

HIGH BUT VARIABLE OUTCROSSING RATES IN THE INVASIVE *GERANIUM CAROLINIANUM* (GERANIACEAE)¹

REBECCA Y. SHIRK² AND J. L. HAMRICK

Department of Plant Biology, University of Georgia, 2502 Miller Plant Sciences, Athens, Georgia 30602 USA

- **Premise of the study:** Mating system plays an important role in population establishment and persistence, maintenance of genetic variation, and adaptive ability, especially for invasive species that colonize new environments to which they may be poorly adapted. In mixed-mating species, population differences in self-fertilization rates often arise due to variation in local ecological conditions (e.g., pollinator or mate availability) or genetic variation in traits promoting selfing or outcrossing. Knowledge of how and why selfing rates vary can help us understand how populations respond to different environments, how this affects patterns of genetic variation, and the role of mating systems in biological invasions.
- **Methods:** We determined outcrossing rates in invasive (China) and native (US) populations of the weedy annual *Geranium carolinianum* in natural populations and an open-pollinated common garden to answer the following questions: To what extent do populations vary in mating system? Do invasive populations differ from native populations? Is interpopulation variation in mating system under genetic control?
- **Key results:** Despite having many characteristics of selfing species, we found high variation in outcrossing rates (t_m from 0.1 to 1.0) resulting from both environmental and genetic variation. Outcrossing rates were generally high and plastic in the Chinese populations.
- **Conclusions:** A delayed selfing mechanism provides reproductive assurance while allowing facultative outcrossing when mates are not limiting. In invasive populations, high outcrossing rates were facilitated by large admixed founding populations, promoting local adaptation and the maintenance of genetic diversity.

Key words: colonization; delayed selfing; Geraniaceae; *Geranium carolinianum*; inbreeding; invasive species; mating system.

Mating systems play a key role in plant population biology by affecting demographic processes, genetic diversity, and the evolutionary potential of plant populations. For weedy annuals that colonize frequently disturbed, heterogeneous environments, the mating system may be crucial to population persistence because annual species have only a single opportunity for reproduction. The best-known hypothesis regarding mating system in colonizing species is Baker's rule (Baker, 1955), which states that pollen limitation in colonizing populations resulting from a lack of nearby mates and/or pollinators should favor self-compatibility over self-incompatibility. In particular, self-compatibility may be especially beneficial for annual, weedy species that frequently colonize disturbed areas (Baker, 1955, 1974).

However, self-compatibility does not necessarily imply high rates of self-fertilization. Indeed, Baker specified that the "ideal weed" would be "self-compatible, but not completely autogamous or apomictic" (Baker, 1974). Mixed-mating species with

intermediate selfing rates are common, and selfing rates often vary among populations. Plasticity in mating system can arise from a variety of factors including mate availability (Smyth and Hamrick, 1984; Kalisz et al., 2004), habitat (Cheptou et al., 2002), and geitonogamous pollination, which can be affected by floral display size (Barrett et al., 1994). Selfing rates can also be controlled by genetic factors, such as heritable variation in reproductive traits (e.g., Motten and Stone, 2000), variation in the prevalence or strength of self-incompatibility mechanisms (Barrett et al., 1989), and the strength of inbreeding depression, which counteracts the automatic transmission advantage of selfing (Lloyd, 1992; Cheptou et al., 2002).

Mating system analyses can be particularly useful in understanding genetic and evolutionary factors influencing plant invasions. Invasive species are colonizers by definition, and are more likely to be self-compatible than obligately outcrossing (Baker, 1955, 1974; van Kleunen et al., 2008; Burns et al., 2011; Hao et al., 2011). Intraspecific studies investigating mating system variation likewise tend to focus on the loss of self-incompatibility in invasive populations. A particularly well-documented example is *Eichhornia paniculata*, which is tristylous and self-incompatible in its native range, but has evolved selfing in di- and monostylous invasive populations (Barrett et al., 1989; Ness et al., 2010). However, there are self-incompatible species that do maintain self-incompatibility during an invasion (e.g., Sun and Ritland, 1998; Li et al., 2012). In contrast, comparisons among native and invasive populations in self-compatible, mixed-mating species are rare. In mixed-mating species, the same factors that favor self-compatible over self-incompatible species may also promote increased selfing rates in colonizing populations. That is, if there is mate limitation in colonizing populations, selfing rates may be higher

¹Manuscript received 19 May 2014; revision accepted 11 June 2014.

The authors thank S.-M. Chang, D. Christopher, C. Deen, B. Foltz, G. Lor, and C. Zhang for assistance in the field and greenhouse. S. Qiang and B. Hu provided travel assistance in China. Two anonymous reviewers provided helpful comments. This material is based upon work supported by the National Science Foundation's PIRE program under Grant No. OISE 0730218, with additional support from the NSF Graduate Research Fellowship Program, the Oberlin Alumni Fellowship, and the University of Georgia Department of Plant Biology.

²Author for correspondence (e-mail: rshirk@wisc.edu)

relative to established populations, and if selfing is advantageous and under genetic control with heritable variation, invasive populations may evolve increased selfing rates.

However, not all colonizing populations experience mate limitation. If reproductive assurance is not a factor, which may often be the case for invaders with large founding populations and/or multiple introductions, high outcrossing rates may provide benefits for weedy species by promoting rapid adaptation to heterogeneous environments (Verhoeven et al., 2011). Theoretical models of metapopulation colonization show that selfing is advantageous only when there are few colonizers. With high densities, colonizers can overcome Allee effects and maintain high outcrossing rates (Pannell and Barrett, 1998; Cheptou, 2012). This may explain the success of many self-incompatible invasive plants (e.g., *Centaurea solstitialis*, Sun and Ritland, 1998; *Crepis sancta*, Cheptou et al., 2002; and *Ambrosia artemisiifolia*, Friedman and Barrett, 2008).

Geranium carolinianum L. (Geraniaceae) is a small, weedy winter annual. While many species of *Geranium* are showy-flowered, outcrossing perennials, annual species tend to be small-flowered, weedy colonizers of disturbed habitats (Aedo, 2000; Fiz et al., 2008), characteristics associated with the evolution of selfing in outcrossing lineages (Ornduff, 1969). *Geranium carolinianum* has small (~5 mm) pink-white flowers, a low pollen to ovule ratio, is capable of setting seed in the absence of pollinators (Aedo, 2000; Fiz et al., 2008), and is therefore expected to be highly selfing under natural pollination conditions, especially where pollinators are rare.

We measured variation in selfing and outcrossing rates for four invasive and four native populations. To determine whether population variation in mating system is under genetic control, we analyzed progeny arrays produced by plants in natural habitats and in an open-pollinated common garden. We address the following questions: (1) What is the mating system of *G. carolinianum*, and how does it vary among populations from different habitats? (2) Does mating system vary among invasive and native populations? (3) Are among-population differences in

the rates of outcrossing due to genetic variation or phenotypic plasticity?

MATERIALS AND METHODS

Study species—*Geranium carolinianum* (Geraniaceae), Carolina cranesbill, is native to North America and is a common weed in fields, lawns, and roadsides in the southeastern United States. Flowers are visited by generalist insect pollinators (Fiz et al., 2008). Fruits are dry, five-seeded schizocarps with explosive dispersal. It is naturalized in the eastern plains region of China, where it grows in similar habitats and is considered a minor invasive with low environmental impacts, although it is a common weed in agricultural fields (S. Qiang, Nanjing Agricultural University, personal communication). The first records of *G. carolinianum* in China are from Nanjing in 1926 (Aedo, 2000; Chinese Academy of Sciences, 2012), which is in the northeastern part of its current range. Genetic data suggest multiple, independent introductions to the Nanjing area, and levels of genetic diversity in these populations are consistent with more than one founder per founding event (Shirk et al., 2014).

Field collections—Seeds were collected during the spring and summer of 2009 from four populations in the native range (southeastern United States [US]) and four populations in the invasive range (eastern China; Fig. 1). Population sizes ranged from >50 to several thousand individuals. Native US populations FL-2, MD-1, and MS-2 and invasive Chinese populations CN-2, CN-3, and CN-4 were collected from weedy, unmanaged roadsides or parks near the putative introduction point of Nanjing. Native population GA-5 and invasive population CN-5 were collected from managed lawns that appeared to be mowed regularly. Between 24 and 50 reproductive individuals per population were selected haphazardly, and all ripe fruits were collected from each plant. Seeds were cleaned from their fruits, pooled by maternal family, and stored in paper envelopes at room temperature. A voucher specimen for population GA-5 has been deposited at the University of Georgia Herbarium (GA accession number 263322).

Common garden—We established an open-pollinated common garden to determine whether there are genetically based differences among populations in outcrossing rate. Eighteen maternal families were haphazardly selected from seed collections of each of the eight field-collected populations. In mid-January 2010, seeds were germinated in a heated greenhouse without supplemental lighting at the University of Georgia Plant Growth Facility in Athens, Georgia.

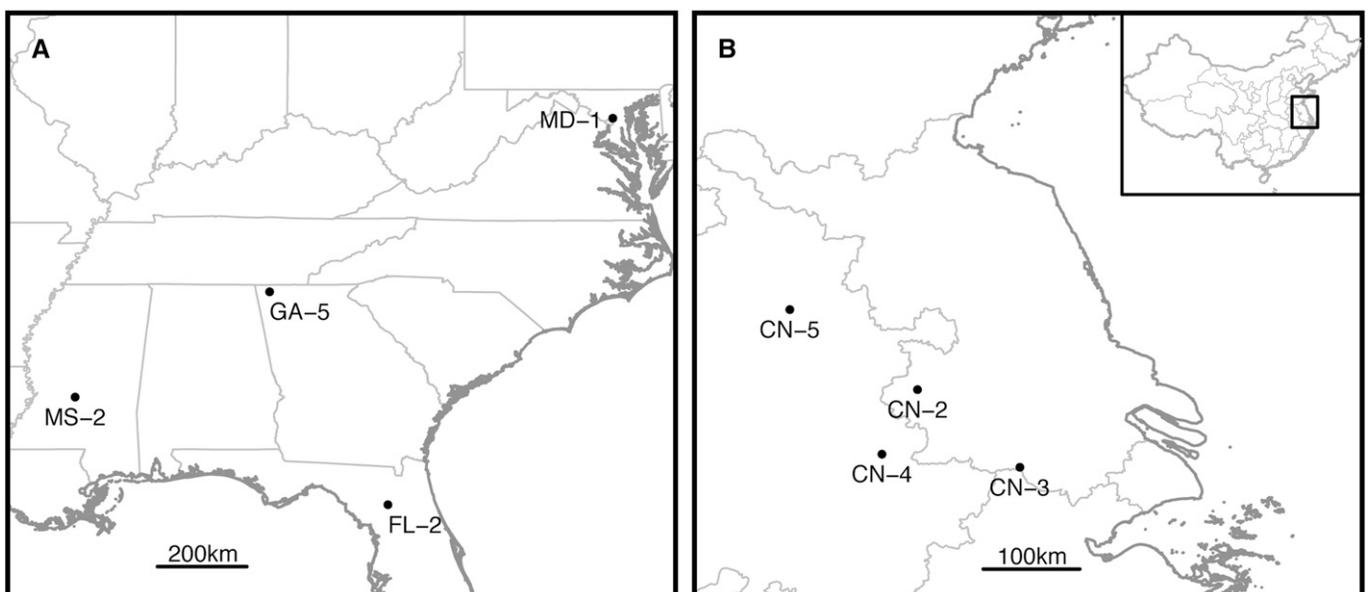


Fig. 1. Map of *Geranium carolinianum* populations sampled for mating system analysis. (A) Native populations in the southeastern United States. (B) Invasive populations in eastern China.

Three seeds per family were planted in flats filled with Fafard 3B soil mix (Sun Gro Horticulture, Agawam, Massachusetts, USA) and placed on a mist bench. After 1 wk, when most of the seeds had germinated, flats were moved to greenhouse benches and watered daily. They were then moved to a cold room in late February to acclimate the plants to late winter temperatures. Between 30 March and 6 April, plants were transplanted into 15 × 15 cm round pots filled with pine bark mix and placed in an outdoor lath house covered in shade cloth to further acclimate them to outdoor conditions.

On 7 April, plants were moved from the lath house to the common garden site, an open field. The few flowers that opened while the plants were in the lath house were pinched off to ensure equal pollinator access to all flowers. One individual per maternal family (18 families/population × 8 populations = 144 individuals) was placed in the main array for use in the mating system analyses. The remaining plants were kept in two randomized, replicate blocks on either side of the main array to increase the size of the experimental population. Because germination was incomplete, the replicate blocks were not full replicates of the main array. Pots were placed edge-to-edge to mimic natural density in a randomized, rectangular array. This rectangular design was used to minimize differential shading across the common garden by a treeline approximately 20 m south of the garden. Plants were watered as necessary throughout the experiment.

All plants were monitored for flowers and fruits every 2 to 3 d from 13 April until the end of the experiment on 16 June, when most plants had senesced. The date of the first flower was recorded, and all ripe fruits were collected from each plant as they matured. Seeds were cleaned from their fruits, pooled by maternal plant, and stored in paper envelopes at room temperature until use.

Allozyme genotyping of progeny arrays—Two progeny arrays were genotyped: one from field-collected seed from the eight natural populations (NAT) and one from seed produced in the experimental common garden (EXP). For the NAT arrays, 12 seeds were genotyped from 18 randomly selected families per population. For the EXP arrays, 12 seeds were genotyped from each of the 144 plants in the main array, which also represented 18 maternal families per population. In total, 216 progeny per population per environment, 3456 individuals, were genotyped.

Seeds were scarified with a razor blade and soaked in water to promote germination. After 1 to 2 wk, when cotyledons were expanded but before true leaves had initiated or expanded, the seed coat was removed, and the entire seedling was crushed in the extraction buffer of Wendel and Parks (1982). The extract was absorbed onto 4 × 6 mm filter paper wicks (Whatman 3 chromatography paper, Piscataway, New Jersey, USA) and stored at -70°C until use. Extracts were run on 11% starch gel electrophoresis and genotyped at seven well-resolved, polymorphic loci. Buffer systems and stains were based on those in Soltis et al. (1983) except where noted: menadione reductase (*Mnr-1*) (Wendel and Weeden, 1989), diaphorase (*Dia-2*) (Manchenko, 1994), and aspartate aminotransferase (*Aat-1*), on buffer system 8-; malate dehydrogenase (*Mdh-4*), 6-phosphogluconate dehydrogenase (*6P-2*), UTP-glucose-1-phosphate (*Ugpp-2*) (Manchenko, 1994), and shikimate dehydrogenase (*Skdh-1*) on buffer system 4. All loci were biallelic except *Skdh-1* and *Aat-1*, which were triallelic. Because allozyme expression from adult leaf tissue is poor in this species (R. Shirk, unpublished data), we were unable to directly obtain maternal genotypes.

Mating system estimation—Mating system parameters were estimated following the correlated-matings model with the program MLTR version 3.2 (Ritland, 2002). Estimates of outcrossing were found using the Newton-Raphson method with pollen and ovule gene frequencies constrained to equal each other. Standard errors were determined based on 5000 bootstraps with maternal family as the resampling unit. Single- and multilocus outcrossing rates (t_s and t_m) were calculated for each population in each environment, using the population × environment combination as a grouping variable. Population values for each environment were averaged by country to obtain estimates of mating system parameters for each country-by-environment grouping.

We performed two-way factorial ANOVAs to determine the effects of source (population or country), pollination environment (NAT vs. EXP), and their interaction on t_m . Tukey's honestly significant difference (HSD) was used for pairwise comparisons among groups. For population-level comparisons, Spearman's rank correlations were used to test associations between EXP and NAT. Significant differences in t_m among groups in EXP and a correlation between EXP and NAT t_m values would indicate that population variation in mating system is under genetic control.

Inbreeding coefficients—Inbreeding coefficients for the maternal generation were calculated based on deviations from Hardy-Weinberg equilibrium for

each NAT population in the program GenAlEx v. 6.5 (Peakall and Smouse, 2006, 2012) using a panel of 19 to 23 polymorphic allozyme loci and between 29 and 50 individuals per population (Shirk et al., 2014). This data set is more appropriate for calculating F because maternal genotypes are known rather than inferred by MLTR, and the larger numbers of individuals and loci should improve precision. F_e , the equilibrium inbreeding coefficient assuming a constant outcrossing rate t , was calculated for each population as $F_e = (1 - t) / (1 + t)$ (Allard et al., 1968), using our estimates of multilocus outcrossing rates (t_m) truncated to $t_m = 1.0$ when they were greater than 100%.

RESULTS

Common garden—The first flowering individuals in the common garden were recorded on 13 April 2010, and the last flowering individual was recorded on 16 May, 33 d later. There was no mortality after plants were moved to the open-pollinated array. Pollinators (mostly bees and other small insects) were observed throughout the experiment visiting the array as well as other spring annuals present in the field (native *Geranium carolinianum*, *G. molle*, *G. pusillum*, *G. dissectum*, *Vicia sativa*, and *V. angustifolia*).

There were significant differences among populations in flowering time. Date of first flower was significantly, positively associated with latitude of the source population (all populations $R^2 = 0.30$, $P < 0.0005$), although the correlation appears to be driven entirely by the US populations (US populations only $R^2 = 0.64$, $P < 0.0005$; Chinese populations only $R^2 = 0.002$, $P = 0.23$).

In the common garden, individuals from population GA-5 were small and did not bolt; rather, flowers were produced close to the ground within the rosette leaves. Mean plant height at maturity measured in a separate greenhouse study was approximately 11 cm for GA-5 plants compared with 30–34 cm for all other populations (Shirk and Hamrick, in press). This low-stature, nonbolting phenotype was also observed in the natural GA-5 population. Maintenance of this phenotype in the common garden indicates that it is under genetic control and possibly indicative of local adaptation to a frequently mowed lawn. Although Chinese population CN-5 was collected from a similarly managed habitat, its individuals bolted in the common garden (mean height = 30 cm), suggesting that they are phenotypically plastic rather than being genetically adapted to their lawn environment.

Mating system analysis—Mating system parameters for all populations are given in Tables 1 and 2. Multilocus outcrossing rates for natural (NAT) populations ranged from 0.14 to >1.0, with China mean $t_m = 0.81$ and US mean $t_m = 0.64$. In the common garden (EXP), t_m ranged from 0.12 to >1.0. Mean outcrossing rates by country for EXP were $t_m = 1.03$ for China and $t_m = 0.68$ for the US. Due to sampling error in the estimation algorithms implemented by MLTR, outcrossing rate estimates greater than 100% are possible. A t_m greater than 1.0 was considered to be fully outcrossed; however, estimates were not truncated at 1.0 to avoid a downward bias for these samples in the ANOVAs. Population-level outcrossing rates in EXP were not correlated with average population flowering time ($F_{1,6} = 0.38$, $P = 0.56$), indicating that differences in t_m were not due to changes in pollinator availability during the common garden experiment. Biparental inbreeding, or mating between relatives, appears low, as $t_m - t_s$ was near zero for most NAT populations. When there is biparental inbreeding, single-locus outcrossing rates should be lower than multilocus outcrossing rates, or $t_m > t_s$.

TABLE 1. Summary of mating system parameters and estimates of inbreeding in natural populations (NAT) of *Geranium carolinianum* in its invasive (China) and native (US) range.

Region	Pop/	t_m	t_s	$t_m - t_s$	F_c	F
China	CN-2	0.50 (0.10)	0.45 (0.09)	0.05 (0.03)	0.34	0.05
China	CN-3	1.03 (0.10)	0.99 (0.09)	0.03 (0.06)	0.00	0.02
China	CN-4	0.87 (0.17)	0.73 (0.14)	0.14 (0.07)	0.07	-0.04
China	CN-5	0.83 (0.11)	0.76 (0.12)	0.07 (0.02)	0.09	0.03
US	FL-2	0.38 (0.09)	0.29 (0.07)	0.09 (0.03)	0.45	0.26
US	GA-5	0.14 (0.04)	0.12 (0.03)	0.02 (0.01)	0.76	0.03
US	MD-1	0.98 (0.09)	0.87 (0.09)	0.11 (0.07)	0.01	0.02
US	MS-2	1.05 (0.06)	0.82 (0.05)	0.23 (0.06)	0.00	0.12

Notes: t_m = multilocus outcrossing rate; t_s = single-locus outcrossing rate; $t_m - t_s$ = rate of biparental inbreeding; F_c = equilibrium inbreeding coefficient estimated from t_m ; F = fixation index.

Differences in mean country outcrossing rates across environments were assessed with a two-way factorial ANOVA. Both the main effects of country (China vs. US) and environment (NAT vs. EXP) were significant, but the interaction was not (Table 3). Mean outcrossing rates in EXP were higher than in NAT. Within each environment, Chinese populations were significantly more outcrossing than US populations (China EXP > US EXP and China NAT > US NAT; Tukey’s HSD $P < 0.05$, Fig. 2). When GA-5, the highly selfing lawn population, was removed from the analysis, the effect of region was only marginally significant ($F_{1,10} = 4.55$, $P = 0.06$), environment remained significant ($F_{1,10} = 15.17$, $P = 0.002$), and the interaction remained nonsignificant.

The main effects of population and environment as well as the interaction term were significant in the population-level ANOVA (Table 4). Variation in outcrossing rates is driven at least partly by genetic differences, as indicated by significant differences among EXP populations (Fig. 3). Genetic control of outcrossing rates is also corroborated by a marginally significant association between outcrossing rates in NAT and EXP (Spearman’s rho = 0.68, $P = 0.06$). Additionally, a significant population-by-environment interaction in the ANOVA implies some degree of plasticity in mating system, but only for the predominately outcrossing populations. The two populations with the lowest outcrossing rates (GA-5 and FL-2) were not significantly different across environments (Tukey’s HSD $P > 0.05$). For populations showing plasticity, most had higher outcrossing rates in the common garden, with the exception of MS-2 (Fig. 3).

Inbreeding coefficients—Inbreeding coefficients for the NAT maternal generation, calculated from a separate population

TABLE 2. Summary of mating system parameters estimated for invasive (China) and native (US) populations (Pop.) of *Geranium carolinianum* grown in an open-pollinated common garden (EXP).

Region	Pop.	t_m	t_s
China	CN-2	0.79 (0.15)	0.72 (0.12)
China	CN-3	1.11 (0.12)	1.19 (0.12)
China	CN-4	1.20 (0.05)	1.01 (0.08)
China	CN-5	1.01 (0.14)	0.92 (0.12)
US	FL-2	0.41 (0.10)	0.38 (0.09)
US	GA-5	0.12 (0.03)	0.09 (0.03)
US	MD-1	1.20 (0.03)	1.01 (0.09)
US	MS-2	0.98 (0.08)	0.80 (0.08)

Notes: t_m = multilocus outcrossing rate; t_s = single-locus outcrossing rate.

genetic data set, ranged from $F = -0.04$ to 0.05 in China with an average of 0.02, and ranged from $F = 0.02$ to 0.26 in the US with an average of 0.11 (Table 1). Lower values for Chinese populations are consistent with results from a broader sampling of 24 Chinese and 16 US populations, where the average inbreeding coefficient was -0.09 in China and 0.10 in the US (Shirk et al., 2014). We also estimated F_c , the inbreeding coefficient calculated under the assumption that the population is at inbreeding equilibrium with a constant outcrossing rate t_m . F_c ranged from 0.00 to 0.34 in China and 0.00 to 0.76 in the US (Table 1). When there is inbreeding depression, selection against selfed progeny will reduce the inbreeding coefficient in the adult cohort relative to juveniles; that is, $F_c > F$ (Ritland, 1990). This pattern was found in three Chinese populations and two US populations. Populations with the greatest differences between F_c and F were GA-5, the low-statured lawn population with the highest selfing rate, and FL-2 and CN-2, which had the second and third highest selfing rates in both environments (Fig. 3). Under assumptions of a constant outcrossing rate and inbreeding equilibrium (constant adult F), these patterns are consistent with the presence of inbreeding depression in these three populations.

DISCUSSION

Mating system analyses of eight populations of *G. carolinianum* show that this species is generally mixed-mating to highly outcrossing. These results were surprising, given that *G. carolinianum* has been described as “generally autogamous” (Fiz et al., 2008) due to its low pollen to ovule ratio and its ability to set fruit when pollinators were excluded. Observed high outcrossing rates despite a clear capacity for self-fertilization likely result from delayed autonomous selfing. *Geranium carolinianum* is incompletely protogynous, with anthers maturing and coming into contact with the stigma a few hours after flower

TABLE 3. Factorial ANOVA testing the effects of region (China vs. US) and environment (common garden vs. natural) on outcrossing rate in native and invasive populations of *Geranium carolinianum*.

Source	df	MS	F
Region	1	4.83	36.45***
Environment	1	1.24	9.37**
Region × environment	1	0.59	4.43
Error	12	0.13	

Notes: ** $P < 0.001$; *** $P < 0.0001$.

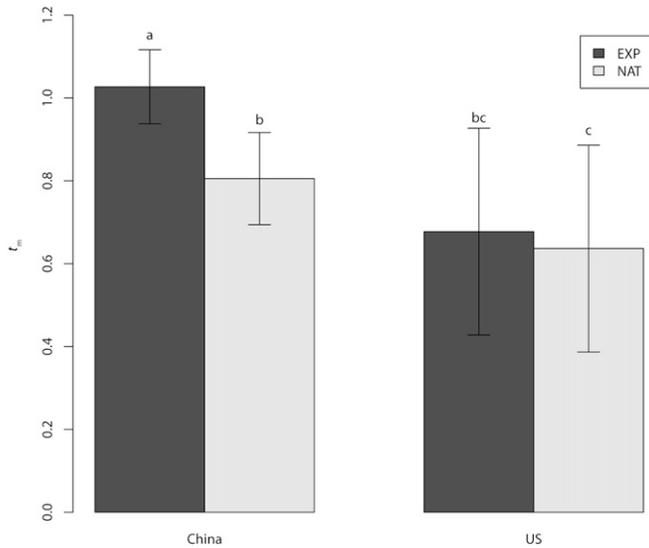


Fig. 2. Mean outcrossing rates (t_m) of *Geranium carolinianum* populations in the invasive (China) and native (US) range based on progeny arrays generated in an open-pollinated common garden (EXP) and natural populations (NAT). Error bars are \pm SE. Bars not sharing a letter differ significantly from each other ($P < 0.05$; Tukey's HSD).

opening (DuBay and Murdy, 1983; R. Shirk and S.-M. Chang, University of Georgia, unpublished data). Thus there is a short, but perhaps variable, period when female stage flowers are available for exclusively outcross pollination, followed by reproductive assurance via delayed self-pollination. If pollinators are abundant, all ovules in each flower may be outcrossed during the female period, but selfing rates will be higher if pollinator visitation is low. Delayed selfing is almost always beneficial, as it provides reproductive assurance without interfering with opportunities for outcrossing (Lloyd, 1992). For colonizing species, delayed selfing assures seed production in situations with one or a few founders, but the advantages of outcrossing are retained when population sizes are larger and pollinators are available (Brys et al., 2011). Our results emphasize the importance of using molecular markers to characterize the mating system, especially in self-compatible species, as the capacity for selfing may not correspond with actual rates of self-pollination.

There was substantial variation among populations in selfing rates for progeny arrays produced in both the natural and garden pollination environments, indicating that among-population differences in mating system have a genetic basis. Adaptive evolution of the mating system has been documented in other species in response to several factors. For example, increased outcrossing in selfing species may be adaptive if increased

TABLE 4. Factorial ANOVA testing the effects of population (pop) and environment (common garden vs. natural; env) on outcrossing rate in native and invasive populations of *Geranium carolinianum*.

Source	df	MS	F
Population	7	4.67	474.20***
Environment	1	1.24	126.09***
Population \times environment	7	0.19	19.64***
Error	272	0.01	

Notes: *** $P < 0.0001$.

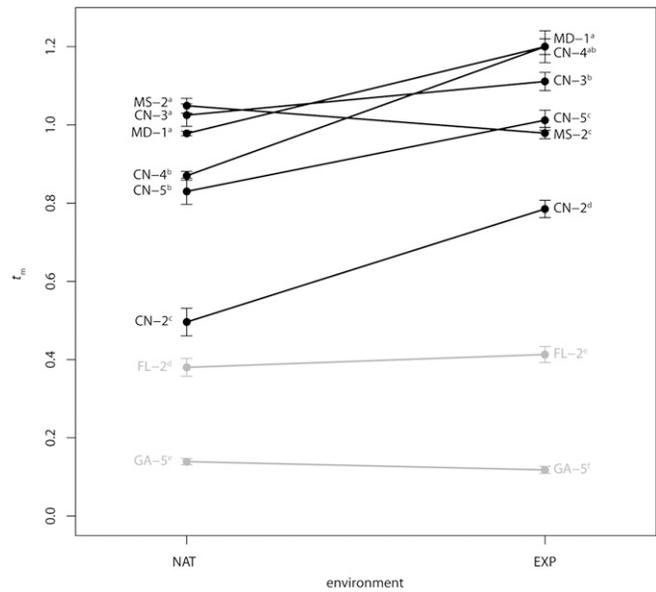


Fig. 3. *Geranium carolinianum* population estimates of outcrossing rates (t_m) based on progeny arrays generated in natural populations (NAT) and a common garden (EXP). Populations with significant differences between NAT and EXP environments ($P < 0.05$; Tukey's HSD) are marked with a black line; those with nonsignificant differences are marked with a gray line. Within each environment, different letters denote significant differences among populations. Error bars are \pm SE.

recombination rates are advantageous (Kahler et al., 1975), although selection must be strong enough to overcome the automatic transmission advantage of selfing (Schemske and Lande, 1985; Lloyd, 1992). Alternatively, increased selfing may evolve in isolated or low-density populations for reproductive assurance. This prediction was supported in *Leavenworthia alabamica* (Busch, 2005) but not in *Crepis sancta* (Cheptou et al., 2002). Selfing may also evolve as a mechanism to minimize maladaptive gene flow (e.g., *Anthoxanthum odoratum* and *Agrostis tenuis* on mine tailings [Antonovics, 1968, 2006]).

We also documented substantial plasticity in the mating system, evidenced by significant differences in outcrossing rates in the two environments (NAT and EXP). However, this plasticity was only demonstrated in highly outcrossed populations; the two populations with low outcrossing rates ($t_m < 0.4$) are genetically differentiated from the others in their mating system but are less plastic. It is possible that variation among populations in the degree of protogyny could explain these results. Like many *Geranium* species, *G. carolinianum* exhibits dichogamy, and the degree of separation between male and female phases varies among species (Philipp, 1985; Aedo et al., 2005), among populations within species, or within a single plant (Philipp, 1985). A preliminary greenhouse study using native populations of *G. carolinianum* suggests both population- and individual-level variation in time to anther dehiscence and stigma contact (R. Shirk and S.-M. Chang, University of Georgia, unpublished data). Populations with delayed selfing would have more opportunity for outcross pollination (= higher t_m), but realized outcrossing rates would depend on pollinator availability (= higher plasticity). Delayed selfing has been proposed to be adaptive in *Collinsia verna* in response to variable pollinator conditions across years (Kalisz and Vogler, 2003). At the other end of the spectrum, populations with prior, competing,

or minimally delayed selfing (Lloyd, 1992; Goodwillie et al., 2005) would have fewer opportunities for outcross pollination (lower t_m) and would be less affected by variation in pollinator availability, leading to less plasticity in outcrossing rates.

The three populations with the lowest estimates of t_m in both the NAT and EXP environments (CN-2, FL-2 and GA-5) are also the only populations that have large differences between their F and F_c values (Table 1). Such differences in inbreeding coefficients appear to be consistent with the action of selection against inbred individuals (i.e., inbreeding depression). This conclusion, however, assumes that the outcrossing rate does not vary across generations and that the populations are at inbreeding equilibrium (Ritland, 1990). Our results do support the assumption of a constant outcrossing rate in these populations, strengthening our evidence for inbreeding depression: mating system estimates for CN-2, FL-2, and GA-5 were very similar in both the NAT and EXP environments (Fig. 3) indicating at least partial genetic control of the mating system for these three populations. In particular, outcrossing rates were not significantly different in the EXP and NAT environments for populations GA-5 and FL-2. Evidence of inbreeding depression in populations with higher selfing rates suggests that in addition to providing reproductive assurance, delayed selfing may be beneficial in *G. carolinianum* by maximizing outcrossing and thus minimizing inbreeding depression. Delayed selfing may also help to explain the unexpectedly high outcrossing rates found in many of the populations sampled.

Adaptation to local habitats may explain some of the observed variation in the mating system. Both lawn populations, GA-5 and CN-5, had low-statured, nonbolting phenotypes in their natural environment, but only GA-5 maintained this phenotype in the common garden. GA-5 was also strongly differentiated in its mating system and had the highest selfing rate. High selfing likely resulted from reduced pollinator visitation because flowers produced by the low-stature phenotype are smaller, closer to the ground, and less visible to pollinators, although opportunities for outcrossing would also be limited if this population exhibits reduced protogyny. Geitonogamous selfing is unlikely to play a large role in the mating system of *G. carolinianum* because plants generally have few simultaneously open flowers. While high selfing rates in GA-5 have a genetic basis, is not clear whether it is adaptive, or if it resulted from strong selection for low stature resulting in smaller plants with lower rates of pollinator visitation. Indeed, evidence for inbreeding depression in this population, as discussed, indicates that selfing could be maladaptive. Alternatively, there may be selection for high selfing rates to reduce gene flow with populations with taller, bolting phenotypes (Antonovics, 1968). More work is needed to assess which traits are under selection (e.g., via phenotypic selection analysis) and to determine the mechanism underlying increased selfing in this population.

In contrast, individuals from CN-5 also did not bolt in their natural habitat (a mowed lawn, similar to GA-5), but this low-stature phenotype does not have a genetic basis since all CN-5 individuals bolted in the common garden. Interestingly, CN-5 had a predominantly outcrossing mating system in its natural environment. The plasticity in mating system observed for this population (higher outcrossing rates in the common garden) may also be explained by plasticity in plant stature, as a low-stature phenotype in the mowed lawn habitat may have contributed to reduced outcrossing rates.

Despite wide variation in mating systems across all eight populations, progeny arrays collected from natural Chinese

populations were significantly more outcrossed than those from the US. The common garden results show that these differences have a genetic basis and are not solely due to environmental factors such as pollinator availability. Thus, apparently it is the outcrossing, not the highly selfing, forms of *G. carolinianum* that were successful invasives, although we cannot determine whether selfing individuals were introduced and failed to establish, or were not introduced. Higher outcrossing rates in China are consistent with results from an earlier genetic diversity study, which found lower inbreeding coefficients in Chinese compared to US populations. Additionally, patterns of genetic diversity in China are consistent with multiple introductions from genetically distinct sources and multiple founders per founder event (Shirk et al., 2014). Thus, initially there may have been little selection for selfing in the newly founded populations, as high propagule pressure, which is characteristic of many invasions, reduces the need for selfing as reproductive assurance. It is also possible that since *G. carolinianum* has been in China for over 80 generations, even if there was selection for selfing early on, outcrossing may have been favored in later generations. In any case, recombination advantages provided by outcrossing would promote local adaptation in colonizing populations. This is especially true for populations that are admixtures of distinct genetic stocks, because outcrossing would produce novel, recombinant genotypes (Verhoeven et al., 2011). Furthermore, high outcrossing would minimize genetic diversity loss due to founder effects.

In summary, despite having many characteristics of a highly selfing species, we found that outcrossing rates in *G. carolinianum* are frequently high, but variable across habitats. A delayed selfing mechanism provides reproductive assurance while allowing facultative outcrossing when mates are not limiting. However, we documented two cases where the populations appear to have evolved high selfing rates that did not vary across environments, most likely as a result of selection in their natural habitats. In the invasive range, high outcrossing rates, which were facilitated by large admixed founding populations, support local adaptation and the maintenance of genetic diversity in invasive populations.

LITERATURE CITED

- AEDO, C. 2000. The genus *Geranium* L. (Geraniaceae) in North America. I. Annual species. *Anales del Jardín Botánico de Madrid* 51: 39–82.
- AEDO, C., O. FIZ, M. L. ALARCÓN, C. NAVARRO, J. J. ALDASORO, AND L. A. ALICE. 2005. Taxonomic revision of *Geranium* sect. *Dissecta* (Geraniaceae). *Systematic Botany* 30: 533–558.
- ALLARD, R. W., S. K. JAIN, AND P. L. WORKMAN. 1968. The genetics of inbreeding populations. *Advances in Genetics* 14: 55–131.
- ANTONOVICS, J. 1968. Evolution in closely adjacent plant populations. V. Evolution of self-fertility. *Heredity* 23: 219–238.
- ANTONOVICS, J. 2006. Evolution in closely adjacent plant populations X: Long-term persistence of prereproductive isolation at a mine boundary. *Heredity* 97: 33–37.
- BAKER, H. G. 1955. Self-compatibility and establishment after 'long-distance' dispersal. *Evolution* 9: 347–349.
- BAKER, H. G. 1974. The evolution of weeds. *Annual Review of Ecology and Systematics* 5: 1–24.
- BARRETT, S. C., M. T. MORGAN, AND B. C. HUSBAND. 1989. The dissolution of a complex genetic polymorphism: The evolution of self-fertilization in tristylous *Eichhornia paniculata* (Pontederiaceae). *Evolution* 43: 1398–1416.

- BARRETT, S. C. H., L. D. HARDER, AND W. W. COLE. 1994. Effects of flower number and position on self-fertilization in experimental populations of *Eichhornia paniculata* (Pontederiaceae). *Functional Ecology* 8: 526–535.
- BRYNS, R., E. DE CROP, M. HOFFMANN, AND H. JACQUEMYN. 2011. Importance of autonomous selfing is inversely related to population size and pollinator availability in a monocarpic plant. *American Journal of Botany* 98: 1834–1840.
- BURNS, J. H., T.-L. ASHMAN, J. A. STEETS, A. HARMON-THREATT, AND T. M. KNIGHT. 2011. A phylogenetically controlled analysis of the roles of reproductive traits in plant invasions. *Oecologia* 166: 1009–1017.
- BUSCH, J. W. 2005. The evolution of self-compatibility in geographically peripheral populations of *Leavenworthia alabamica* (Brassicaceae). *American Journal of Botany* 92: 1503–1512.
- CHEPTOU, P. O. 2012. Clarifying Baker's Law. *Annals of Botany* 109: 633–641.
- CHEPTOU, P. O., J. LEPART, AND J. ESCARRE. 2002. Mating system variation along a successional gradient in the allogamous and colonizing plant *Crepis sancta* (Asteraceae). *Journal of Evolutionary Biology* 15: 753–762.
- CHINESE ACADEMY OF SCIENCES. 2012. Chinese virtual herbarium. Available at http://www.cvh.org.cn/index_en.php. [accessed 9 September 2009]
- DUBAY, D. T., AND W. H. MURDY. 1983. Direct adverse effects of SO₂ on seed set in *Geranium carolinianum* L.: A consequence of reduced pollen germination on the stigma. *Botanical Gazette* 144: 376–381.
- FIZ, O., P. VARGAS, M. ALARCÓN, C. AEDO, J. L. GARCÍA, AND J. J. ALDASORO. 2008. Phylogeny and historical biogeography of Geraniaceae in relation to climate changes and pollination ecology. *Systematic Botany* 33: 326–342.
- FRIEDMAN, J., AND S. C. H. BARRETT. 2008. High outcrossing in the annual colonizing species *Ambrosia artemisiifolia* (Asteraceae). *Annals of Botany* 101: 1303–1309.
- GOODWILLIE, C., S. KALISZ, AND C. G. ECKERT. 2005. The evolutionary enigma of mixed mating systems in plants: Occurrence, theoretical explanations, and empirical evidence. *Annual Review of Ecology, Evolution and Systematics* 36: 47–79.
- HAO, J. H., S. QIANG, T. CHROBOCK, M. VAN KLEUNEN, AND Q. Q. LIU. 2011. A test of Baker's law: Breeding systems of invasive species of Asteraceae in China. *Biological Invasions* 13: 571–580.
- KAHLER, A., M. CLEGG, AND R. W. ALLARD. 1975. Evolutionary changes in the mating system of an experimental population of barley (*Hordeum vulgare* L.). *Proceedings of the National Academy of Sciences, USA* 72: 943–946.
- KALISZ, S., AND D. W. VOGLER. 2003. Benefits of autonomous selfing under unpredictable pollinator environments. *Ecology* 84: 2928–2942.
- KALISZ, S., D. W. VOGLER, AND K. M. HANLEY. 2004. Context-dependent autonomous self-fertilization yields reproductive assurance and mixed mating. *Nature* 430: 884–887.
- LI, X.-M., W.-J. LIAO, L. M. WOLFE, AND D.-Y. ZHANG. 2012. No evolutionary shift in the mating system of North American *Ambrosia artemisiifolia* (Asteraceae) following its introduction to China. *PLoS ONE* 7: e31935.
- LLOYD, D. G. 1992. Self- and cross-fertilization in plants. II. The selection of self-fertilization. *International Journal of Plant Sciences* 153: 370–380.
- MANCHENKO, G. P. 1994. Handbook of detection of enzymes on electrophoretic gels. CRC Press, Ann Arbor, Michigan, USA.
- MOTTEN, A. F., AND J. L. STONE. 2000. Heritability of stigma position and the effect of stigma-anther separation on outcrossing in a predominantly self-fertilizing weed, *Datura stramonium* (Solanaceae). *American Journal of Botany* 87: 339–347.
- NESS, R. W., S. I. WRIGHT, AND S. C. BARRETT. 2010. Mating-system variation, demographic history and patterns of nucleotide diversity in the tristylous plant *Eichhornia paniculata*. *Genetics* 184: 381–392.
- ORNDUFF, R. 1969. Reproductive biology in relation to systematics. *Taxon* 18: 121–133.
- PANNELL, J. R., AND S. C. H. BARRETT. 1998. Baker's law revisited: Reproductive assurance in a metapopulation. *Evolution* 52: 657–668.
- PEAKALL, R., AND P. E. SMOUSE. 2006. GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6: 288–295.
- PEAKALL, R., AND P. E. SMOUSE. 2012. GenAIEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics* 28: 2537–2539.
- PHILIPP, M. 1985. Reproductive biology of *Geranium sessiliflorum*. 1. Flower and flowering biology. *New Zealand Journal of Botany* 23: 567–580.
- RITLAND, K. 1990. Inferences about inbreeding depression based on changes of the inbreeding coefficient. *Evolution* 44: 1230–1241.
- RITLAND, K. 2002. Extensions of models for the estimation of mating systems using *n* independent loci. *Heredity* 88: 221–228.
- SCHEMSKE, D. W., AND R. LANDE. 1985. The evolution of self-fertilization and inbreeding depression in plants. II. Empirical observations. *Evolution* 39: 41–52.
- SHIRK, R. Y., AND J. L. HAMRICK. In press. Multivariate adaptation but no increase in competitive ability in invasive *Geranium carolinianum* L. (Geraniaceae). *Evolution*.
- SHIRK, R. Y., J. L. HAMRICK, C. ZHANG, AND S. QIANG. 2014. Patterns of genetic diversity reveal multiple introductions and recurrent founder effects during range expansion in invasive populations of *Geranium carolinianum* (Geraniaceae). *Heredity* 112: 497–507.
- SMYTH, C., AND J. L. HAMRICK. 1984. Variation in estimates of outcrossing in musk thistle populations. *The Journal of Heredity* 75: 303–307.
- SOLTIS, D. E., C. H. HAUFLE, D. C. DARROW, AND G. J. GASTONY. 1983. Starch gel electrophoresis of ferns: A compilation of grinding buffer, gel and electrode buffers, and staining schedules. *American Fern Journal* 73: 9–27.
- SUN, M., AND K. RITLAND. 1998. Mating system of yellow starthistle (*Centaurea solstitialis*), a successful colonizer in North America. *Heredity* 80: 225–232.
- VAN KLEUNEN, M., J. C. MANNING, V. PASQUALETTO, AND S. D. JOHNSON. 2008. Phylogenetically independent associations between autonomous self-fertilization and plant invasiveness. *American Naturalist* 171: 195–201.
- VERHOEVEN, K. J., M. MACEL, L. M. WOLFE, AND A. BIERE. 2011. Population admixture, biological invasions and the balance between local adaptation and inbreeding depression. *Proceedings of the Royal Society, B, Biological Sciences* 278: 2–8.
- WENDEL, J. F., AND C. R. PARKS. 1982. Genetic control of isoenzyme variation in *Camellia japonica* L. *The Journal of Heredity* 73: 197–204.
- WENDEL, J. F., AND N. F. WEEDEN. 1989. Visualization and interpretation of plant isozymes. In D. E. Soltis and P. S. Soltis [eds.], *Isozymes in plant biology*, 5–45. Dioscorides Press, Portland, Oregon, USA.