

Evaluating Possible Reinforcement in *Campanula americana*:
Response to Postzygotic Isolation by Increasing Selfing?

Hanqin Wu
Jingdezhen, Jiangxi, China

Bachelor of Science, Beijing Normal University, 2015

A Thesis presented to the Graduate Faculty of the University of
Virginia in Candidacy for the Degree of
Master of Science

Department of Biology

University of Virginia
May, 2018

Abstract

Allopatry leads to postzygotic isolation through the accumulation of genetic incompatibilities between different lineages. Consequently, when lineages come into secondary contact, the fitness of hybrids between lineages will be reduced. Then a process called reinforcement may occur, where selection to avoid hybridization leads to the evolution of greater prezygotic isolation between lineages. Selfing is reported as a mechanism of prezygotic isolation between sympatric sister species that contributes to reinforcement, but whether selfing also is involved in reinforcement in the early stages of speciation is still unknown. To examine whether selfing increases in secondary contact as a mechanism of reinforcement in the early stages of speciation, I estimated the levels of selfing in populations from different lineages in contact zones and allopatry in the hermaphroditic herb, *Campanula americana*. This species has asymmetric cytonuclear incompatibilities between lineages that cause unfit hybrids. In addition, selfing is evolutionarily flexible for *C. americana*, since populations vary in the degree of autonomous selfing. Selfing was measured by determining autonomous fruit set of plants in the greenhouse as well as by estimating selfing rates from wild-collected seeds. I found that populations in contact zones do not have higher autonomous fruit set or selfing rates than allopatric populations, and populations that experience stronger cytonuclear incompatibility also do not have higher autonomous fruit set or selfing rates. These findings indicate that reinforcement is not the cause of observed variation in selfing. However, there was a linear increase of selfing rates from eastern populations to western populations, indicating that a geographical cline, rather than reinforcement, explains the variation in selfing among populations. Despite the evolutionary flexibility of autonomous selfing in *C. americana*, there is no evidence that selfing evolves in response to postzygotic isolation as a prezygotic isolating mechanism.

Acknowledgements

I would first like to thank my thesis advisor Prof. Laura Galloway for the patient guidance, encouragement and advice she has provided throughout these two years. I also would like to thank all Galloway labbies: Karen Barnard-Kubow, Matthew Koski, Brittany Sutherland and Catherine Debban, as well as Alyssa Bangerter and Erin Fegley, who provided so much help for both my work and my life. Then, I would like to thank my committee members, Prof. Deborah Roach and Prof. Robert Cox, for their comments on my paper and their general enthusiasm and openness to discussion about the work. Finally, I would like also thank to my parents who gave me unconditional love and support during the past twenty-four years.

Introduction

Understanding the mechanisms of speciation is a primary goal in evolutionary biology.

Reinforcement, a process restricting interspecific gene flow between coexisting lineages, is thought to be the latter step of a two-step speciation process (Servedio and Noor 2003). In the first allopatric step, unique adaptations or mutations accumulate in each lineage, leading to postzygotic isolation between the two lineages that reduce hybrid fitness. In the second sympatric step, allopatric lineages come into secondary contact and reinforcement, a process in which prezygotic isolation between coexisting lineages is strengthened by the selection to avoid producing unfit hybrids, completes speciation (Schluter 2001). Empirical evidence of reinforcement is reported in many taxa, including fruit flies (Coyne and Orr 1989), frogs (Gerhardt 1994), birds (Sætre et al. 1999) and flowering plants (Fishman and Wyatt 1999). Since reinforcement reveals how natural selection, a primary microevolution force, might lead to speciation, a macroevolution change, exploration of when and how it occurs contributes to our understanding of species diversification (Servedio 2004).

Reinforcement is usually detected by looking for its signature: greater prezygotic isolation in sympatry than in allopatry (Servedio and Noor 2003). Since selection against hybridization only occurs where populations from two lineages have gene flow, stronger prezygotic isolation should be found between sympatric populations than allopatric populations (Ortiz-Barrientos et al. 2009). A primary mechanism of sympatric prezygotic isolation is nonrandom mating, which can be achieved either through assortative mating or mating preference (Kirkpatrick and Ravigne 2002). Assortative mating occurs when individuals select mates phenotypically similar to themselves (e.g. similar body size), while mating preference occurs when individuals from different lineages prefer mates with different traits (e.g. different body colors) (Servedio 2000). For plants, selfing is an assortative mating mechanism (Fishman and Wyatt 1999, Servedio and Noor 2003), while divergence in intensity of flower color (Hopkins and Rausher 2012), floral morphology (Whalen 1978) and flowering time (McNeilly and Antonovics 1968) are mating

preference mechanisms (Servedio 2009).

Theoretical studies predict that assortative mating mechanisms will contribute more to reinforcement than mating preference mechanisms, since the later has more restricted conditions (Liou and Price 1994, Servedio 2000, Kirkpatrick and Ravigne 2002). In the mating preference mechanisms, recombination might breakdown linkage-disequilibrium between mate preference genes affecting prezygotic isolation and genes affecting postzygotic isolation, leading to a failure of reinforcement. In contrast, for assortative mating mechanisms, the gene causing premating isolation, such as selfing genes, is identical in both populations, so recombination does not affect the genetic association between pre- and postzygotic isolation (Felsenstein 1981). However, the relative frequencies of these two mechanisms in natural populations is still unknown (Servedio and Noor 2003).

Empirical studies that find reinforcement signatures in plants typically demonstrate mating preference mechanisms (Armbruster et al. 1994, Kay 2006, Hopkins and Rausher 2012), though an assortative mating mechanism, like selfing, is predicted to be more prevalent (Servedio and Noor 2003). The shift in mating system, especially from outcrossing to selfing, is one of the most common evolutionary transitions in plants (Runquist et al. 2014). Moreover, mixed mating systems, where reproduction occurs by both selfing and outcrossing, are also frequent in hermaphroditic plants (Goodwillie et al. 2005). In plants with mixed mating systems, autonomy (within-flower self-pollination without vectors, thus providing reproductive assurance) and geitonogamy (between-flower self-pollination by vectors) together contribute to selfing (Eckert 2000). Most studies on the evolution of mating systems focus on the role of inbreeding depression and reproduction assurance (Epinat and Lenormand 2009), therefore the contribution of selfing to reinforcement needs further exploration.

Increasing evidence indicates that the transition to selfing may contribute to reproductive

isolation between coexisting sister species. Sympatric populations are more self-compatible than allopatric populations for sister species pairs with overlapping ranges (Levin 1985, Matallana et al. 2010). Sister species pairs including at least one selfing species are more likely to coexist (Grossenbacher and Whittall 2011, Brys et al. 2013). Moreover, “selfing syndromes”, such as smaller flowers, reduced herkogamy (temporal separation of male and female parts), reduced dichogamy (spatial separation of male and female parts) and reduced pollen production, are found in sympatric populations (Smith and Rausher 2007, Runquist et al. 2014). However, direct measurement and comparison of selfing rates between wild sympatric and allopatric populations is rare. Moreover, increased selfing is seldom reported as a mechanism of reinforcement in the early stages of speciation when the divergence is less strong. Models predict that initial postzygotic isolation between lineages is necessary for reinforcement to occur (Liou and Price 1994). Empirical investigation of the role of selfing in reproductive isolation within a species will help to determine when reinforcement occurs.

Here, I test whether selfing increases to avoid producing unfit hybrids in the early stages of speciation in the hermaphroditic herb, *Campanula americana*. Recent genomic work reveals a division of *C. americana* into three major genetic lineages that are also geographically distinct: the Western lineage, the Appalachian lineage and the Eastern lineage (Barnard-Kubow et al. 2015, Figure 1). The ranges of the Western lineage and the Appalachian lineage overlap in North Carolina and Pennsylvania (Figure 1). The regions of overlap are called contact zones, since populations from different lineages are close enough to have gene flow. The prerequisite of reinforcement, costly hybridization, is found in *C. americana*. Asymmetric cytonuclear incompatibilities between lineages cause strong reductions in germination and seedling survival in between-lineage hybrids, with more severe reductions in hybrids with a Western lineage mother (Barnard-Kubow et al. 2016). These incompatibilities are less severe between populations in the North Carolina contact zone than those in the Pennsylvania contact zone or in allopatry (C. Debban, unpublished data). This species has a mixed mating system and shows

large variation in autonomous selfing among populations. A previous study that surveyed 24 Western lineage populations found that autonomy increases from the east to the west and from the south to the north (Koski et al. 2017), indicating that the mating system of *C. americana* is evolutionarily flexible and can perhaps evolve to avoid postzygotic isolation.

To examine possible reinforcement during secondary contact, I determined autonomous fruit set of greenhouse plants and estimated selfing rates from wild-collected seeds for different *C. americana* lineages in contact zones and allopatry. The former method evaluates selfing without pollinators, while the latter evaluates the frequency of selfing when outcrossing pollen is provided by pollinators. To test for a signature of reinforcement, I compared autonomous fruit set and selfing rates of populations in contact zones and allopatry, and hypothesized that (1) populations in contact zones have higher autonomy and selfing rates than allopatric populations. To test whether reinforcement is the cause of any increase in selfing, I compared the autonomous fruit set and selfing rates of populations experiencing different levels of postzygotic isolation, hypothesizing that (2) populations from the Western lineage that experience stronger cytonuclear incompatibility have higher autonomy and selfing rates than those from the Appalachian lineage; and (3) populations in the North Carolina contact zone where hybrids are more fit have lower selfing rates than populations in the Pennsylvania contact zone. To evaluate an alternative explanation for variation in selfing other than reinforcement, I determined the association between autonomous fruit set and selfing rates with both longitude and latitude, testing the alternative hypothesis that a geographical cline influences autonomy and selfing rate of *C. americana* populations.

Methods

System

The American Bellflower (*Campanula americana* L. = *Campanulastrum americanum* Small) is an autotetraploid, monocarpic herb that grows on road sides and forest edges in eastern North America. It is either annual or biennial, requiring vernalization to flower. Flowers are

hermaphroditic and located in indeterminate inflorescences at reproductive nodes on the main stem and also on the side branches of larger individuals. It is insect-pollinated and largely outcrossing, while self-compatibility and limited inbreeding depression permit selfing (Galloway et al. 2003). Both dichogamy and herkogamy are exhibited. Upon anthesis, flowers are in male phase with pollen held on pollen collecting hairs along the side of the style. Pollen removal reduces the duration of male phase (Evanhoe and Galloway 2002). After one or two days, stigmatic lobes at the top of the style open, initiating female phase. However, pollen can remain on the style even when plants transition into female phase (Koski et al. 2018). Reduced dichogamy and longer pollen viability are associated with high autonomy (Leibman et al. 2018, Koski et al. 2018). Autonomy varies among populations and demonstrates a geographic cline that increases from south to north and from east to west (Koski et al. 2017).

Campanula americana is divided into three major chloroplast lineages that are geographically distinct. The eastern lineage is located in the Blue Ridge Mountains and east to the Atlantic Ocean, the Appalachian lineage only occurs in the Appalachian Mountains and the Western lineage is found throughout the species range to the west of the Appalachian Mountains (Barnard-Kubow et al. 2015). The ranges of the Appalachian and Eastern lineage overlap in Virginia, while the ranges of the Western and Appalachian lineages overlap in North Carolina and Pennsylvania (Figure 1). Since populations from different lineages are close enough to hybridize in these overlapping regions, they form contact zones.

Substantial postzygotic isolation in *C. americana* is observed in hybrids between lineages as reduced germination and survival (Barnard-Kubow et al. 2016). Hybrids with Western or Eastern cytoplasm experience chlorosis (insufficient chlorophyll) due to cytonuclear incompatibility and are often inviable (Etterson et al. 2007, Barnard-Kubow and Galloway 2017), while the hybrids from Appalachian maternal plants are typically viable. Hybrids from the North Carolina contact zone have higher germination and survival than hybrids from the Pennsylvania contact zone or

allopatric populations (C. Debban, unpublished data).

Plant Material

This study compared plants from two different lineages, the Western and Appalachian lineages, in three different regions, the North Carolina and Pennsylvania contact zones, as well as an “allopatric region”. In each contact zone, I selected two sympatric populations from each lineage. One allopatric population from each lineage at a similar latitude as each contact zone was also selected. Allopatric populations of each lineage were a similar distance from the nearest contact zone. In summary, four North Carolina contact-zone populations, four Pennsylvanian contact-zone populations and four allopatric populations were selected (Table 1, Figure 1). The selfing rates and autonomy of PA29, KY51 and OH64 were measured in other studies (Koski et al. unpublished; Koski et al. 2017).

Field-collected seeds were grown in the greenhouse to measure autonomy and selfing rate. All seeds that were collected from the same plant comprised a maternal family. I randomly selected 15 families from each population (except only 12 families from PA104) and 20 seeds per family. Four groups of five seeds from each family were individually sown in $2.54 \times 2.54 \text{ cm}^2$ cells in a 9×18 plug tray filled with 3:1 metromix (Sungro Horticulture, Agawam, Massachusetts, USA) / turface (Profile Products LLC, Buffalo Grove, Illinois, USA) and germinated under near-optimal conditions in a growth chamber (21 °C day/14 °C night, 12 h days, daily watering) at the University of Virginia. After 30 days, seedlings were transferred to a cold room (5 °C day/5 °C night, 12 h days) for 7 weeks to promote vernalization and cue flowering. Then one seedling from each family was randomly chosen for transplant into containers and moved to a greenhouse with supplemental light, increasing day length to 16 hours, until fruits matured. On average 11.2 individuals per population, 106 individuals total were transplanted in the greenhouse.

Autonomy measurement

Autonomy was measured as the fruit set without pollinators in the greenhouse. Plants flowered undisturbed in the greenhouse. After fruits matured, I collected two flowering nodes from each plant (one from the middle of the stem, the other from the top) and stored them in coin envelopes. When fruits were dry, I counted the number of flowers per node and the number of fruits per node. Big and round ovaries were counted as a fruit, while flat and small ovaries were not. Some flowers were dissected to observe whether there were seeds inside. Autonomous fruit set for each individual was calculated as the ratio of total fruit number to total flower number.

Selfing rate measurement

To measure selfing rates of wild *C. americana* populations, I genotyped seedlings grown from field-collected seed using four microsatellite loci (158730, 56849, 72306, and 12305) that were identified and tested in previous study (Leibman et al. 2018). The forward primer for each locus was labelled with TET (56849), HEX (158730), or FAM (12305, 72306). Primers of 158730 and 72306 were paired in a PCR reaction, as were primers of 56849 and 12305. For DNA extraction, about 0.1mg leaf tissue was submerged in 50uL 1.25% w/v SDS solution and incubated at 95C for 5 minutes to expedite lysis. 1uL leaf extract, 25 uL MyTaq Plant-PCR Mix (Bioline, Taunton, MA, USA) and 1.3uL of each primer pair were mixed well for PCR reaction. Microsatellite fragments were then amplified in a Mastercycler® EP384 Thermocycler (Eppendorf, Hauppauge, NY, USA) using the following temperature-cycle profile: an initial melting step at 95 °C for 3 min, followed by 40 cycles of amplification (95 °C for 15 sec, 58 °C for 15 sec, 72 °C for 45 sec). Fragment analysis was carried out on a 3730x1 96-Capillary Genetic Analyzer with Rox500 size standard at Yale Genome Sequencing Center (New Haven, CT USA). PCR reactions for NC114 (North Carolina contact zone, Appalachian lineage), NC91 (North Carolina contact zone, Western lineage) and VA73 (Allopatry, Appalachian lineage) failed, so there are no selfing rate data for these three populations.

GeneMapper Software 5 (Applied Biosystems, Foster City, CA, USA) was used to determine the genotype of each seedling. The program MLTET (Ritland, University of British Columbia) was used to calculate the family- and population-level outcrossing rates according to seedling genotype. The family- and population-level selfing rate was calculated as 1 minus the outcrossing rate.

Statistical analysis

Both autonomy and selfing data were analyzed by ANOVA with lineage (A/W), region (NC/PA/ALLO), and the interaction between lineage and region as fixed factors, while population nested in the interaction between lineage and region was a random factor. Autonomy data was arcsine transformed to meet the normality assumption, while selfing rate data did not require transformation. To test my three hypotheses, a priori contrasts were conducted within different factors of the ANOVA. For hypothesis 1, all allopatric populations were compared with all sympatric populations (SYM vs ALLO) within the factor region. For hypothesis 2, all Appalachian lineage populations in the two contact zones were compared with all Western lineage populations in the contact zones (A vs W) within the interaction of lineage and region. For hypothesis 3, all populations in the North Carolina contact zone were compared with the Pennsylvanian contact-zone populations (NC vs PA) within the region factor. The correlation of autonomy and selfing with latitude and longitude was determined separately to test the alternative hypothesis.

Results

Variation explained by region and lineage

Mean population autonomous fruit set varied from 0.267 to 0.562 (Figure 2A) and the population-level selfing rate varied from 0.369 to 0.710 (Figure 2B). Neither lineage nor region explain the variation in autonomous fruit set, however the interaction between lineage and region was significant (Table 2). In allopatry, Western lineage populations have higher autonomous

fruit sets than Appalachian lineage populations, while in the North Carolina contact zones the Appalachian lineage has higher fruit set than the Western lineage, which caused the significant effect of the interaction between lineage and region (Figure 3). Lineage and the interaction between lineage and region influence the variation in selfing rates (Table 2). Western lineage populations have higher selfing rates than Appalachian lineage populations, but the pattern is driven by the difference between the two lineages in allopatry rather than in the contact zones (Figure 3). Since the differences in selfing between lineages are higher in allopatry than sympatry, geographical locations of populations rather than reinforcement in contact zone might be the cause of the difference in selfing.

Contrasts were conducted to test the three hypotheses (Table 2). There was no difference in autonomous fruit set and selfing rate between allopatric and sympatric populations (Hypothesis 1). Among contact-zone populations, populations from the Appalachian and Western lineages showed no significant difference in autonomous fruit set or selfing rate (Hypothesis 2). Also, North Carolina contact-zone populations did not show higher autonomous fruit set or selfing rates than the Pennsylvania contact zones populations (Hypothesis 3).

Variation explained by geographical cline

Autonomy increased linearly from the east to the west with longitude (Fig. 4B, $R = -0.50$, $P = 0.05$), while latitude had no effect ($R = -0.27$, $P = 0.50$). Similarly, populations in the west have higher selfing rates than those in the east (Fig. 4D, $R = -0.68$, $P = 0.04$). However, population-level selfing rate was not associated with latitude ($R = -0.20$, $P = 0.61$). The alternative hypothesis that selfing shows a geographical cline is supported.

Discussion

Theory predicts that when hybridization between two sympatric populations causes a reduction in fitness, prezygotic isolating mechanisms will be promoted by selection against unfit hybrids

(Noor 1999). This speciation mechanism is called reinforcement. Shifting from outcrossing to selfing, an evolutionary transition that reduces interspecific gene flow, is therefore predicted to be more frequent where divergent taxa are in secondary contact (Grossenbacher et al. 2016). However, there was no support for selfing as a mechanism of prezygotic isolation in *C. americana*. There was no evidence of higher autonomy or selfing rates in contact zone populations than allopatric populations, expected as a signature of reinforcement. Similarly, sympatric Western lineage populations that experience greater postzygotic isolation did not have higher autonomy and selfing rates than sympatric Appalachian lineage populations. Finally, populations in the North Carolina contact zone where hybrids experience less postzygotic isolation did not have lower autonomy and selfing rates than Pennsylvania contact-zone populations. Indeed, there was not an association between pre- and postzygotic isolation. In summary, these results indicate that the shift in mating system is not a mechanism of reinforcement in *C. americana*.

There are two potential reasons why reinforcement via elevated selfing was not observed. First, selection against hybrids between the Appalachian and Western lineages might not be strong enough to promote prezygotic isolation. An important requirement for the occurrence of reinforcement is enough initial postzygotic isolation (Liou and Price 1994). In *C. americana*, the intrinsic genetic incompatibility is not complete. Though the postzygotic isolation between the Western and Appalachian lineages can be large in F1 generation hybrids (78%), biparental inheritance helps to mitigate cytonuclear incompatibility, leading to recovery of fitness in the F2 generation hybrids (Etterson et al. 2007, Barnard-Kubow et al. 2017). Second, the age of contact zones might not be old enough for the evolution of increased selfing. The only trait indicating a selfing syndrome in high autonomy populations is reduced dichogamy (Koski et al. 2018). This may imply that the duration of secondary contact in the *C. americana* lineage might not be long enough for mating system evolution. Though reinforcement is thought to be a mechanism that contributes to the divergence of nascent species, evidence for increasing selfing as a

reinforcement mechanism is typically found between well-established sister species (Smith and Rausher 2007, Grossenbacher and Whittall 2011, Runquist et al. 2014) My results shows that despite prediction the mechanism of increasing selfing to avoid hybridization is not found in incipient species.

The fact that reinforcement is not achieved by increasing selfing rates in *C. americana* does not mean that reinforcement does not occur in this species. Aside from selfing, nonrandom mating can also be achieved by divergence of floral traits (Servedio and Noor 2003). The advantages of increasing selfing over floral trait divergence as a mechanism of reinforcement is that it reduces pollinator competition and provides reproductive assurance (Grossenbacher et al. 2016).

However, due to the cost of inbreeding depression and pollen discounting brought by selfing, unless the cost of finding a mate is high under floral trait divergence, reinforcement may be less likely to evolve via selfing (Lenormand 2012). For *C. americana*, usually all the pollen is removed from pollen collecting hairs before the initiation of female phase (Koski et al. 2017), which means available pollen for autonomy is limited. Thus autonomy is not efficient and reproductive assurance is not effective in this species (Koski et al. 2017). Under these conditions, other reinforcement mechanisms will be favored by selection. This might explain why an increase in selfing is not observed as a mechanism of reinforcement. Also, a two-week difference in flowering time between Pennsylvania contact-zone populations was observed in the greenhouse, which suggests that strategies other than a shift in mating system may be selected to avoid hybridization (C. Debban, unpublished data).

The linear increase in autonomy and selfing rate from the east to the west is similar to the results of a previous study that measured autonomous fruit set of 24 Western lineage populations (Koski et al. 2017). In that study, a linear increase in autonomy was observed from the southern to the northern edge and from eastern to western populations. This pattern was thought to result from a northward and westward migration of the Western lineage from Gulf Coast refugia after the Last

Glacial Maximum period (Koski et al. 2017). But migration history cannot explain my observed results since the Appalachian lineage has a different refugium and its distribution is restricted to the Appalachian Mountains. The reproductive assurance hypothesis, a common explanation for selfing, was also rejected in *C. americana* (Koski et al. 2017). Consequentially, the presence of a longitudinal pattern in my results might be caused by other biotic or abiotic selection agents that have spatial structures, such as pathogen-mediated selection (Levin, 1975) and selection pressure brought by drought (Opedal et al. 2016). The absence of a latitudinal pattern in my results might come from the lack of latitudinal variation in sampling. More exploration is needed to determine the major driver of shifts in mating system in *C. americana*, though reinforcement via selfing is excluded by this study.

If the mating system of *C. americana* populations is mainly determined by their geographical location, comparing autonomy and selfing rate of contact-zone populations with the local “background” allopatric populations near them may be more meaningful than simply comparing autonomy and selfing rate of all contact-zone populations with all allopatric populations. Some patterns can be gleaned by plotting the autonomous fruit set of my populations with the populations from the previous study (Koski et. al. 2017, Figure 5). Without contact zone populations (Figure 5A), autonomy increases from east to west and from south to north, indicated by three color bands. The blue band indicates a low-autonomy region, while the green is medium autonomy and the red is high autonomy. However, new patterns emerge in Figure 5B, when the autonomy of contact zones is compared with their adjacent regions. The North Carolina contact zone is colored green with medium autonomy, while it sits in low-autonomy blue band and its surrounding regions are also blue in color (Figure 5A). This means populations in the North Carolina contact zone have higher autonomy than we would predict from to the geographical cline. In contrast, the Pennsylvania contact zone is the same blue color as adjacent regions (Figure 5A, 5B), indicating populations there do not have higher autonomy than predicted by their location. Therefore, populations in the North Carolina contact zone might have

increased autonomous fruit set as a mechanism of reinforcement, a shift not shown in the previous analysis. This provides a new direction for future study and would require greater local sampling of allopatric populations.

There are several possible outcomes when isolated, divergent populations come into secondary contact: extinction of one population, introgression between populations, fusion of the two populations or additional divergence of the populations (Lemmon et al. 2004). When hybrids between two populations are of lower fitness than parental types, increased prezygotic isolation is predicted to reinforce isolation, leading to further divergence (Dobzhansky 1936). However, greater autonomy and selfing rate in sympatric populations than allopatric populations were not observed in *C. americana*. Populations whose hybrids experience stronger postzygotic isolation did not have higher autonomy and selfing rates. Instead, autonomy and selfing rate linearly increased from the east to the west. The results suggest that a shift in mating system is not a method to increase prezygotic isolation in *C. americana*. Instead the shift in mating system was associated with a geographical cline, which indicates the observed variation of selfing in *C. americana* is caused by a larger scale selective force than reinforcement.

Table 1. Populations used to study selfing rates and autonomy in *Campanula americana*. Lineage A indicates the population belongs to Appalachian lineage and W indicates the population belongs to Western lineage. Region NC means the population comes from North Carolina contact zone, PA means the population comes from Pennsylvania contact zone and ALLO means the population comes from an allopatric region. # indicates the data for these populations were obtained from another study (Koski et al. 2017). See Figure 1.

Population	Lineage	Region	Latitude	Longitude
NC91	A	NC	35.5862	-83.0663
TN92	A	NC	35.6758	-83.5259
NC114	W	NC	35.4362	-83.0480
NC105	W	NC	35.7031	-82.8333
PA104	A	PA	40.8021	-80.0547
PA102	A	PA	40.3225	-80.1109
PA103	W	PA	40.5503	-80.3112
PA27 [#]	W	PA	41.0079	-80.0833
VA73	A	ALLO	37.3534	-80.5522
KY51 [#]	W	ALLO	37.9340	-84.2595
PA95	A	ALLO	40.4752	-78.2808
OH64 [#]	W	ALLO	41.1147	-81.5181

Table 2: Linear models testing if region and/or lineage cause variation in autonomous fruit set and selfing rates among *Campanula americana* populations. Population is nested within the interaction between region and lineage as a random factor. F values of each factor are shown. Three contrasts testing a priori hypotheses are also shown. * indicates $P < 0.05$.

Effect	Num DF	Den DF	Autonomy	Selfing
Region	2	6	0.82	0.26
Lineage	1	6	0.19	3.69*
Region × Lineage	2	6	3.52*	2.84*
Pop (Region × Lineage)	1	6	0	0.12
Contrast				
(1) ALLO vs SYM	1	6	0.55	0.52
(2) PA vs NC	1	6	0.85	0.04
(3) A vs W	1	6	1.03	0.05

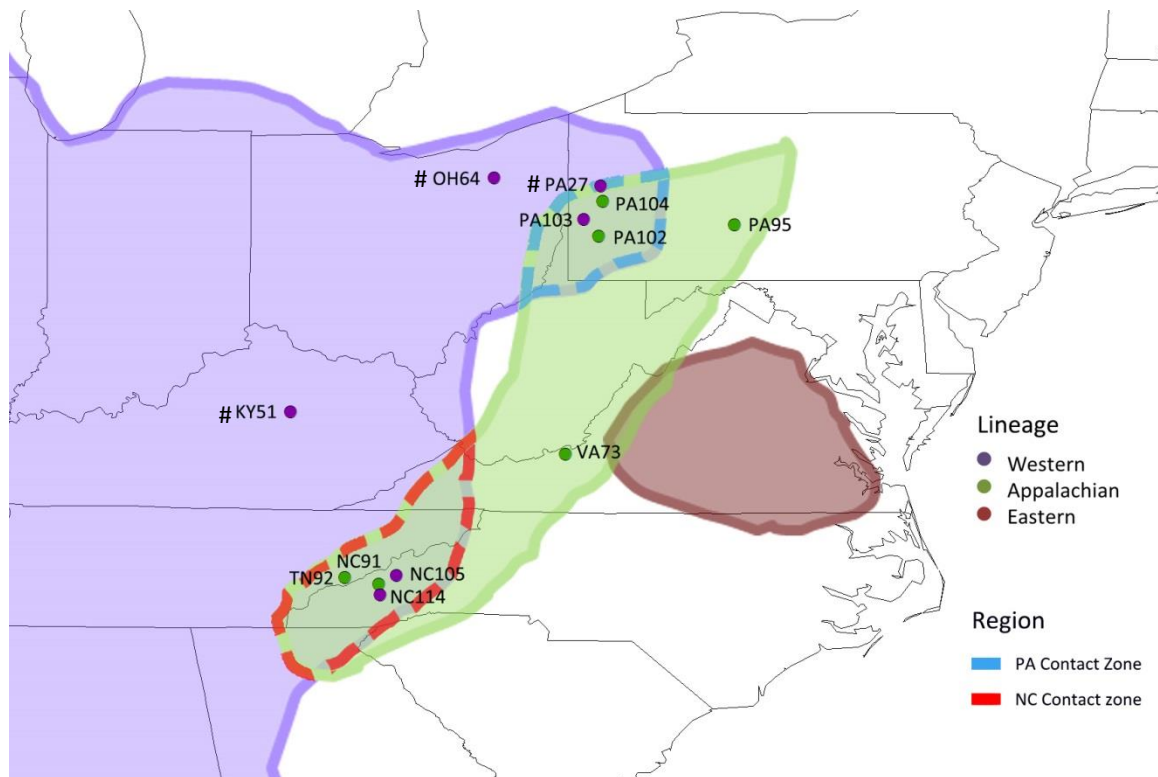


Figure 1. The range of Western, Appalachian and Eastern lineages of *Campanula americana* and populations used to evaluate autonomy and selfing rate. The range of Western and Appalachian lineages overlap in the Pennsylvania and North Carolina contact zones. Two populations from the range of Western lineage and two populations from the range of Appalachian lineage were selected to represent allopatry. Two populations from each lineage were selected within each contact zone. Data of populations with a # symbol were gathered in another study (Koski et al. 2017).

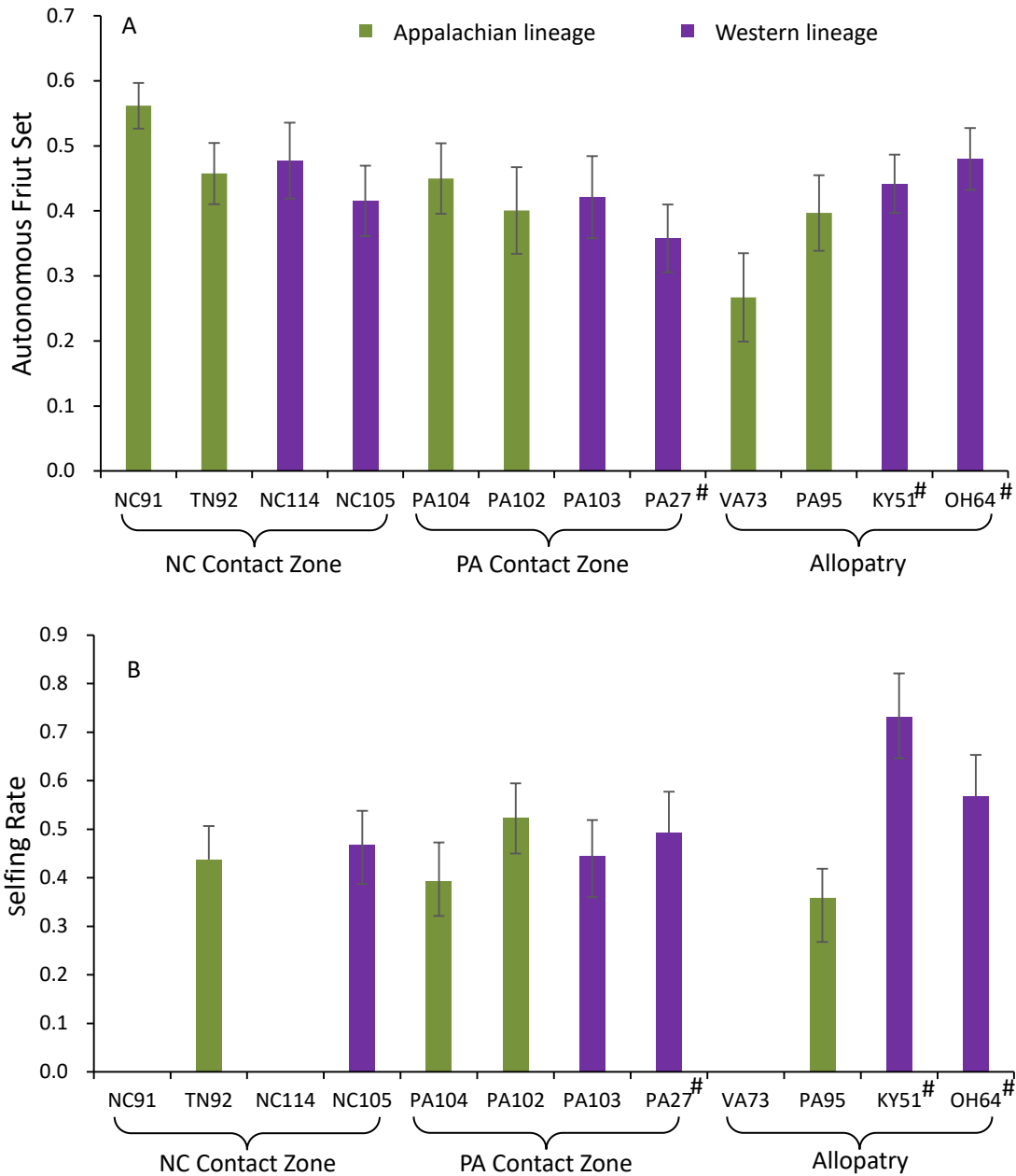


Figure 2. Mean autonomy (A) and population-level selfing rates (B) of *Campanula americana* populations from Appalachian and Western lineages in two contact zones and allopatry. Error bars are standard error. Blank bars are missing data. # indicates the data for these populations were obtained from another study (Koski et al. 2017).

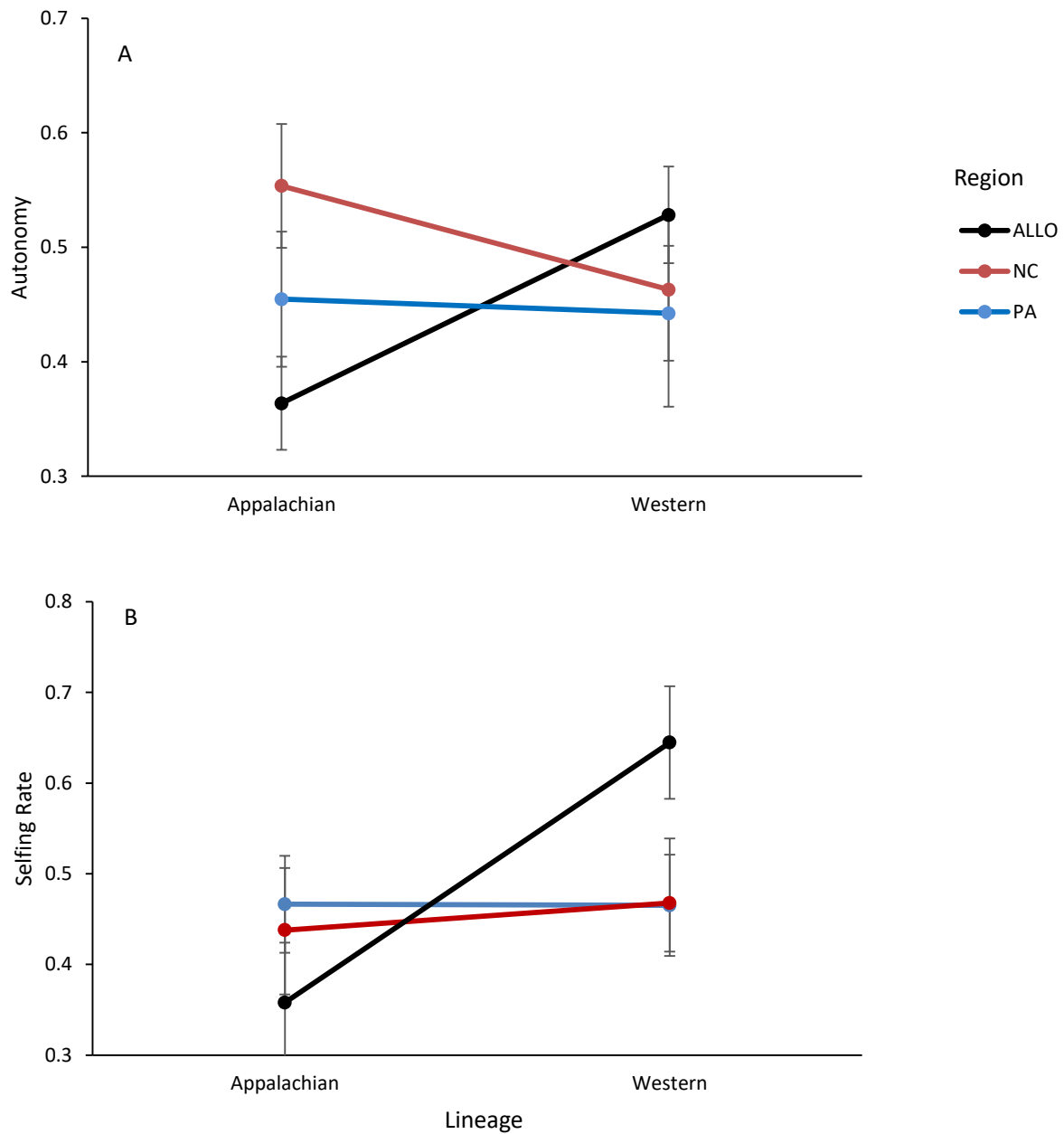


Figure 3. The difference in autonomy (A) and selfing rates (B) between Appalachian and Western lineages of *Campanula americana* populations in North Carolina and Pennsylvania contact zones as well as allopatry shown by lsmeans and standard errors.

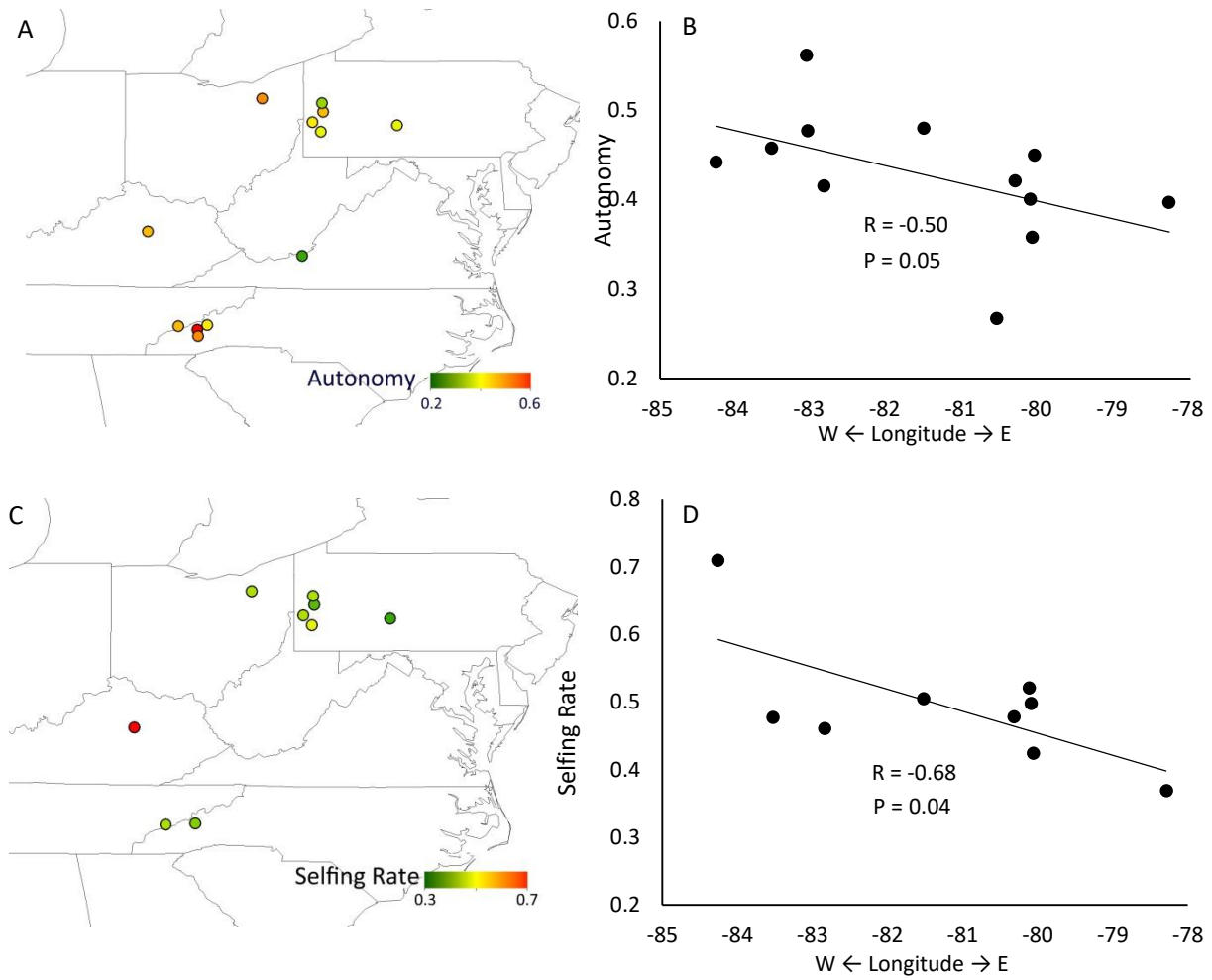


Figure 4. Geographic variation of autonomy (A) and selfing rates (C) for *Campanula americana* populations sampled in two contact zones and allopatric region. Significant effects of longitude on autonomy (B) and selfing rate (D) are shown by best fit lines.

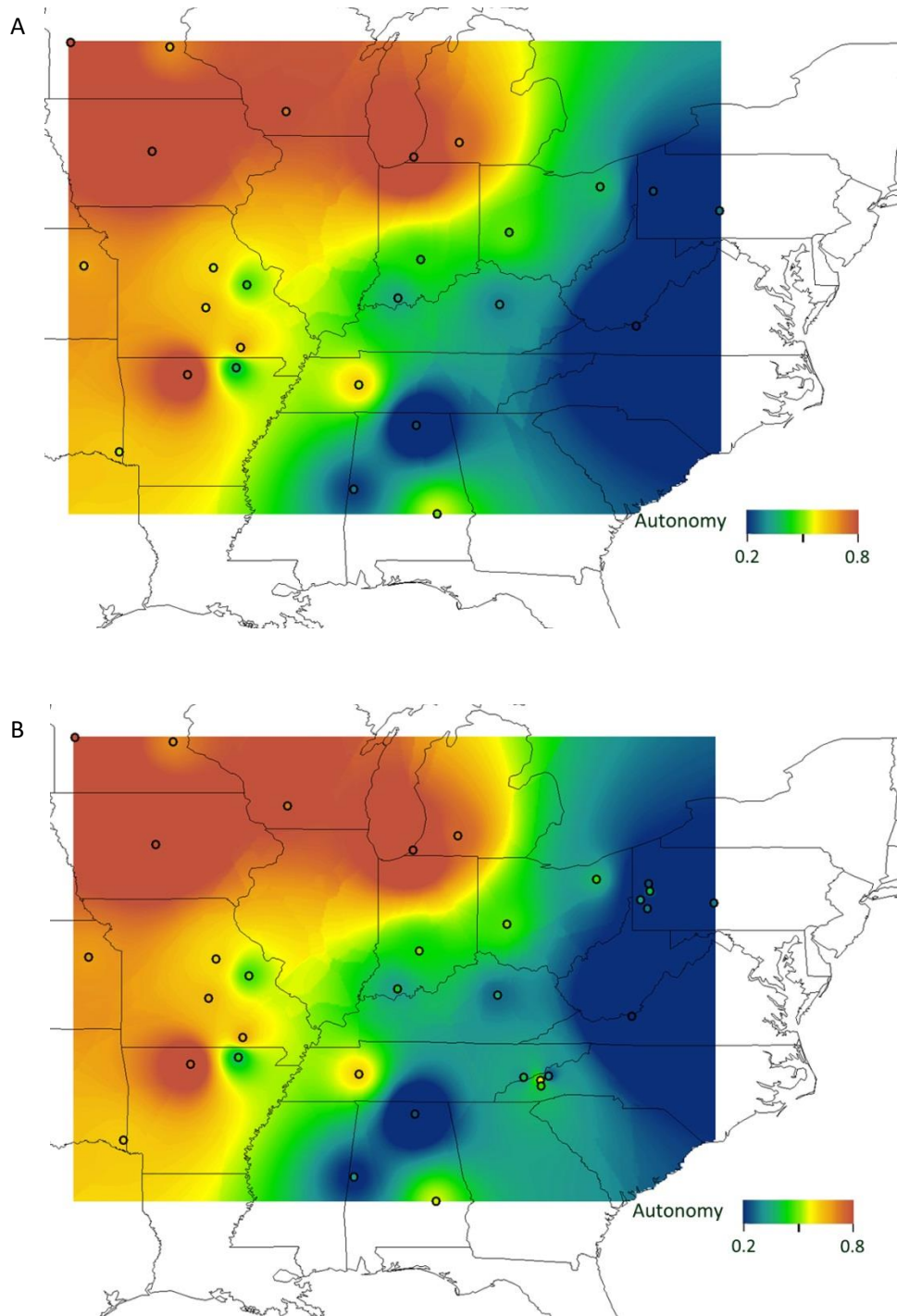


Figure 5. Heat maps showing autonomy for *Campanula americana* populations across the range without (A) and with (B) the contact-zone populations. Autonomy measured in this study and a previous one (Koski et al. 2017) were used to generate the raster and were plotted on the map. Populations in the North Carolina contact zone have higher autonomy than surrounding ones, while those in Pennsylvania do not.

References

- Armbruster, W. S., M. E. Edwards, and E. M. Debevec. 1994. Floral character displacement generates assemblage structure of Western Australian triggerplants (*Stylidium*). *Ecology* 75:315–329.
- Barnard-Kubow, K. B., C. L. Debban, and L. F. Galloway. 2015. Multiple glacial refugia lead to genetic structuring and the potential for reproductive isolation in a herbaceous plant. *American Journal of Botany* 102:1842–1853.
- Barnard-Kubow, K. B., and L. F. Galloway. 2017. Variation in reproductive isolation across a species range. *Ecology and Evolution* 7:9347–9357.
- Barnard-Kubow, K. B., M. A. McCoy, and L. F. Galloway. 2017. Biparental chloroplast inheritance leads to rescue from cytonuclear incompatibility. *New Phytologist* 213:1466–1476.
- Barnard-Kubow, K. B., N. So, and L. F. Galloway. 2016. Cytonuclear incompatibility contributes to the early stages of speciation. *Evolution* 70:2752–2766.
- Brys, R., A. Vanden Broeck, J. Mergeay, and H. Jacquemyn. 2013. The contribution of mating system variation to reproductive isolation in two closely related *Centaureum* species (Gentianaceae) with a generalized flower morphology. *Evolution* 68:1281–1293.
- Coyne, J. A., and A. H. Orr. 1989. Patterns of speciation in *Drosophila*. *Evolution* 43:362–381.
- Dobzhansky, T. 1936. Studies on hybrid sterility. II. Localization of sterility factors in *Drosophila pseudoobscura* hybrids. *Genetics* 21:113–135.
- Eckert, C. G. 2000. Contributions of autogamy and geitonogamy to self-fertilization in a mass-flowering, clonal plant. *Ecology* 81:532–542.
- Epinat, G., and T. Lenormand. 2009. The evolution of assortative mating and selfing with in- and outbreeding depression. *Evolution* 63:2047–2060.
- Etterson, J. R., S. R. Keller, and L. F. Galloway. 2007. Epistatic and cytonuclear interactions govern outbreeding depression in the autotetraploid *Campanulastrum americanum*. *Evolution* 61:2671–2683.
- Evanhoe, L., and L. F. Galloway. 2002. Floral longevity in *Campanula americana* (Campanulaceae): a comparison of morphological and functional gender phases. *American Journal of Botany* 89:587–591.
- Felsenstein, J. 1981. Skepticism towards Santa Rosalia, or why are there so few kinds of animals? *Evolution* 35:124–138.
- Fishman, L., and R. Wyatt. 1999. Pollinator-mediated competition, reproductive character displacement, and the evolution of selfing in *Arenaria uniflora* (Caryophyllaceae). *Evolution* 53:1723–1733.

- Galloway, L. F., J. R. Etterson, and J. L. Hamrick. 2003. Outcrossing rate and inbreeding depression in the herbaceous autotetraploid, *Campanula americana*. *Heredity* 90:308.
- Gerhardt, H. C. 1994. Reproductive character displacement of female mate choice in the grey treefrog, *Hyla chrysoscelis*. *Animal Behaviour* 47:959–969.
- 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.
- Grossenbacher, D. L., and J. B. Whittall. 2011. Increased floral divergence in sympatric monkeyflowers. *Evolution* 65:2712–2718.
- Grossenbacher, D., R. D. B. Runquist, E. E. Goldberg, and Y. Brandvain. 2016. No association between plant mating system and geographic range overlap. *American Journal of Botany* 103:110–117.
- Hopkins, R., and M. D. Rausher. 2012. Pollinator-mediated selection on flower color allele drives reinforcement. *Science* 335:1090–1092.
- Kay, K. M. 2006. Reproductive isolation between two closely related hummingbird-pollinated neotropical gingers. *Evolution* 60:538–552.
- Kirkpatrick, M., and V. Ravigne. 2002. Speciation by natural and sexual selection : models and experiments. *The American Naturalist* 159:s22–s35.
- Koski, M. H., D. L. Grossenbacher, J. W. Busch, and L. F. Galloway. 2017. A geographic cline in the ability to self-fertilize is unrelated to the pollination environment. *Ecology* 98:2930–2939.
- Koski, M. H., K. Liao, K. M. Niedermaier, and L. F. Galloway. 2018. Timing is everything: Dichogamy and pollen germinability underlie variation in autonomous selfing among populations. *American Journal of Botany* 105:241–248.
- Leibman, L., A. Rowe, M. H. Koski, and L. F. Galloway. 2018. Populations with greater flexibility in floral traits modify mating system in response to the pollinator environment. *Functional Ecology*. in press.
- Lemmon, A. R., C. Smadja, and M. Kirkpatrick. 2004. Reproductive character displacement is not the only possible outcome of reinforcement. *Journal of Evolutionary Biology* 17:177–183.
- Lenormand, T. 2012. From local adaptation to speciation: specialization and reinforcement. *International Journal of Ecology*. doi:10.1155/2012/508458
- Levin, D. A. 1985. Reproductive character displacement in *Phlox*. *Evolution* 39:1275–1281.
- Liou, L. W., and T. D. Price. 1994. Speciation by reinforcement of premating isolation. *Evolution* 48:1451–1459.

- Matallana, G., M. A. S. Godinho, F. A. G. Guilherme, M. Belisario, and T. S. Coser. 2010. Breeding systems of *Bromeliaceae* species: evolution of selfing in the context of sympatric occurrence. *Plant Systematics and Evolution* 289:57–65.
- McNeilly, T., and J. Antonovics. 1968. Evolution in closely adjacent plant populations. *Heredity* 23:205–218.
- Noor, M. A. F. 1999. Reinforcement and other consequences of sympatry. *Heredity* 83:503–508.
- Opedal, Ø. H., J. Listemann, E. Albertsen, W. S. Armbruster, and C. P. Åabon. 2016. Multiple effects of drought on pollination and mating-system traits in *Dalechampia scandens*. *International Journal of Plant Sciences* 177:682–693.
- Ortiz-Barrientos, D., A. Grealy, and P. Nosil. 2009. The genetics and ecology of reinforcement implications for the evolution of prezygotic isolation in sympatry and beyond. *Annals of the New York Academy of Sciences* 1168:156–182.
- Runquist, R. D. B., E. Chu, J. L. Iverson, J. C. Kopp, and D. A. Moeller. 2014. Rapid evolution of reproductive isolation between incipient outcrossing and selfing *Clarkia* species. *Evolution* 68:2885–2900.
- Sætre, G. P., K. Krå, S. Bures, and R. A. Ims. 1999. Dynamics of a clinal hybrid zone and a comparison with island hybrid zones of flycatchers (*Ficedula hypoleuca* and *F. albicollis*). *Journal of Zoology* 247:53–64.
- Schluter, D. 2001. Ecology and the origin of species. *Trends in Ecology & Evolution* 16:372–379.
- Servedio, M. 2000. Reinforcement and the genetics of nonrandom mating. *Evolution* 54:21–29.
- Servedio, M. R. 2004. The evolution of premating isolation: local adaptation and natural and sexual selection against hybrids. *Evolution* 58:913–924.
- Servedio, M. R. 2009. The role of linkage disequilibrium in the evolution of premating isolation. *Heredity* 102:51–56.
- Servedio, M. R., and M. Noor. 2003. The role of reinforcement in speciation: theory and data. *Annual Review of Ecology, Evolution, and Systematics* 34:339–364.
- Smith, R. A., and M. D. Rausher. 2007. Close clustering of anthers and stigma in *Ipomoea hederacea* enhances prezygotic isolation from *Ipomoea purpurea*. *New Phytologist* 173:641–647.
- Whalen, M. D. 1978. Reproductive character displacement and floral diversity in *Solanum* section *Androceras*. *Systematic Botany* 3:77–86.