position of Mediterranean Astereae and character evolution of daisies (*Bellis*, Asteraceae) inferred from nrDNA ITS sequences. *Molecular Phylogenetics and Evolution* **25**: 157–171.

Fox J, Weisberg S. 2019. *An R companion to applied regression*. Thousand Oaks, CA: Sage.

Friedman J. 2020. The evolution of annual and perennial plant life-histories: ecological correlates and molecular mechanisms. *Annual Reviews of Ecology, Evolution and Systematics* **51**: 461–81.

Friedman J, Rubin MJ. 2015. All in good time: understanding annual and perennial strategies in plants. *American Journal of Botany* **102**: 497–499.

Goodwillie C, Sargent RD, Eckert CG, *et al.* 2010. Correlated evolution of mating system and floral display traits in flowering plants and its implications for the distribution of mating system variation. *New Phytologist* **185**: 311–321.

Grierson A. 1961. A revision of the genus *Incarvillea*. *Notes from the Royal Botanical Garden, Edinburgh* **23**: 303–354.

Grossenbacher D, Briscoe Runquist R, Goldberg EE, Brandvain Y. 2015. Geographic range size is predicted by plant mating system. *Ecology Letters* **18**: 706–713.

Han Y, Dai C, Yang CF, Wang QF, Motley TJ. 2008. Anther appendages of *Incarvillea* trigger a pollen-dispensing mechanism. *Annals of Botany* **102**: 473–479.

Hargreaves AL, Eckert CG, Bailey J. 2014. Evolution of dispersal and mating systems along geographic gradients: implications for shifting ranges. *Functional Ecology* **28**: 5–21.

Henslow G. 1879. On the self-fertilization of plants. *Transactions of the Linnean Society of London. 2nd Series: Botany* **1**: 317–398.

Herlihy CR, Eckert CG. 2007. Evolutionary analysis of a key floral trait in *Aquilegia canadensis* (Ranunculaceae): genetic variation in herkogamy and its effect on the mating system. *Evolution* **61**: 1661–1674.

Husband BC, Schemske DW. 1996. Evolution of the magnitude and timing of inbreeding depression in plants. *Evolution* **50**: 54–70.

Kenward MG, Roger JH. 2009. An improved approximation to the precision of fixed effects from restricted maximum likelihood. *Computational Statistics and Data Analysis* **53**: 2583–2595.

van Kleunen M, Ritland K. 2004. Predicting evolution of floral traits associated with mating system in a natural plant population. *Journal of Evolutionary Biology* **17**: 1389–1399.

Koelling VA, Monnahan PJ, Kelly JK. 2012. A Bayesian method for the joint estimation of outcrossing rate and inbreeding depression. *Heredity* **109**: 393–400.

Lande R, Schemske DW. 1985. The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. *Evolution* **39**: 24–40.

Lesaffre T, Billiard S. 2019. The joint evolution of lifespan and self-fertilization. *Journal of Evolutionary Biology* **33**: 41–56.

Levin DA. 2012. Mating system shifts on the trailing edge. *Annals of Botany* **109**: 613–620.

Lloyd DG. 1965. Evolution of self-compatibility and racial differentiation in *Leavenworthia* (Cruciferae). *Contributions from the Gray Herbarium of Harvard University* **195**: 3–134.

Lloyd DG. 1979. Some reproductive factors affecting the selection of self-fertilization in plants. *American Naturalist* **113**: 67–79.

Lloyd DG. 1980. Demographic factors and mating patterns in angiosperms. In: Solbrig OT, ed. *Demography and evolution in plant populations*. Oxford: Blackwell, 67–88.

Lloyd DG. 1987. Allocations to pollen, seeds and pollination mechanisms in self-fertilizing plants. *Functional Ecology* **1**: 83–89.

Mitchell RJ, Ashman TL. 2008. Predicting evolutionary consequences of pollinator declines: the long and short of floral evolution. *New Phytologist* **177**: 576–579.

Moeller DA, Briscoe Runquist RD, Moe AM, *et al.* 2017. Global biogeography of mating system variation in seed plants. *Ecology Letters* **20**: 375–384.

Morgan MT. 2001. Consequences of life history for inbreeding depression and mating system evolution in plants. *Proceedings of the Royal Society of London B* **1478**: 1817–1824.

Morgan MT, Barrett SCH. 1989. Reproductive correlates of mating system variation in *Eichhornia paniculata* (Spreng.) Solms (Pontederiaceae). *Journal of Evolutionary Biology* **2**: 183–203.

Morgan MT, Schoen DJ, Bataillon TM. 1997. The evolution of self-fertilization in perennials. *American Naturalist* **150**: 618–638.

Munoz F, Violle C, Cheptou PO. 2016. CSR ecological strategies and plant mating systems: outcrossing increases with competitiveness but stress-tolerance is related to mixed mating. *Oikos* **125**: 1296–1303.

van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* **4**: 535–538.

Opedal ØH. 2018. Herkogamy, a principal functional trait of plant reproductive biology. *International Journal of Plant Sciences* **179**: 677–687.

Opedal ØH, Bolstad GH, Hansen TF, Armbruster WS, Pélabon C. 2017. The evolvability of herkogamy: quantifying the evolutionary potential of a composite trait. *Evolution* **71**: 1572–1586.

Ornduff R. 1969. Reproductive biology in relation to systematics. *Taxon* **18**: 121–133.

Peakall R, Smouse PE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**: 288–295.

Qu RM, Li XJ, Luo YB, *et al.* 2007. Wind-dragged corolla enhances self-pollination: a new mechanism of delayed self-pollination. *Annals of Botany* **100**: 1155–1164.

Reynolds LK, Stachowicz JJ, Hughes AR, Kamel SJ, Ort BS, Grosberg RK. 2017. Temporal stability in patterns of genetic diversity and structure of a marine foundation species (*Zostera marina*). *Heredity* **118**: 404–412.

Ritland K. 2002. Extensions of models for the estimation of mating systems using n independent loci. *Heredity* **88**: 221–228.

Roels SA, Kelly JK. 2011. Rapid evolution caused by pollinator loss in *Mimulus guttatus*. *Evolution* **65**: 2541–2552.

Scofield DG, Schultz ST. 2006. Mitosis, stature and evolution of plant mating systems: low-Φ and high-Φ plants. *Proceedings of the Royal Society of London B* **1584**: 275–282.

Shore JS, Barrett SCH. 1990. Quantitative genetics of floral characters in homostylous *Turnera ulmifolia* var. *angustifolia* Willd. (Turneraceae). *Heredity* **63**: 105–112.

Stebbins GL. 1950. *Variation and evolution in plants*. London, UK: Oxford University Press.

Stebbins GL. 1974. *Flowering plants: evolution above the species level*. Cambridge, MA: Belknap Press of Harvard University.

Takebayashi N, Wolf DE, Delph LF. 2006. Effect of variation in herkogamy on outcrossing within a population of *Gilia achilleifolia*. *Heredity* **96**: 159–165.

Tank DC, Olmstead RG. 2008. From annuals to perennials: phylogeny of subtribe Castillejinae (Orobanchaceae). *American Journal of Botany* **95**: 608–625.

Venables WN, Ripley BD. 2002. *Modern applied statistics with S*. New York: Springer.

Verma S, Kaul V, Magotra R, Koul AK. 2008. Pollinator-induced anther dehiscence in *Incarvillea emodi* (Bignoniaceae). *Current Science* **94**: 1372–1374.

Wang WT, Pan KY, Zhang ZY, Li ZY, Tao DD, Yin WQ. 1990. *Incarvillea* Juss. In: Wang WT, ed. *Flora Reipublicae Popularis Sinicae* **69**: 36. Beijing: Science Press.

Whitehead MR, Lanfear R, Mitchell RJ, Karron JD. 2018. Plant mating systems often vary widely among populations. *Frontiers in Ecology and Evolution* **6**: 38.

Yin G, Barrett SC, Luo YB, Bai WN. 2016. Seasonal variation in the mating system of a selfing annual with large floral displays. *Annals of Botany* **117**: 391–400.

Yu HY, Gao J, Luo YB, Bai WN. 2011. Development of polymorphic microsatellite markers for *Incarvillea sinensis* (Bignoniaceae). *American Journal of Botany* **98**: e224–e225.

Zhang DY. 2000. Resource allocation and the evolution of self-fertilization in plants. *American Naturalist* **155**: 187–199.