The role of mating system in colonization and morphological variation

Hanna Makowski

B.S. Saint Mary's College, Indiana

A dissertation presented to the Graduate Faculty of the University of Virginia in Candidacy for the Degree of Doctor of Philosophy

Department of Biology

University of Virginia
July 2023

Acknowledgements

Dissertations are never the work of a single individual. The introduction to my dissertation is written in the singular possessive tense. However, you'll find that the individual chapters contained within are written in plural possessive tense. When I use the word "we," I am referring to my co-authors for each study who made this work possible. This, of course, includes the contributions from my fellow lab members, Keric Lamb, Austin Kim, and Emily Scott as well as my advisor, Dr. Laura Galloway.

Laura has been a supportive mentor and advisor. As a professional mentor, she has honed my ability to develop relevant questions, taught me how to design experiments to answer these questions, and helped me navigate the often inevitable reality of data when conducting experiments with biological organisms. Under her guidance, I am incredibly proud of the experiments and data I produced for my dissertation. Laura fostered and supported my creativity but also forced me to channel it and turn my good ideas into good science. I learned a lot through this process and am looking forward to incorporating my newfound knowledge into future endeavors. As a personal mentor, Laura has been incredibly supportive of me as an individual over these past five years. Graduate school is an interesting mix of people at all different stages of life. I came into graduate school directly from college, and Laura has been there to guide my burgeoning personal growth and intellectual maturity.

Keric Lamb is a fellow graduate student in the Galloway Lab. He has been an enormous help throughout this process, and as a result, he will be a co-author on every paper that comes out of this dissertation. My first summer conducting dissertation work, Keric was out there in the field with me helping to set up and run experimental arrays. For the pollen-collecting hair

research, he oversaw the initial development of the AI image detection model and guided me through its completion. Anything coding related in my third chapter, Keric was either involved in developing the initial stages or providing much-needed assistance as I confusedly barraged though it, as I do most coding endeavors. We worked closely together in the field, road tripped to field sites/collection trips, lived in a cabin together in the "ghost town" of Mountain Lake Biological Station during summer 2020, and somehow, still choose to hang out at social events outside of work.

Austin Kim and Emily Scott are two individuals who I worked closely with and mentored as undergraduates performing their own independent research in the Galloway Lab. My dissertation would not be anywhere near completion today if it was not for their work. Most of my planned dissertation experiments became impossible when the global pandemic prevented me from traveling to field sites and a cold room failure killed thousands of my plants. Thankfully, Austin's and Emily's projects became a larger part of my dissertation as I expanded on their own interesting findings. Austin and I were both in the second years of our respective programs when we started working together. I learned a lot about mentoring through working with Austin, and I am incredibly proud of him as he is currently starting medical school this year. Emily is just about to start her fourth year at UVA, and I cannot believe the amount she has accomplished before even starting her distinguished major project. Our project fortunately gave us the opportunity to visit incredible botanic gardens, both domestically and abroad, and I will never forget the trips we took together. Emily's drive and commitment to her work was incredibly motivating as I was finishing up my dissertation. I am thankful I had someone as

intelligent and kind as Emily to sample flowers with, and when we had time to spare, search (unsuccessfully) for the Loch Ness Monster.

I would like to thank my committee members Dr. Alan Bergland, Dr. Dave Carr, Dr. Amanda (Mandy) Gibson, and Dr. Jessamyn Manson for their input and support. From formalized committee meetings to impromptu office visits to ask unscheduled questions, their perspectives have guided and improved my work.

Additionally, many current and former lab members have contributed to ideas, helped with planting or dissecting, or provided feedback and edits on various stages of my work. Matt Koski and Catherine Debban were lab members when I started, and I was so lucky to have their mentorship. Antoine Perrier and Alfredo Lopez are current postdocs who were always willing to lend a hand as I approached the finish line. Samuel Adu was a student worker when I started and helped me plant thousands of plants – I enjoyed getting to know him as we spent hours in the greenhouse.

I would like to thank Chris Robinson, for his academic support and friendship. Chris was the only other EEBIO student in my year, so we became close by circumstance, but I'd like to think we'd be friends in a cohort of 100. As we went through classes, Chris helped teach me how to code in R, was always willing to help troubleshoot something or bounce ideas off of, and gave me the confidence to keep going when I was doubting myself. Chris is non-judgmental, but he will still call you out on your bias or something he doesn't think is right. That is the ideal quality to have in a scientist and a friend. I am a better scientist and person through my friendship with Chris.

Thank you to the multiple funding sources that helped make this dissertation possible, through either direct grants to myself or to Austin and Emily. I received grants from the Botanical Society of America, The Society for the Study of Evolution, The Raven Society at the University of Virginia, the Center for Global Innovation and Inquiry at the University of Virginia, and the Graduate School of Arts and Sciences. Austin and Emily were both funded by the Schwager Summer Research Scholarship. Austin was a Harrison award recipient and was co-funded with me as a Raven fellow. Emily also received the Minerva Award through UVA to fund her work.

Now, for the personal side of things. My family. My dad, Denise, and siblings—both old (Emma and Keenan) and new (Grace and Sam)—have all given me love and support throughout this whole process. I would characterize my father, Matt Makowski, as both a science and personal support. From the science side of things, my dad allowed me to use his property as a personal field station to set up experimental colonization sites. Even though the experiments never happened due to uncontrollable circumstances, when I was planning the long-distance field work across the U.S., I was looking forward to being around a supportive family while conducting my first field season. That comfort helped me get through the stress of writing my dissertation proposal and defending my qualifying exams.

I am especially grateful for a new closeness with my grandma, Francis Makowski, who went through the dissertation process herself. She was one of the first women admitted to the graduate program at Notre Dame and has been an inspiration. I was honored to be gifted her graduation tam at my public defense, and I wear it with pride. My grandpa, Richard Makowski,

has always provided love and encouragement. My Aunt Theresa has provided kindness and generous support for me as well.

I was lucky to have met my partner, Michael Testa, during the last year of our respective programs at UVA. His calm presence has provided a respite from the stress of completing a dissertation, and his dedication to his own studies provided the inspiration and motivation I needed to finish mine. I love him a lot and this last year of my dissertation would not have been the same without his love and support.

Adopting a dog as a field biologist was an interesting logistical decision, though I couldn't be happier to have Monty in my life. He is an incredibly goofy and loving dog who has been my walking partner around Charlottesville the past four years. Multiple people have made dog ownership possible as a graduate student with an unpredictable field travel schedule. First and foremost, Karen Riggs. I met Karen through the greyhound adoption group when I adopted Monty, and she offered to help watch him for holidays. Since then, Monty has spent a fair number of holidays and a couple of summer months at Karen's place. My dad has also watched Monty for a summer while I did field work. Keric, and his partner Anna, and Michael have also happily watched Monty for me.

Lastly, thank you to *Campanula americana*. I couldn't have done it without you.

Table of Contents

Abstract

Mating system, the tendency to reproduce via outcrossing or self-fertilization, varies widely across flowering plants. About a third of plant species both outcross and self-fertilize in mixed mating systems, which allows them to realize the benefits of both reproductive modes. Understanding the drivers of mating system evolution is important because mating system affects patterns of genetic diversity, floral traits, and the ability of populations to adapt. While the factors that influence the evolution of outcrossing and selfing, as well as their respective influence on floral phenotypes, are understood independently, less is known about the environments that select for mixed mating and its phenotypic consequences. In Chapter 1 of my dissertation, I tested the benefit of within-flower selfing in colonization of a mostly outcrossing herb, *Campanula americana.* Using experimental colonization experiments, I found a benefit of selfing in mate-limited environments through a decrease in pollen limitation. In Chapter 2, I characterized variation in the morphological structures, pollen-collecting hairs, involved in reproduction in *C. americana* to determine how outcrossing ability is maintained when selfing potential evolves. Pollen-collecting hairs hold pollen along the style and retract overtime to release pollen for pollination. I found that selfing ability was associated with an increased length of the hairs suggesting longer hairs aid in pollen retention and allow for pollen to remain for selfing. The longer hairs also retracted in a way that did not influence outcrossing potential, showing evidence for a stable mixed mating system. Finally, in Chapter 3 I stepped outside of mating-system variation to explore alternative hypotheses for variation in pollen-collecting hair traits across 39 species from the Campanulaceae family. I found that pollen-collecting hair length scaled allometrically across Campanulaceae and the variation in C.

americana spanned a majority of the interspecific variation in the family, showing the strength of mating system as a selective agent. Taken together, the findings presented in my dissertation advance our understanding of the factors that select for mating system variation and in turn mating system variations effect on reproductive mechanisms and morphology.

Introduction

Plant mating systems, the frequency of reproduction through outcrossing versus self-fertilization (Barrett 2002), are variable at both the intra- and interspecific levels (Schemske and Lande 1985, Barrett and Husband 1990, Ness *et al.* 2010). Angiosperms are basally outcrossing (Allen and Hiscock 2008), and transitions to self-fertilization are the most common evolutionary transition and dominant direction of changes in reproductive-mode in plants (Stebbins 1974, Barrett *et al*. 1996). Understanding the drivers of mating system evolution is important because mating system affects patterns of genetic diversity, floral traits, and the ability of populations to adapt.

The negative genetic effects of self-fertilization, known as inbreeding depression, often selects for mechanisms to promote outcrossing in hermaphroditic organisms (Lande and Schemske 1985, Charlesworth and Charlesworth 1987). However, if a population has high levels of homozygosity, then there is no reduction in fitness of inbreeding compared to outcrossing (Kirkpatrick and Jarne 2000; Angeloni *et al.* 2011). This can be seen in a decreased cost of inbreeding at range edges, which have undergone population bottlenecks resulting in decreased genetic diversity (Pujol *et al.* 2009, Barringer *et al.* 2012). Decreased interactions with pathogens at range edges that create a relaxation of selection for outcrossing and extreme environments that select for local adaptation to prevent maladaptive gene flow and fix beneficial genes can also favor increased self-fertilization (Antonovics 1968, Bell 1982, Mazer *et al.* 2004, Elle *et al.* 2010). Finally, self-fertilization can be advantageous in environments without conspecifics or the ability to move gametes between individuals.

When mates and pollinators are limited, obligate outcrossing can lead to decreased reproductive output due to lack of pollen receipt. Self-fertilization, specifically within-flower selfing, should allow for density-independent reproduction, mitigating the effect of mate-limitation and pollinator availability in colonization. Range edges, again, exemplify a situation where both mates and pollinators are likely limited (Karron *et al.* 1995; Moeller 2006; Hargreaves and Eckert 2014). The evolution of self-fertilization in mate-limited environments is considered a foundational theory in plant mating system evolution (Baker's law, Baker 1955), with biogeographic support indicated by increased selfing at range margins and on islands compared to range core and mainland populations (Randle *et al.* 2009, Grossenbacher *et al.* 2015, 2017, Griffin and Willi 2014). While such biogeographic correlations are compelling, it is difficult to disentangle the causality and strength of the effect of mate limitation as a selective agent with only biogeographic evidence.

Mating system is enforced through floral traits. Selection to promote outcrossing has pushed the presentation of male and female gametes apart in space and time. When transitions to selfing occur, we often see a relaxation of selection to promote outcrossing and a reversal in direction of those morphological and phenological distances (i.e., selfing syndrome, Belaoussoff and Shore 1995, Totland and Schulte-Herbrüggen 2003, Delph and Ashman 2006, Sicard and Lenhard 2011, Toräng *et al.* 2017). When genes that promote self-pollination arise, theory predicts the transition to high selfing rates will occur rapidly due to purging of deleterious mutations, i.e., genetic load, and subsequent reduction of inbreeding depression, in addition to an inherent genetic transmission advantage (Lande and Schemske 1985, Charlesworth *et al.* 1990, Stone *et al.* 2014). Despite this, about 33% of species employ mixed

mating systems (Vogler and Kalisz 2001). Mixed mating systems allow for the 'best of both worlds.' Where the genetic advantage of outcrossing is retained while within-flower selfing (hereafter, autonomous selfing) can provide assured seed set when outcross pollen is limited, i.e., reproductive assurance (Kalisz and Vogler 2003). While the changes in traits that evolve as part of the selfing syndrome are generally understood, less is known about how morphological changes in gamete presentation promote autonomous selfing and also maintain outcrossing in mixed mating systems, allowing for the 'best of both worlds.' Understanding the evolution of structures that promote both outcrossing and allow selfing when needed can help to determine whether mixed mating represents a stable evolutionary strategy in contrast to a transition state to full selfing.

In my dissertation, I advanced our understanding of mating system from the phenotype to the phylogeny. I used mating system variation in the mixed-mating herb *Campanula americana* to test the benefit of autonomous selfing in colonization. Additionally, I explored whether variation in reproductive structures involved in pollen-presentation underlie the variation in autonomous self-fertilization we see across populations of *C. americana*. Finally, I expanded the characterization of the pollen-presentation phenotype to multiple species across the Campanulaceae family to contextualize variation of this mating trait in a macroevolutionary perspective.

In Chapter 1, I tested whether populations of *C. americana* with high rates of autonomous self-fertilization performed better than population with low autonomous selfing ability in experimental colonization scenarios. I created experimental populations that varied in autonomous selfing ability and population size and compared their reproductive output. I found

that single plants and small founding populations of high autonomous selfing ability performed consistently, while low autonomous selfing populations had smaller seed output in single-plant populations relative to small, multiple plant founding populations. This difference demonstrates the benefit of self-fertilization in colonization and provides an empirical confirmation of Baker's Law. The benefit of selfing in colonization provides a mechanism for the geographic variation in mating system we see across the range in *C. americana* and more broadly demonstrates the influence of historic colonization pressures on contemporary mating systems.

In Chapter 2, I characterized morphological variation in pollen-presentation structures across *C. americana* populations that varied in their autonomous selfing ability. *C. americana* is protandrous, with flowers opening in male phase and transitioning to female over time. For autonomous selfing to occur, pollen must remain from the male phase into the female phase. Plants in the Campanulaceae family exhibit a unique form of pollen presentation, where pollen is displayed on pollen-collecting hairs along the style that retract and release pollen over time

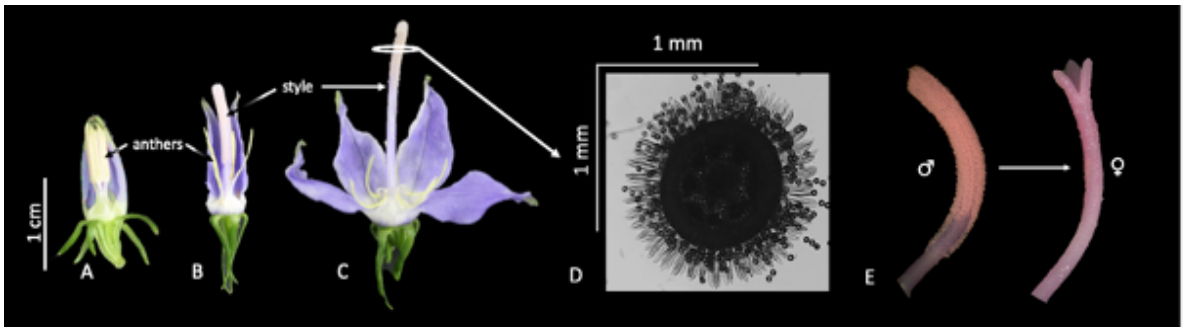


Figure 1. Secondary pollen presentation in Campanulaceae, shown in *Campanula americana.* First, (A) pollen is deposited from the anthers onto hairs along the style while the flower is in bud. The flower opens and the style begins to elongate (B and C). You can see the pollen present along a cross section of the style (D). Over time, the hairs retract and the stigmatic lobes open and the flower becomes functionally female (E).

(Figure 1). Variation in the length of pollen-collecting hairs and the timing of retraction may influence the amount of pollen available for autonomous selfing. I found pollen-collecting hairs were longer at floral opening in populations with high autonomous selfing rates compared to those with low autonomous selfing rates. However, more rapid retraction of pollen-collecting hairs in high autonomy populations resulted in similar hair length across autonomy levels within six hours of a flower's opening. Through my characterization of pollen-collecting hairs across multiple populations of *C. americana* that varied in their autonomous selfing ability, I found support for pollen-collecting hairs as a novel mechanism of autonomous self-fertilization. Longer initial hairs are associated with increased self-fertilization ability and through their fast retraction outcross function is potentially not affected, suggesting a stable mixed mating system.

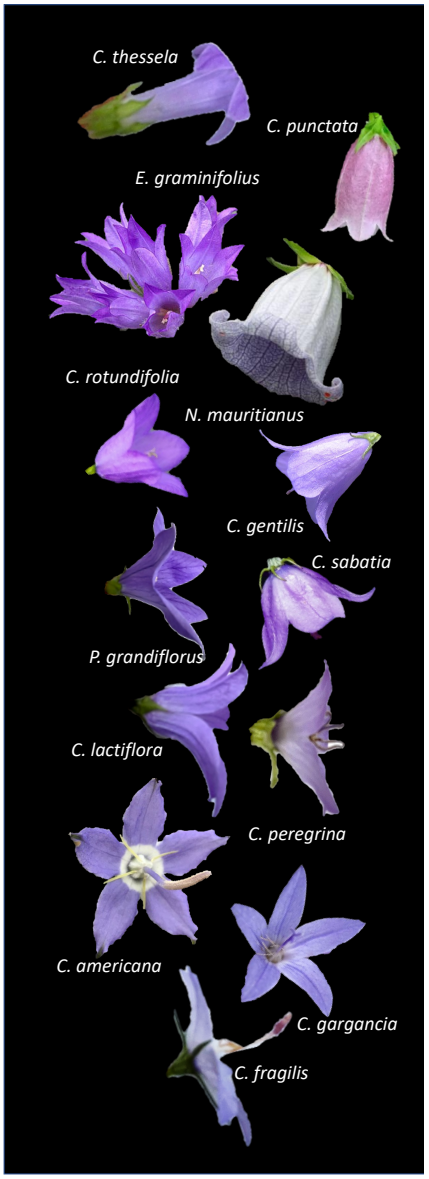


Figure 2. Floral shape variation across Campanulaceae. From top to bottom, flowers get less bell shaped and more open and rotate, which influences the amount of pollen that is exposed to environmental factors such as wind, rain, and UV.

In Chapter 3, I looked beyond intraspecific variation in mating system and used phylogenetic comparative methods to explore alternative hypotheses for variation in pollen-collecting hair morphology across the Campanulaceae family. Campanulaceae is largely outcrossing and

mostly perennial, which removes mating system variation as a potential selective agent that has structured variation in pollen-collecting hairs. Flowers in species across Campanulaceae vary from bell shaped to open (Figure 2), so I tested whether pollen-collecting hair variation was related to the amount of pollen that was exposed to environmental factors such as wind and rain. I anticipated that the pollen-collecting hairs would have a protective function and be longer when pollen was exposed to environmental factors compared to pollen that was protected within a flower. I found that pollen-collecting hair length was phylogenetically constrained and associated with flower size. However, it was not associated with the amount of environmental exposure the pollen received. Furthermore, the intraspecific variation in *C. americana* spanned a wide range of the interspecific variation, suggesting selection on mating system in range expansion can break phylogenetically constrained allometric size relationships.

Taken together, the findings presented in my dissertation advance our understanding of the factors that select for mating system variation and in turn mating system variations effect on reproductive mechanisms and morphology. I demonstrated support for Baker's law, uncovered morphological variation underlying autonomous self-fertilization ability in *C. americana* and found that the morphological variation is unusually variable in *C. americana* compared to the rest of Campanulacaeae. My morphological results show how mechanisms to facilitate autonomous selfing in *C. americana* are not necessarily product of outcross trait degradation. Self-fertilization ability can be selected on independently and exist in congruence with the ability to outcross, which allows for the 'best of both worlds' and supports mixed-mating as a stable evolutionary strategy.

Literature Cited

Allen, A.M., Hiscock, S.J. 2008. Evolution and phylogeny of self-incompatibility systems in angiosperms. In: Franklin-Tong VE, ed. *Self-incompatibility in flowering plants: evolution, diversity, and mechanisms*. Berlin: Springer. 73–101.

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

Antonovics J. 1968. Evolution in closely adjacent plant populations: V Evolution of self-fertility. *Heredity* 23: 219–238.

Baker, H.G. 1955. Self-compatibility and establishment after "long-distance" dispersal. *Evolution* 9: 347-348.

Belaoussoff, S., Shore, J.S. 1995. Floral correlates and fitness consequences of mating-system variation in *Turnera ulmifolia. Evolution* 49: 545–556.

Bell G. 1982. The masterpiece of nature: the evolution and genetics of sexuality. Berkeley, CA, USA: University of California Press.

Barrett, S.C.H., 2002. The evolution of plant sexual diversity. *Nature Reviews Genetics* 3: 274–284.

Barrett, S.C.H., Harder, L.D., Worley, A.C. 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.

Barrett, S.C.H., Husband, B.C., 1990. Variation in outcrossing rates in *Eichhornia paniculata*: the role of demographic and reproductive factors. *Plant Species Biology* 5: 41–55.

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

Charlesworth, D., Morgan, M.T., Charlesworth, B. 1990. Inbreeding depression, genetic load, and the evolution of outcrossing in a multilocus system with no linkage. *Evolution* 44: 1469-1498.

Delph, L.F., Ashman, T-L. 2006. Trait selection in flowering plants: how does sexual selection contribute? *Integrative and Comparative Biology* 46: 465– 472.

Elle, E., Gillespie, S., Guindre-Parker, S., Parachnowitsch, A.L. 2010. Variation in the timing of autonomous selfing among populations that differ in flower size, time to reproductive maturity, and climate. *American Journal of Botany* 97: 1894–1902.

Griffin, P.C., Willi, Y. 2014. Evolutionary shifts to self-fertilization restricted to geographic range margins in North American *Arabidopsis lyrata. Ecology Letters* 17: 484-490.

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

Grossenbacher *et al.* 2017. Self-compatibility is overrepresented on islands. *New Phytologist* 215: 469-478.

Hargreaves, A.L. and Eckert, C.G. 2014. Evolution of dispersal and mating systems along geographic gradients: implications for shifting ranges. *Functional Ecology* 28: 5-21.

Karron, J.D., Thumser, N.N., Tucker, R., Hessenauer, A.J. 1995. The influence of population density on outcrossing rates in *Mimulus ringens. Heredity* 75: 175–180.

Kirkpatrick, M., Jarne, P. 2000. The effects of a bottleneck on inbreeding depression and the genetic load. *The American Naturalist* 155: 154–167.

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

Mazer, S.J., Paz, H., Bell, M.D. 2004. Life history, floral development, and mating system in *Clarkia xantiana* (Onagraceae): do floral and whole-plant rates of development evolve independently? *American Journal of Botany* 91: 2041–2050.

Moeller, D.A., 2006. Geographic structure of pollinator communities, reproductive assurance, and the evolution of self-pollination. *Ecology* 87: 1510–1522.

Ness, R.W., Wright, S.I., Barrett, S.C.H. 2010. Mating-system variation, demographic history and patterns of nucleotide diversity in the tristylous plant *Eichhornia paniculata*. *Genetics* 184: 381–392.

Pujol, B., Zhou, S.R., Sahchez-Vilas J., Pannell, J.R. 2009. Reduced inbreeding depression after species range expansion. *Proceeding of the National Academy of Sciences USA* 106: 15379–15383.

Schemske, D.W., Lande, R., 1985. The evolution of self-fertilization and inbreeding depression in plants II. empirical observations. *Evolution* 39: 41–52.

Sicard, A., Lenhard, M. 2011. The selfing syndrome: a model for studying the genetic and evolutionary basis of morphological adaptation in plants. *Annals of Botany* 107: 1433–1443.

Stone, J.L., VanWyk, E.J., Hale, J.R. 2014. Transmission advantage favors selfing allele in experimental populations of self-incompatible *Witheringia solanaceae* (Solanaceae). Evolution 68: 1845-1855.

Toräng, P., Vikström, L., Wunder, J., Wötzel, S., Coupland, G., Ågren, J. 2017. Evolution of the selfing syndrome: Anther orientation and herkogamy together determine reproductive assurance in a self-compatible plant: evolution of the selfing syndrome. *Evolution* 71: 2206–2218.

Vogler, D.W., Kalisz, S. 2001. Sex among the flowers: the distribution of plant mating systems. *Evolution* 55: 202-204.

CHAPTER 1:

Evaluating the benefit of self-fertilization in colonization

Abstract

Range shifts proceed through colonization of new habitats and are a well-documented response to global change. The ability to self-fertilize is predicted to provide an advantage in colonization because a single individual can establish a next generation in a new location. While there is theoretical and correlative support for this idea, it has not been experimentally tested. Here, I explore how a plants' ability to self-fertilize can mitigate density-dependent reproduction and impact colonization success using known mating system variation in the American Bellflower (*Campanula americana).* I established experimental populations that emulated isolated colonization and compared the reproductive output of single individuals to small populations, as well as plants that differed in their ability to self-fertilize. I found that experimental populations of plants that readily self-fertilized had consistent seed set across experimental population sizes, whereas populations with lower ability to self-fertilize had density-dependent reproduction with greater seed production in small populations with multiple plants. Together, these patterns support the benefit of self-fertilization in colonization and help to explain the prevalence of self-fertilization at range edges.

Introduction

Colonization of new habitats through range shifts is a well-documented response to global climate change (Davis & Shaw 2001, Parmesan 2006, Moritz *et al.* 2008, Chen *et al.* 2011, Steinbauer *et al.* 2018). Identifying traits that determine colonization potential is a central problem of ecology and evolution that has implications for understanding contemporary species distributions and forecasting shifts in distributions with climate change. Much of the research to date on colonization potential focuses on dispersal ability and the extent to which ecological interactions allow taxa to persist in a habitat (Angert *et al.* 2011, Sunday *et al.* 2015, MacLean & Beissinger 2016). In particular, examinations of plant reproduction often focus ecological generalization of pollinator assemblages. However, mating system, the propensity to outcross or self-fertilize, is also likely to have a substantial impact on a species ability to establish in new habitats. Geographic patterns of mating system suggest self-fertilization may be beneficial in colonization of new habitats.

Typically only one or a few individuals make it to a new location during colonization of a new habitat. With only a few individuals around, reproductive success may be reduced due to a lack of mates (Karron *et al.* 1995, Hargreaves & Eckert 2014). This density-dependent fitness is termed an Allee effect, or a positive association between population density and fitness (Stephens *et al.* 1999). An individual must overcome density-dependent limitations to reproduction to establish the next generation. One possible mechanism to overcome mate-limitation is through self-fertilization (Gascoigne 2009). In plants, autonomous self-fertilization, or the ability to self-fertilize in the absence of mates and pollinators, provides reproductive assurance in a mate-limited environment. Therefore, self-fertilization is expected to be favored

in range expansion after long distance dispersal, an idea colloquially known as Baker's law (Baker 1955, 1967; Pannell *et al.* 2015).

Correlative examinations of species distributions and mating system support the theoretical prediction of Baker's law. For instance, a comparison of sister-taxa pairs finds that species with the ability to self have larger range sizes than species that require outcrossing (Randle *et al.* 2009), suggesting that self-fertilization provides a range expansion benefit. Additionally, self-compatible species are overrepresented on islands compared to mainland habitats (Grossenbacher *et al.* 2015, 2017). Finally, populations at the edges of species ranges tend to have a higher incidence of self-fertilization (Griffin & Willi 2014). While these studies provide correlative support for Baker's law, there are other hypotheses for why range expansion may select for increased self-fertilization that have nothing to do with mate-limitation. For example, lower inbreeding depression that accompanies decreases in genetic diversity with range expansion (Pujol *et al*. 2009), reduced interactions with pathogens (Bell, 1982), and local adaptation to extreme environments (Antonovics 1968, Mazer *et al.* 2004, Elle *et al.* 2010), can all select for increased self-fertilization at range margins. The potential for multiple factors to influence geographic patterns in self-fertilization motivates an experimental test of Baker's Law to isolate the effect of mate-limitation in the evolution of selfing during colonization.

We experimentally evaluate Baker's law using variation in autonomous self-fertilization among populations of *Campanula americana*. Specifically, we ask: *(1) Does autonomous self-fertilization decrease pollen limitation and provide reproductive assurance in congruence with Baker's law? (2) Does autonomous self-fertilization prevent an Allee effect?*

Methods

Study System

Campanula americana L. (= *Campanulastrum americanum* Small) is a widespread self-compatible herb common in Eastern North America pollinated mainly by bumblebees (*Bombus* spp.) (Koski *et al*. 2017). The protandrous flowers open in male phase and then transition to female phase following pollen removal (Evanhoe and Galloway 2002). Within the contemporary range, pollinators visit frequently and as a result the male phase is brief, with most pollen removed within 3 h of anthesis (Evanhoe and Galloway 2002). Male phase can persist for 1-3 days if the flower is left unmanipulated in the greenhouse (Koski *et al.* 2018b).

While natural populations are largely outcrossing (Galloway *et al*. 2003, Koski *et al.* 2019a), autonomous selfing can occur when pollen is retained from male phase and deposited on the stigmatic surface in female phase (Koski *et al.* 2018b). There is intraspecific variation in autonomous selfing ability, hereafter autonomy, within *C. americana*. Populations that have a high rate of autonomy have flowers with shorter male phases than low autonomy populations (Koski *et al*. 2018b. Additionally, upon manipulation of the style to emulate a pollinator visit, high autonomy populations speed up their sexual phase progression and reach female phase faster than low autonomy populations, showing increased touch sensitivity (Koski *et al.* 2018b). Beyond timing, the mechanism of autonomous selfing ability is not known.

Previous greenhouse work has shown a clinal pattern in autonomy in *C. americana* (Koski *et al.* 2017,) that tracks the historic post-glaciation expansion route (Koski *et al.* 2019b). Specifically, there is low autonomy in populations close to the glacial refugia in Kentucky and high autonomy in populations that have expanded north and westward towards Minnesota and

Kansas. Autonomy is not correlated with current levels of pollen limitation of reproduction in natural populations (Koski *et al.,* 2017).

Experimental populations and floral manipulations

We grew individuals from high and low autonomy populations of *C. americana* and transported them to the field to emulate colonization events. We define autonomy as the portion of flowers that become fruits in a pollinator free environment. Four populations of *C. americana* were selected based on their autonomy level, two high (61% and 76% autonomous fruit set) and two low (26% and 34% autonomous fruit set) autonomy populations (Table S1). Seeds of these populations were randomly located in flats with 3 parts PGX soilless growth media and 1-part turface. Flats were placed in growth chambers for germination (12h light/dark cycle; 21C days and 19C nights) for one month. Seedlings were then moved to a cold room for vernalization at 5C with 12-hour days. After six weeks, plants were transplanted to cone-tainers and placed in a greenhouse at the University of Virginia where lights extended day length to 16 hours. In late June, when plants were near/at flowering, they were transported to Mountain Lake Biological Station, Pembroke, VA for use in the field experiments from June 30th- August 1st. Mountain Lake Biological station is within the contemporary range of *C. americana*.

We selected 10 sites at least 1.6 km away from each other and from known natural *C. americana* populations to emulate isolated colonization events. We placed an experimental population at each site. An experimental population had either a single plant with an average of 8 flowers or multiple plants (2-7, mean 4.25) with an average of 80 flowers to create differences in mate availability. While ten times larger than the single plant experimental

populations, the multiple plant experimental populations are still relatively small in terms of population size and represent a small founding population. Each experimental population was comprised of either high or low autonomy plants from a single source population. Ten to fifteen temporal replicates of each combination of population size and autonomy were carried out for 53 total experimental populations. All experimental populations were initiated with a 1:1 ratio of male- to female-phase flowers and the sex ratio of the flowers was recorded each day. A single experimental population was left at one of the ten sites, watered daily, and then returned to the greenhouse to set fruit after the tagged flowers senesced (x = 4 days at a site).

We measured pollen limitation and reproductive assurance within each experimental population using standard floral manipulations (Eckert *et al.* 2010). When the plants were placed in the field, we tagged three open flowers on each plant within an experimental population. One was left untouched (control, C), a second was supplemented (S) with outcrossed pollen from a member of the same population, and the final one was emasculated (E), with its pollen removed in male phase so it could not self-fertilize. The degree of pollen limitation was measured as a difference of seed set between the supplemented and control flowers (i.e. S-C). If seed set was higher in supplemented flowers than in control flowers, this indicated the control flower's seed number was limited by outcross pollen receipt. Emasculated flowers cannot self-fertilize, so the extent to which seed set in the control flowers was greater than the emasculated flowers (i.e., C-E), indicated the degree of autonomous self-fertilization. Fruits from tagged flowers were collected and seeds counted. In total, 353 fruits were tagged, the multiple-plant small populations had an average of 66 fruits per source population and the single plant experimental populations had an average of 23 fruits tagged per source population.

Pollinator observations

A multiple plant display can be more visually and chemically attractive to pollinators than a single plant display. We assessed pollinator visitation to determine if potential differences in seed set were due to differences in pollinator visitation. Two 15-minute pollinator observations were conducted on each experimental population the first two days it was in the field, totaling 23.5 hours of pollinator observations across all experimental populations. For each pollinator, we counted the number of floral visits across all of the flowers in the experimental population and noted pollinator type (bumblebee, small bee, and medium bee). A visit was defined as an insect touching the style or stigma of a flower; repeated touches within a single floral visit were not counted as unique visits. Return visits from a single pollinator after leaving were recorded. Observations occurred between 11am and 3pm, the time of peak pollinator visitation (Galloway *et al.* 2002).

Statistical Analysis

To measure the effect of autonomy level and population size on pollen limitation and reproductive assurance, we compared seed set across floral treatments. We used average seeds per treatment for each experimental population rather than seeds per fruit because there was only a single fruit in the single plant population size whereas the small experimental populations had multiple fruits. We used generalized mixed linear models ("glmer" function in the lme4 R package; Bates *et al.* 2014) with average number of seeds per pollination treatment per experimental population as the response variables, indicating a Poisson distribution. The models all had normal residuals with non-significant Shaprio-Wilk's p-values. The models

included the relevant pollination treatment (Pollen limitation model: C and S (n = 105), and Reproductive assurance model: C and E (n = 103)), autonomy level (High, Low), experimental population size (Single, Small), and their interactions as fixed effects. Source population was also included as a fixed effect nested in autonomy level, as such interactions with population were not included. Random effects consisted of site and date an experimental population was placed in the field. Because the number of pollinator visits were consistent across experimental population size and autonomy interactions (see results), they were not included in the model.

To assess differences in pollinator visitation, we compared total visits and visits per flower across experimental populations. We combined all pollinator types into one total visit metric due to sample size. I used a generalized linear model with either total number of visits or visits per flower as the response variable, again specifying a Poisson distribution. The fixed effects included experimental population size, autonomy level, and their interactions as well as source population nested within autonomy as fixed effects. Random effects included site and date.

Results

Pollen limitation

Pollen limitation was present across both experimental population types as evidenced by pollen supplemented flowers producing more seeds than the control flowers that only received natural pollination (Figure 1A, S1, S2, Table 1, *S2*). There was an overall density-dependent relationship in pollen limitation for low but not high autonomy populations (3-way interaction, Table 1). On average, high autonomy populations had consistent pollen limitation

across the different population sizes, but there was a decrease in pollen limitation with the addition of mates for the low autonomy populations (Figure 1A).

Reproductive assurance

Reproductive assurance was only present on average in the small, high autonomy experimental populations (Figure 1B, Table 1B, S3), as evidenced by the control flowers producing more seeds than the emasculated flowers that could not autonomously self-fertilize due to the removal of self-pollen. There was no reproductive assurance in either of the single plant populations or the small populations with low autonomy.

Pollinators

Small multiple plant experimental populations had three-times more floral visits than single plants (31.5 vs. 9.3 total visits, Wald Chisquare = 151.70, p = <0.001), though on average single plant experimental populations received almost three-times more visits per flower (1.1 vs. 0.4 visits per flower, Chisquare = 146.60, p =<0.001). There was no difference in total visitation or visits per flower for the interaction between autonomy level and size (Table S3); however, autonomy and population nested within autonomy was significant (Table S3) for both visitation metrics. Populations differed slightly, but the only population that varied significantly with other populations at the same population size was a low autonomy population, AL79. This low autonomy population had on average more total visits and also visits per flower than the other low autonomy single plant population. In addition, its small multiple plant population size

had slightly more visits per flower than the other low autonomy population and one of the high autonomy multiple plant populations. All other contrasts were nonsignificant.

Sex ratio

Populations were initiated with 1:1 ratio and on day 2 only 10% of the open flowers were male, regardless of autonomy level or population size.

Discussion

This work identifies a benefit of pollinator-independent self-fertilization in colonization, showing support for Baker's law. The ability to self-fertilize decreased pollen limitation and prevented an Allee effect. All combinations of experimental population size and self-fertilization ability showed evidence for pollen limitation. However, pollen limitation increased as population size decreased for low autonomy populations but on average high autonomy populations had consistent pollen limitation regardless of experimental population size. However we did not find density-dependent reproduction associated reproductive assurance.

Self-fertilization is predicted to provide an advantage in colonization by mitigating mate-limitation (Baker 1955, 1967; Pannell *et al.,* 2015). A number of studies find increased selfing at range margins (Griffin & Willi 2014), including in *C. americana* (Koski *et al.* 2017). Our study is the first to experimentally isolate the effect of mate-limitation in colonization and test whether mate-limitation alone can select for increased self-fertilization. The populations we used are from different geographic locations and have different expansion histories but when we isolate the effect of mate-limitation in a common environment, we find a benefit of self-fertilization.

Increased selfing ability decreased the dependence of reproduction on density, mitigating an Allee effect.

While the capacity for self-fertilization differs among populations, high outcrossing rates are maintained in natural populations of *C. americana* (Koski *et al.,* 2019a). The maintenance of outcrossing suggests that the contemporary cline of autonomous selfing ability across the range in *C. americana,* is due to the historic benefit of self-fertilization in colonization. When selection on self-fertilization ability occurs in a mate-limitation context it is driven by density rather than the benefit of decreased genetic diversity. The cost of selfing (inbreeding depression) is not gone in these populations and therefore outcrossing ability is still beneficial and maintained once the population reaches a certain density. The benefit of outcrossing is lost with the other forms of selection for selfing, such as decreased pathogen interactions and local adaptation to extreme environments, because in those cases the benefit/result of selfing is the decreased genetic diversity (Pannell *et al.* 2015).

We expected differences in pollen limitation to be driven by reproductive assurance; however, evidence for reproductive assurance was only present in multiple plant, high autonomous populations and not in single plant populations. Therefore reproductive assurance is not likely to be the mechanism that decreases pollen limitation at small population sizes. One potential reason for a lack of reproductive assurance for high autonomy plants in single population sizes is pollen availability. Pollinators stripped most of the pollen within the first day, as evident from the percentage of open males decreasing from 50% to 10%. Single plant experimental populations received more pollinator visits per flower, therefore it is possible that the pollinators depleted available pollen and there was no pollen available for self-

reproduction. With fewer visits per flower in the multiple plant populations and therefore more pollen present, the high autonomy populations were able to use the remaining pollen in self-reproduction and show reproductive assurance. However, this justification alone lacks a rational explanation for the consistent level of pollen limitation across population size in the high autonomy populations. To explain this, we speculate that the expedited transition to female phase in high autonomy source populations created more overlap of male and female flowers on the same plant, allowing for more fertilizations between flowers on the same plant. This would explain the two-fold difference seed set in the high x single control compared to the low x single control seed set. Alternatively, this unexpected pattern could be an artifact of our experimental design. To measure reproductive assurance, we emasculated flowers before putting them out in experimental populations. We know that manipulating the style of *C. americana* speeds up the sexual phase progression and female phase occurs earlier than non-manipulated styles (Koski 2018b). This would cause emasculated flowers to turn female before the control flowers, and thereby receive outcross pollen before it is depleted in contrast to the control flowers. Later emasculation, such as at the end of the first day of an experimental population, would have provided more comparable treatments. Unfortunately, we do not have data with which to evaluate this possibility. Further work to explore this unexpected pattern of reproductive assurance would be aided by a complete understanding of the physical mechanism of autonomous selfing in *C. americana*.

In conclusion, we show that on average the interaction of autonomous selfing ability and population size underlies a population's ability to set seed, shedding light on the role colonization plays in the evolution of mating system. We suggest that selection for increased

self-fertilization ability as a mechanism to mitigate pollen limitation during historic expansion has shaped the contemporary potential mating system of *C. americana*. It would be of interest to determine how selfing ability is maintained in the face of high outcrossing rates.

Literature Cited

Angert, A.L., *et al*. 2011. Do species' traits predict recent shifts at expanding range edges? *Ecology Letters* 14: 677-689.

Antonovics J. 1968. Evolution in closely adjacent plant populations: V Evolution of self-fertility. Heredity 23: 219–238.

Baker, H.G. 1955. Self-compatibility and establishment after "long-distance" dispersal. *Evolution* 9: 347-348.

Baker, H.G. 1967. Support for Baker's law – as a rule. *Evolution* 21: 853-856.

Bell G. 1982. The masterpiece of nature: the evolution and genetics of sexuality. Berkeley, CA, USA: University of California Press.

Chen, I.C., Hill, J.K., Ohlemüller, R., Roy, D.B., Thomas, C.D. 2011. Rapid range shifts of species associated with high levels of climate warning. *Science* 333: 1024-1026.

Davis, B.M & Shaw, R.G. 2001. Range shifts and adaptive responses to Quaternary climate change. *Science* 292: 673-679.

Eckert *et al.* 2010. Plant mating systems in a changing world. *Trends in Ecology and Evolution* 25: 35-43.

Elle, E., Gillespie, S., Guindre-Parker, S., Parachnowitsch, A.L. 2010. Variation in the timing of autonomous selfing among populations that differ in flower size, time to reproductive maturity, and climate. American Journal of Botany 97: 1894–1902.

Evanhoe, L., Galloway, L.F., 2002. Floral longevity in *Campanula americana* (Campanulaceae): a comparison of morphological and functional gender phases. *American Journal of Botany* 89: 587–591.

Galloway, L. F., T. Cirigliano, and K. Gremski. 2002. The contribution of display size and dichogamy to potential geitonogamy in *Campanula americana*. *International Journal of Plant Sciences* 163: 133– 139.

Galloway, L.F., Etterson, J.R., Hamrick, J.L., 2003. Outcrossing rate and inbreeding depression in the herbaceous autotetraploid, *Campanula americana*. *Heredity* 90: 308–315.

Gasgoine, J. Berec, L., Gregory, S., Courchamp, F. 2009. Dangerously few liaisons: a review of mate-finding Allee effects. *Population Ecology 51: 355-372.*

Griffin, P.C., Willi, Y. 2014. Evolutionary shifts to self-fertilisation restricted to geographic range margins in North American *Arabidopsis lyrata. Ecology Letters* 17: 484-490.

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

Grossenbacher *et al.* 2017. Self compatibility is overrepresented on islands. *New Phytologist* 215: 469-478.

Hargreaves, A.L. and Eckert, C.G. 2014. Evolution of dispersal and mating systems along geographic gradients: implications for shifting ranges. *Functional Ecology* 28: 5-21.

Karron, J.D., Thumser, N.N., Tucker, R., Hessenauer, A.J. 1995. The influence of population density on outcrossing rates in *Mimulus ringens. Heredity* 75: 175-180.

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., Ison, J.L., Padilla, A., Pham, A.Q., Galloway, L.F., 2018a. Linking pollinator efficiency to patterns of pollen limitation: small bees exploit the plant-pollinator mutualism. *Proceedings of the Royal Society B: Biological Sciences* 285.

Koski, M.H., Kuo, L., Niedermaier, K.M., Galloway, L.F., 2018b Timing is everything: Dichogamy and pollen germinability underlie variation in autonomous selfing among populations. *American Journal of Botany.* 105: 241–248.

Koski, M.H., Layman, N.C., Prior, C.J., Busch, J.W., Galloway, L.F., 2019a Selfing ability and drift load evolve with range expansion. *Evolution Letters.* 3: 500–512.

Koski, M.H., Galloway, L.F., Busch,J.W., 2019b. Pollen limitation and autonomous selfing inteact to shape variation in outcrossing rate across a species range. *American Journal of Botany* 106: 1240-1247.

Lau, J.A., Galloway, L.F., 2004. Effects of low-efficiency pollinators on plant fitness and floral trait evolution in *Campanula americana* (Campanulaceae). *Oecologia* 141: 577–583.

MacLean, S.A., Beissinger, S.R. 2016. Species' traits as predictors of range shifts under contemporary climate change: a meta-analysis. *Global Change Biology* 23: 4094– 4105.

Mazer, S.J., Paz, H., Bell, M.D. 2004. Life history, floral development, and mating system in *Clarkia xantiana* (Onagraceae): do floral and whole-plant rates of development evolve independently? *American Journal of Botany* 91: 2041–2050.

Mortiz, C. *et al.* 2008. Impact of a century of climate change on small-mammal communities in Yosemite National Park, USA. *Science* 322: 261-264.

Pannell, J.R., *et al*. 2015. The scope of Baker's Law. *New Phytologist 208*: 656- 667.

Parmesan, C. 2006. Ecological and Evolutionary Responses to Recent Climate Change. *Annual Review of Ecology, Evolution, and Systematics 37*: 637-669.

Pujol, B., Zhou, S.R., Sahchez-Vilas J., Pannell, J.R. 2009. Reduced inbreeding depression after species range expansion. *Proceeding of the National Academy of Sciences USA* 106: 15379– 15383.

Randle, A.M., Slyder, J.B., Kalisz, S. 2009. Can differences in autonomous selfing ability explain differences in range size among sister-taxa pairs of *Collinsia* (Plantaginaceae)? An extension of Baker's Law. *New Phytologist* 183: 618-629.

Steinbauer *et al*. 2018. Accelerated increase in plant species richness on mountain summits is linked to warming. *Nature* 556:231-234.

Stephens, P.A., Sutherland, W.J., Freckleton, R.P. 1999. What is the Allee effect? *Okios* 87: 185-190.

Sunday *et al.* 2015. Species traits and climate velocity explain geographic range shifts in an early ocean-warming hotspot. *Ecology Letters 18*: 944-953

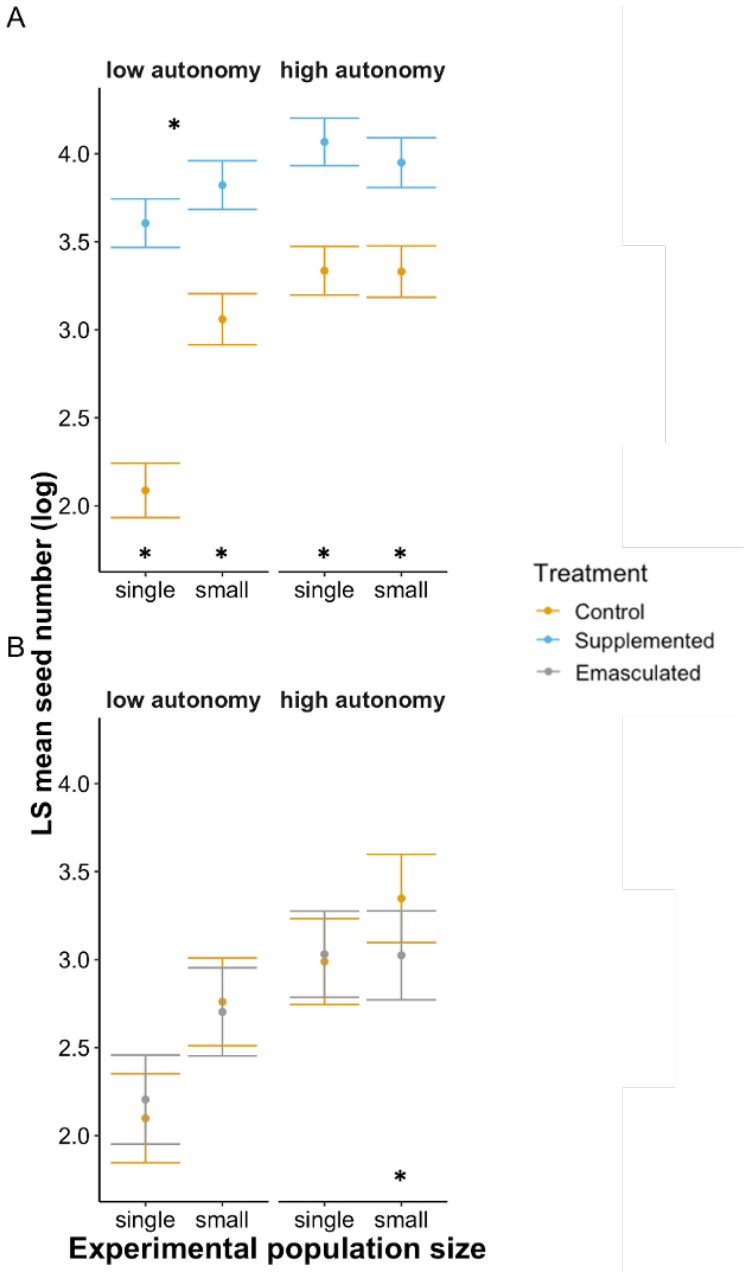


Figure 1. Least square means of seed number for experimental populations of *Campanula americana* placed in isolated natural environments to emulate colonization. Populations differed in their autonomous selfing ability (high vs. low) and their size (single plant vs. small population). I measured (A) pollen limitation and (B) reproductive assurance using three different pollination treatments; an unmanipulated control flower, a flower supplemented with additional pollen, and an emasculated flower. Points indicate average seed number and error bars show SE. Asterisks below means indicate significant difference between treatments, representing either (A) pollen limitation or (B) reproductive assurance (Table S2). The asterisk centered between columns in (A) indicates a significant difference in the amount of pollen limitation occurring between single plants and small populations of low autonomy experimental populations (Table S2).

Table 1. Linear mixed models testing whether pollen limitation and reproductive assurance in *Campanula americana* varies with population size and autonomy level.

A. Pollen limitation		
Fixed effects	Wald chisquare	p
pollination treatment (C vs. S)	632.70	**<0.001**
autonomy (Low vs. High)	70.06	**<0.001**
population size (Single vs. Small)	38.59	**<0.001**
source population nested within autonomy	23.60	**<0.001**
pollination treatment: autonomy	41.45	**<0.001**
pollination treatment: population size	36.21	**<0.001**
autonomy: population size	35.38	**<0.001**
pollination treatment: autonomy: population size	19.88	**<0.001**
B. Reproductive assurance		
Fixed effects	Wald chisquare	p
pollination treatment (C vs.E)	1.61	0.20
autonomy (Low vs. High)	86.33	**<0.001**
population size (Single vs. Small)	44.95	**<0.001**
source population nested within autonomy	3.21	0.20
pollination treatment: autonomy	3.36	0.07
pollination treatment: population size	8.62	**<0.001**
autonomy: population size	6.51	**0.01**
pollination treatment: autonomy: population size	1.21	0.27

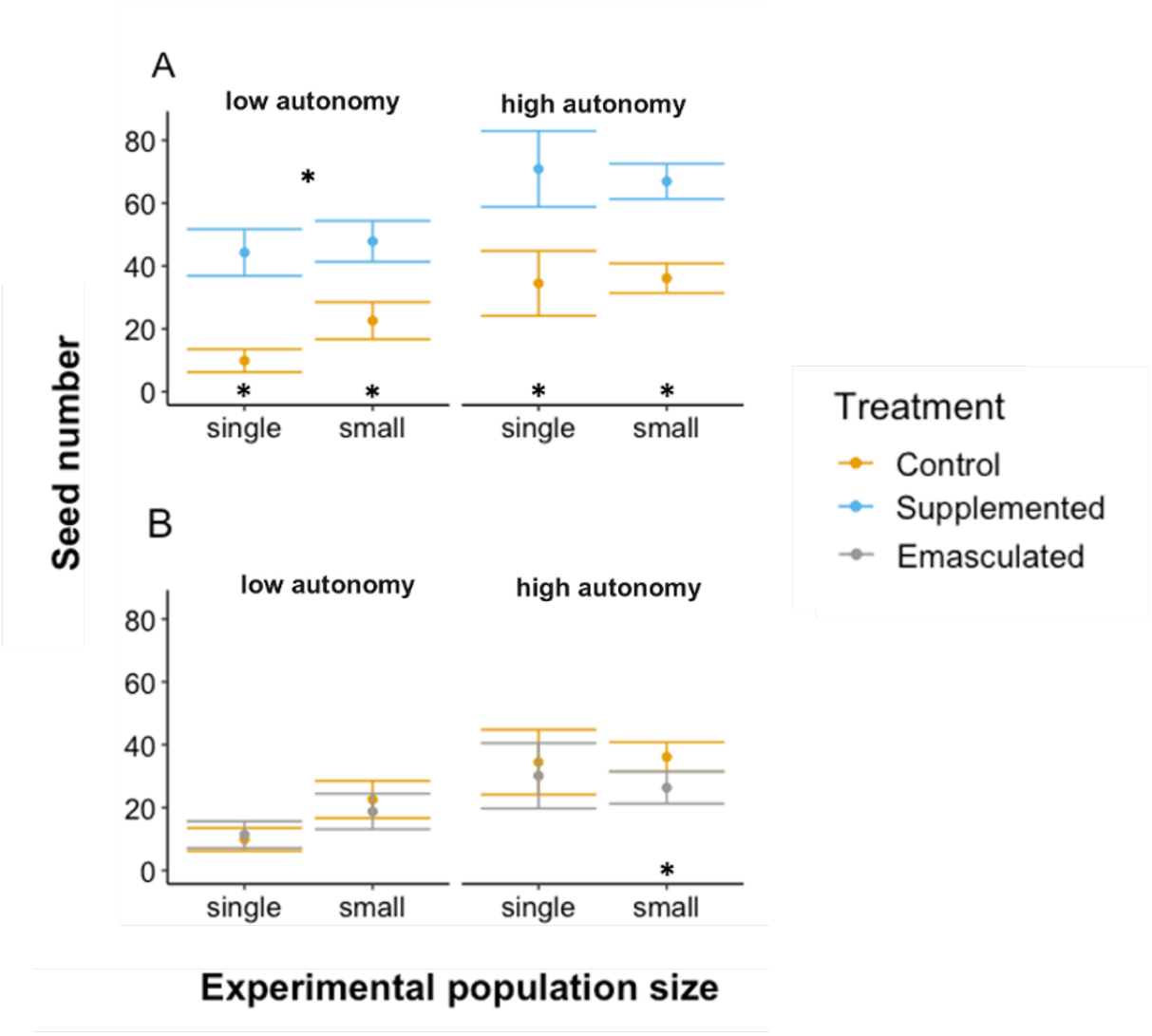


Figure S1. Raw seed number for experimental populations of *Campanula americana* placed in isolated natural environments to emulate colonization. Populations differed in their autonomous selfing ability (high vs. low) and their size (single plant vs. small population). I measured (A) pollen limitation and (B) reproductive assurance using three different pollination treatments; an unmanipulated control flower, a flower supplemented with additional pollen, and an emasculated flower. Points indicate average seed set and error bars show SE. Asterisks below means indicate significant difference between treatments, representing either (A) pollen limitation or (B) reproductive assurance (Table S2). The asterisk centered between columns in (A) indicates a significant difference in the amount of pollen limitation occurring between single plants and small populations of low autonomy experimental populations (Table S2).

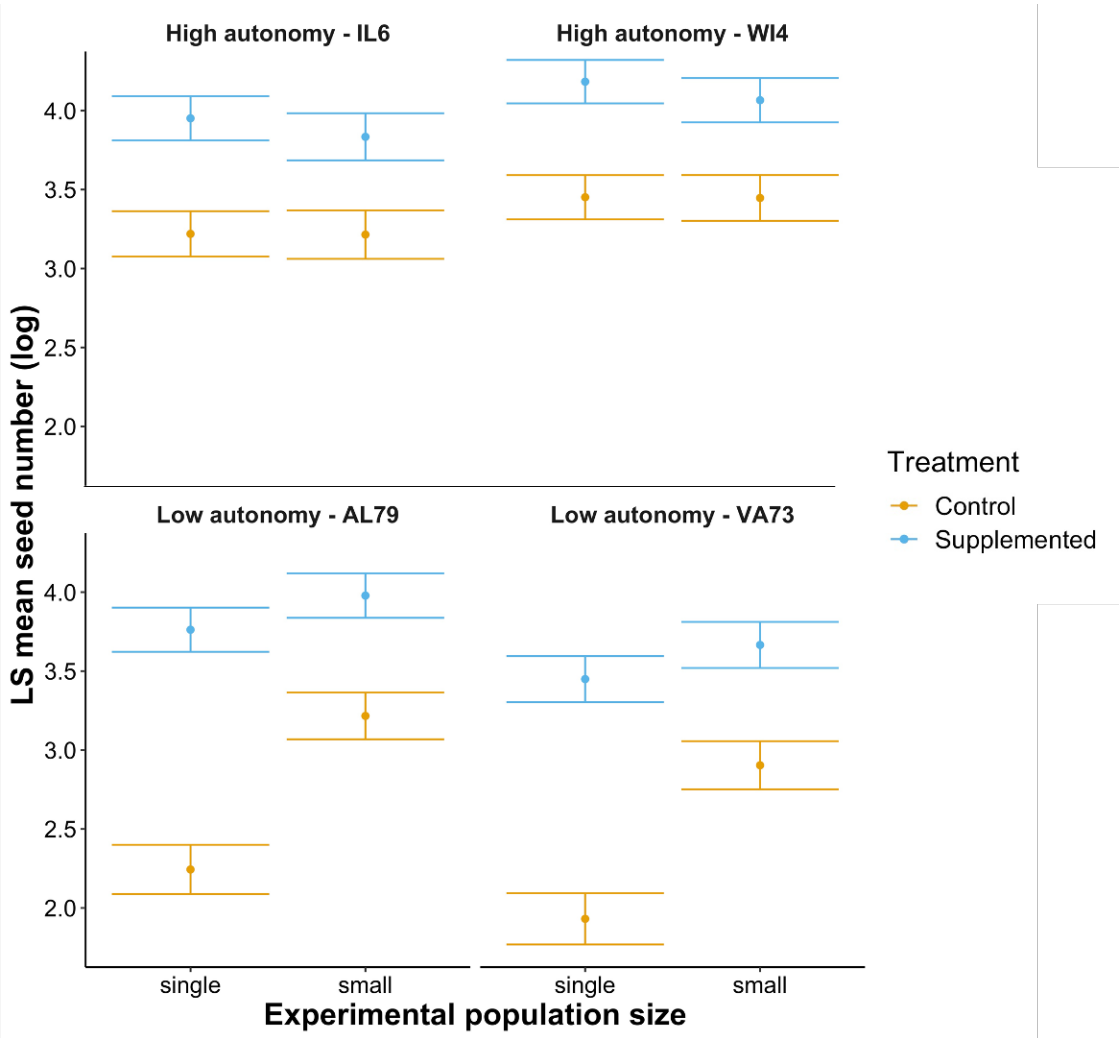


Figure S2. Seed number of pollination treatments to assess pollen limitation broken up by source population of *Campanula americana*. These source populations were used to create single plant and small multiple-plant experimental populations placed in isolated natural environments to emulate colonization. Points indicate LS mean of seed set and error bars show SE.

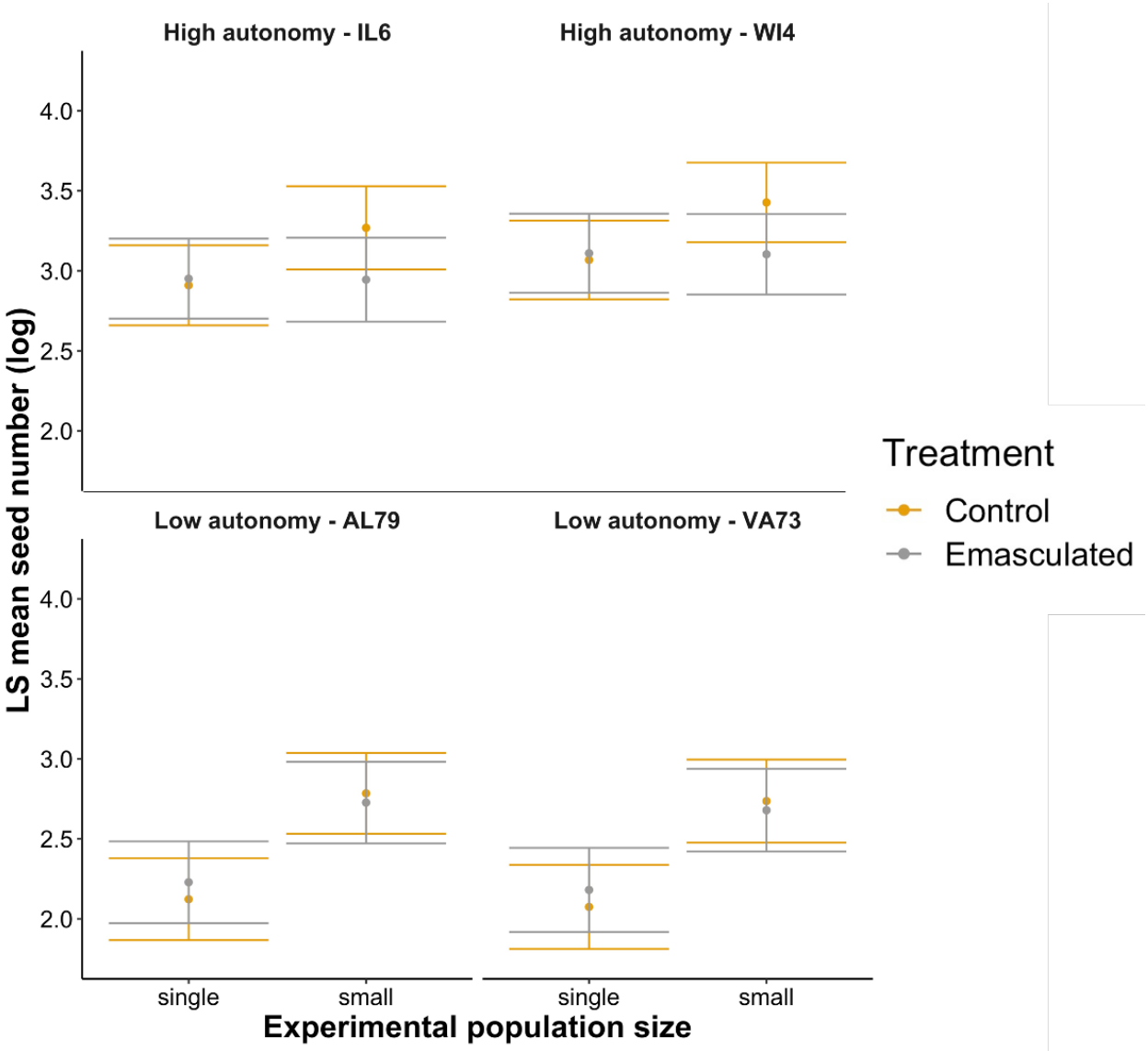


Figure S3. Seed set of pollination treatments to assess reproductive assurance broken up by source population of *Campanula americana*. These source populations were used to create single plant and small multiple plant experimental populations placed in isolated natural environments to emulate colonization. Points indicate LS mean of seed set and error bars show SE.

Table S1. Source population location and autonomy levels

Population	Latitude	Longitude	Autonomy	Autonomy level
AL79	32.93	-88.21	0.26	low
VA73	37.35	-80.55	0.34	low
WI4	43.41	-89.64	0.61	high
IL6	41.71	-87.96	0.76	high

Table S2. Post-hoc pairwise Tukey contrasts

Pollen limitation contrasts: Control - Supplemented				
Autonomy	High		Low	
Size	Small	Single	Small	Single
estimate	-0.62	-0.73	-0.76	-1.52
SE	0.07	0.06	0.07	0.09
p value	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Size		Small	Single	
estimate		0.14	0.79	
SE		0.10	0.11	
p value		0.15	**< 0.001**	
Reproductive assurance contrasts: Control - Emasculated				
Autonomy	High		Low	
Size	Small	Single	Small	Single
C-E contrast estimate	0.32	-0.04	0.06	-0.11
SE	0.08	0.07	0.10	0.11
p value	**<0.001**	0.55	0.55	0.34
Size		Small	Single	
estimate		0.27	0.06	
SE		0.13	0.13	
p value		**0.03**	0.62	

Table S3. Pollinator analyses

	Fixed effects	Wald Chisquare	p
A. Visits per flower			
	population size	151.70	**<0.001**
	autonomy	7.34	**0.007**
	source population nested within autonomy	17.91	**<0.001**
	population size: autonomy	2.20	0.14
B. Total visits			
	population size	146.60	**< 0.001**
	autonomy	30.40	**< 0.001**
	source population nested within autonomy	61.05	**< 0.001**
	population size: autonomy	3.49	0.06

CHAPTER 2:

Morphological and phenological variation in pollen-collecting hairs and their association with mating-system in *Campanula americana*

Abstract

Transitions from outcrossing to selfing often drive the evolution of floral traits. Secondary pollen presentation, the relocation of pollen from the anthers to elsewhere in the flower, occurs in a number of plant families and is thought to have evolved as to enhance outcrossing. We characterized the morphology and retraction phenology of pollen-collecting hairs, a structure involved in secondary pollen presentation within Campanulaceae, and hypothesize its association with mating system. We test whether variation in pollen-collecting hairs in 15 *C. americana* populations is associated with within-flower selfing ability. We found two-fold variation in the length of pollen-collecting hairs across populations, and that variation is associated with within-flower selfing ability. Populations with greater within-flower selfing ability had longer hairs that retracted quickly early in floral anthesis. Longer hairs may allow flowers to acquire more pollen so it is available for within-flower selfing if pollinator failure occurs. This study supports pollen-collecting hairs as a novel mechanism to facilitate reproductive assurance through selfing while maintaining outcrossing ability.

Introduction

About 33% of plant species both outcross and self in mixed mating systems (Vogler and Kalisz 2001). Mixed mating systems confer reproductive assurance via selfing without sacrificing the potential benefits of outcrossing (Kalisz and Vogler 2003). Floral traits, including attractive structures such as petals and reproductive structures such anthers and styles, evolve in response to selection favoring outcrossing as well as selection to increase reproductive assurance (Belaoussoff and Shore 1995, Belaoussoff and Shore 1995). Because floral traits contribute to and are influenced by mating system, mixed-mating systems provide an ideal system to test hypotheses about whether floral traits are selected for both outcrossing and reproductive assurance or whether some attributes of floral structures may be selected for outcrossing and others for reproductive assurance.

Self-fertilization within a flower without an insect visitor (i.e., autonomous selfing) is a common form of reproductive assurance. For within-flower self-fertilization to occur, the sexual phases must overlap within a flower. Morphological and phenological differences in structures associated with pollen-presentation can influence the amount and timing of pollen available for selfing and therefore its potential contribution to reproductive assurance. For example, transitions to selfing are often associated with a reduction in the spatial (Fishman *et al.* 2002, Takebayashi *et al.* 2006, Belaoussoff and Shore 1995, Toräng *et al.* 2017) and temporal separation of the sexes (Kalisz *et al.* 2011, Koski *et al.* 2018) within a flower. Outcrossing rates have been found to be significantly correlated with anther-stigma distances and orientation (Belaoussoff and Shore 1995, Motten and Stone 2020, Fishman *et al.* 2002, Toräng *et al.* 2017). Increased selfing rates have been found with increased sexual phase overlap (Totland and

Schulte–Herbrüggen 2003, Brys et al. 2013). However, less is known about whether floral traits may promote selfing and maintain outcrossing in mixed-mating systems. These mechanisms to promote outcross pollination and autonomous selfing may respond independently to different selection pressures (Armbruster 1988, Fenster and Martén- Rodríguez 2007), allowing outcrossing ability to be maintained while tandemly increasing self-fertilization ability.

Secondary pollen presentation is the relocation of pollen from the anthers to elsewhere in the flower. It is hypothesized to have evolved promote outcrossing (Westerkamp and Weber 1997) through extending the length of male phase, optimizing the placement of pollen on biotic vectors, prolonging pollen viability, and preventing intrasexual conflict (Howell *et al.* 1993, Nyman 1993a). Species in the Campanulaceae family exhibit a unique form of pollen presentation, where pollen is relocated from the anthers to hairs along the style (Yeo 1993, Howell *et al*. 1993). These hairs retract overtime, releasing pollen for transfer. While most species in the Campanulaceae are self-incompatible and obligately outcrossing, mixed-mating has repeatedly evolved in the family (Roquet 2008). Flowers in the family are typically protandrous, starting off in male phase and transitioning to female phase, requiring retention of pollen from the male to the female phase for autonomous selfing to occur. Pollen-collecting hair morphology varies across the family (Jost 1918, Shetler 1979, Nyman 1993a), and the potential for hairs to influence pollen retention into the female phase has led to speculation that pollen-collecting hairs contribute to mating system (Nyman 1993b). However, we lack a test of the hypothesis that variation in pollen-collecting hair's morphology or phenological variation in retraction (but see Vranken *et al.* 2013) is associated with mating system.

The goal of this study was to determine the extent of intraspecific variation in pollen-collecting hairs, the phenology of hair retraction over floral anthesis, and the role that pollen-collecting hairs may play in autonomous selfing. Using multiple *Campanula americana* populations, a largely outcrossing species with variation in autonomous selfing ability, we ask: (Q1) *Do pollen-collecting hairs vary in length among populations* and (Q2) *what is the timing of their retraction? Is any variation in pollen-collecting hair (Q3) length or (Q4) retraction associated with variation in autonomous selfing?* We hypothesize that hair length will vary among populations, with high autonomous selfing ability associated with longer hairs and/or hairs that retract more slowly, allowing them to retain more pollen into stigmatic receptivity and thereby self-fertilize.

Methods

Study system

In the Campanulaceae family pollen is presented on pollen-collecting hairs present along the style. In this secondary presentation method, anthers dehisce in the bud and pollen is deposited onto pollen-collecting hairs along the outer surface of the style (Fig. 1A, 1D, and 1E). The style then elongates and displays the pollen sub-terminally along the style upon floral anthesis (Fig. 1B and 1C, Leins and Erbar 1990). The pollen-collecting hairs gradually retract into the style, pollen is released for transfer, and the stigmatic lobes open and become receptive after most of the hairs have retracted (Fig. 1E).

Campanula americana L (= *Campanulastrum americanum* Small) is a self-compatible but largely outcrossing, insect-pollinated herb common in eastern North America (Galloway *et al.*

2003, Koski *et al.* 2019). The protandrous flowers open in male phase around noon (Evanhoe and Galloway 2002). The stigmatic surface is inaccessible during male phase. When the female phase begins, the tip of the style splits into three lobes, the sigma becomes accessible, and overtime the stigmatic lobes curl towards the style. If pollinators are not present, the transition to female phase occurs in 2-4 days (Evanhoe and Galloway 2002, Koski *et al.* 2018).

There is a clinal increase in autonomous selfing potential across the range (Koski *et al.* 2017), with populations in the northern and western reaches having the greatest selfing potential. Populations with a higher frequency of autonomous selfing transition to female phase faster, have more pollen present on their stigmatic lobes at the beginning of female phase, and have an increased deposition of pollen onto the stigma the longer the flower is in female phase (Koski *et al.* 2018). The curling of the stigmatic lobes, hypothesized to be a method of autonomous selfing in Campanulaceae by increasing proximity of stigmas to pollen on the outer surface of the style (Stephenson *et al.* 2000, Vranken *et al* 2014), is not associated with autonomous selfing rate in *C. americana* (Koski *et al*. 2018).

Sampling scheme and collection

To assess variation in pollen-collecting hair in *C. americana*, we sampled 15 populations from a latitudinal cline across the natural range. These populations varied in their tendency to autonomously self, measured as the number of fruits set per flower in a pollinator free environment, with values ranging from 0.28-0.68 (Table S1). In 2021 and 2022, seeds from multiple maternal families from each population were sown in a 3:1 combination of PGX soilless growth media and turface. Germination occurred in a growth chamber at 21°C/14°C (day/night)

with 12-hour days. Six weeks after sowing, seedlings were transferred to a cold room set to 5°C and 12-hour days for seven weeks of vernalization to cue flowering. After vernalization, individuals were transplanted into cone-tainers (Stuewe & Sons, Inc., Tangent, OR, USA) and placed in the University of Virginia greenhouse where lights extended day length to 16 hours. We sampled an average of 4.6 maternal families in 11 populations (range 1-10) in 2021, and multiple individuals from 7.5 maternal families in 12 populations (range 1-14) in 2022.

We sampled styles at flower opening to assess initial pollen-collecting hair length and at a series of times after opening to evaluate retraction pattern. Sampled flowers were tagged in bud in the morning before 9am and then collected at flower opening (time 0) and 6, 9 and 21 hours after flowers opened. Collected styles were fixed in FAA solution (2:10:1:5 v:v:v:v, 37% formaldehyde: 95% ethanol: 100% acetic acid: H_2O). The 2021 samples include only the initial timepoint where pollen-collecting hairs were fully extended (0 hours after opening) from 10 populations (52 samples; 5.2 samples/population on average). The 2022 collection included 11 populations sampled at the initial timepoint as well as 6, 9, and 21 hour timepoints across 9 of the 11 populations (448 samples; 12.4 samples/population/timepoint on average). Two populations had little to no replication at later timepoints and were dropped from phenological analyses. Six populations overlapped between the 2021 and 2022 datasets (Table S1). For the 2021 dataset, if any individual had repeated samples, they were averaged so that individual was only represented once. In the 2022 dataset, there were repeated measures over timepoints of different flowers on the same individual (see sampling scheme below). There were 14-21 unique individuals sampled in each population. Occasionally there was additional replication of the same individual on a different day. Individuals were sampled from one to eleven times, with

a mean and median of three which shows that most individuals were only sampled once at a single timepoint.

Sample dissection and imaging

Style samples were dissected and imaged to view the pollen-collecting hairs. Styles were first vortexed and rinsed with additional fixative to remove pollen. Three to four thin (< 0.5mm) cross-sectional slices were made just below the split of the three stigmatic lobes with a 15° stab knife on a dissecting scope. The thinnest dissection with the least amount of pollen in view was imaged on a standard light microscope. Alternative methods for more precise dissection were explored, such as using a cryostat or microtome, but these methods compromised the integrity of the pollen-collecting hairs. For the initial timepoint samples in 2021, we imaged pollen-collecting hairs at 200x using acamera attached to a light microscope and captured approximately 25% of the circumference of each style in the image (Fig. 2A-C). In 2022, we also included 40x images with the entire style circumference in addition to the 200x images (Fig. 2E). A ruler was imaged at each magnification to convert pixels to metric units.

Image processing using computer vision and object detection

To obtain data on pollen-collecting hair count and length, we trained two models to detect hairs, one for 40x images and the other for 200x images. The models were computer vision algorithms trained on extra images taken while we were collecting our experimental image dataset. We performed model training and object detection using Detecto (Detecto n.d.), a python package. Detecto implements PyTorch (Paszke *et al.* 2019) for custom model

development using Faster Region-based Convolutional Neural Networks. To train the two models, we used 107 images at 40x and 506 images at 200x, and drew bounding boxes around each pollen-collecting hair to create an annotated image set using Make Sense AI (Make Sense n.d.; Fig 2, SI Fig 1). For the 40x model, where the full style was in view, we also trained the model to recognize the style so we could filter that information out. After image annotation, we applied contrast-limited adaptive histogram equalization to make hairs more visually prominent, then divided the annotated images for each model, i.e., 40x and 200x, in two: one set (70%) was used to train the models; the other set (30%) was for validation during model training. We ran the models for 25 epochs, with a learning step size of 5 and a learning rate of $1x10^{-3}$.

In our training set, we drew boxes around each pollen-collecting hair based on what we wanted our two models to identify and measure and used that set to train the models. After developing the two image detection models, we used a small subset of the experimental dataset (around 30 images) to determine the accuracy of both the 40x and 200x computer vision models. We did this by comparing the model output to the data we could get from a human doing counts in a standard image viewer. We regressed hand-counts of pollen-collecting hair number in each image against model outputs. The relationships between hand counted and model counted hairs was strong, $R^2 = 0.87$ for the 40x model and $R^2 = 0.80$ for the 200x model. We also tested the correlation between hand-counts done by separate individuals in the lab and found humans were more consistent in the 40x image assessment ($R^2 = 0.96$) and less consistent in the 200x assessment ($R^2 = 0.74$). We visually evaluated the differences between human and model pollen-collecting hair identification and determined that the model was

performing well and perhaps made better calls than a human in some cases. All experimental images (below) were visually confirmed with model output to check for erroneous data (e.g. detached hairs, extra style tissue with hairs).

We ran the models on the experimental image dataset of 448 40x and 144 200x images to obtain data for analyses. For both the 40x and 200x models, the output was the number and dimensions of boxes drawn around individual pollen-collecting hairs. Because the 40x and 200x images showed different proportions of the style, we used different approaches to get metrics for analysis. For the 40x images, where the full style cross-section was in view, we used the number of bounding boxes as the number of pollen-collecting hairs per timepoint per style. We used hypotenuse of each pollen-collecting hair's bounding box (Fig. 2) as the raw hair length. Lengths were converted from pixels to metric units using Image-J to assess the ratio of pixels to millimeters (40x = 15.26 pixels/ 0.05 mm; 200x = 13.14 pixels/ 0.01 mm). Average hair length was calculated for each sample in the 200x image dataset. The 40x images, with the full circumference of the style, were used to assess the length of pollen-collecting hairs as retraction occured. To do this, it was necessary to account for hairs that had fully retracted. We did this by calculating an estimated average pollen-collecting hair length by summing the length of the hairs per image and then dividing that sum by the population average number of hairs at the initial timepoint.

Analysis

We determined if populations were differentiated from each other, the phenology of retraction, and the association of hair traits with autonomous selfing ability using the length

and number of pollen-collecting hairs. Residuals of all variables met assumptions of normality and homoscedasticity. To determine if the length of pollen-collecting hairs varied across populations (Q1), we used the 200x initial pollen-collecting hair length data (time 0) from both years of data collection. This included 144 samples from 15 populations with six populations replicated across the years. We modeled the individual average pollen-collecting hair length as a function of population (R, "lm"). To determine if the initial number of pollen-collecting hairs varied across populations, we used timepoint 0 values from the 2022 dataset at 40x with 103 individuals measured across 11 populations. We used the same samples to determine if hair number and length at floral opening (timepoint 0) was correlated.

We assessed retraction phenology (Q2) using the number and length of pollen-collecting hairs in the nine populations from the 2022 dataset with 40x images across all 4 timepoints. Because our calculation of estimated pollen-collecting hair length at later timepoints used initial average number of hairs per population in the denominator, we used population as our level of replication, i.e. calculated averages for each population and time. We compared populations at specific sets of timepoints by modeling the effect of timepoint in an ANOVA using population averages for pollen-collecting hair number and estimated average hair length as response variables. We used post-hoc Tukey tests to compare timepoints.

To evaluate whether pollen-collecting hair length or number was associated with autonomous selfing ability (Q3), we used the 200x initial hair length dataset for hair length and the 40x dataset for hair number. Autonomous selfing values are a population level metric so we used population means of traits. We modeled the population average pollen-collecting hair length and number as a function of the population's autonomous selfing ability. To assess

differences in retraction across autonomy levels (Q4), we used an ANOVA with the estimated average pollen-collecting hair length from the 40x timing data set (same as Q2) as a response variable (R, "lm"). We only included the first two timepoints after finding that most of the retraction happens between 0 and 6 hours (Q2). We included an interaction between timepoint and autonomous-selfing ability.

Results

The average length of an individual's pollen-collecting hairs at flower opening significantly differed two-fold among *C. americana* populations, ranging from 0.18 to 0.44 mm ($F_{14, 129} = 11.55$, $p < 0.001$). The number of hairs in the circumference of the cross-section of the style at flower opening also varied two-fold among populations, ranging from 49 to 95 hairs ($F_{10, 92} = 2.17$, $p = 0.03$). However post-hoc Tukey tests showed only 2 of the 11 populations were significantly different from each other for hair number. Despite similarity in the range of difference among populations, pollen-collecting hair number and length were not correlated with each other ($R = 0.09$, $F_{1,101} = 0.75$, $p = 0.39$).

The number of pollen-collecting hairs decreased sharply during the initial six hours after flower opening with approximately one-third of all hairs fully retracted. This was followed by a more gradual decrease in pollen-collecting hair number across later timepoints ($F_{3,32} = 46.05$, $p < 0.001$, Fig. 3A), with half of the hairs fully retracted by the final timepoint. The estimated average hair length, which accounts for zeros for fully retracted hairs, follows a similar pattern. Average hair length decreased 50% between zero and six hours and then did not change across later timepoints ($F_{3,32} = 40.12$, $p < 0.001$, Fig. 3B)

Average pollen-collecting hair length increased with autonomous selfing ability (Figure 4A, $R = 0.69$, $F_{1,13} = 12.28$, $p = 0.004$). However, there was no correlation between pollen-collecting hair number and autonomy ($R = 0.07$, $F_{1,7} = 0.03$, $p = 0.86$). While populations with high levels of autonomous selfing started off with longer hairs, they retracted faster than those with lower selfing rates during the first 6 hours, and so were the same length at 6 hours after floral anthesis (Figure 4B, timepoint x autonomy interaction $F_{1,14} = 11.39$, $p = 0.005$).

Discussion

Secondary pollen presentation is thought to have evolved to enhance outcrossing. We characterized the extent of intraspecific variation in pollen-collecting hairs, a key trait in secondary pollen presentation in the Campanulaceae, and tested the trait's association with autonomous selfing ability in *Campanula americana*. We found longer pollen-collecting hairs in populations with higher levels of autonomous selfing (Fig 4A). We also found that pollen-collecting hairs in high-autonomy populations had a faster retraction rate than those in low autonomy populations. As a result, six hours after floral anthesis, pollen-collecting hairs were a similar length across all populations (Fig 4B), and populations did not differ in the degree to which pollen was available for outcrossing. These results suggest that initial pollen-collecting hair length is driving differences in autonomous selfing ability across populations. In total, we demonstrate that traits that are thought to have evolved to promote outcrossing may be selected on to facilitate selfing while still retaining outcrossing function.

The longer initial pollen-collecting hair length in high-autonomy populations compared to low-autonomy populations may allow for more pollen retention through additional

accumulation. *C. americana* populations with elevated levels of autonomous selfing have been found to have more pollen on the stigmatic lobes at the beginning of female phase in the greenhouse (Koski *et al.* 2018), and greater pollen remaining following exposure to pollinators (Leibman *et al.* 2018) than populations with lower rates of autonomous selfing. Longer pollen-collecting hairs could retain pollen better by offering greater surface area and a more complex matrix in which to hold pollen. This is supported by the observation that pollen-collecting hairs make it harder for insect visitors to collect pollen (Shetler 1979, Nyman 1993a, Makowski personal observation). In contrast, low-autonomy populations have shorter pollen-collecting hairs and potentially accumulate less pollen in bud or don't hold on to it as well, which decreases their ability to retain pollen into female phase. Information on the amount of pollen retained through male phase in unvisited flowers in high and low autonomy populations is needed to evaluate this hypothesis of how longer pollen-collecting hairs may facilitate autonomous selfing.

Flowers in the high-autonomy populations started off with longer pollen-collecting hairs, but they retracted quickly such that they were the same length as low-autonomy population flowers within six hours of anthesis (Fig 4B). We anticipated that if hairs in the high-autonomy populations were the same length as those in the low-autonomy populations, they would retract more slowly than low-autonomy populations, allowing pollen to be retained over a longer time window. Alternatively, if high-autonomy populations had longer hairs than low-autonomy populations, as we found, we predicted a comparable retraction rate would also allow for longer pollen retention. However, these hypotheses were not supported. The more rapid retraction that we found may be a byproduct of the shortened male phase found in high

autonomy populations (Koski *et al.* 2017). In natural settings, most pollen is removed from the flower within the first four hours following anthesis (Evanhoe and Galloway 2002). Since pollen-collecting hairs hold onto pollen and make it more difficult for pollinators to remove, similar hair length six hours after anthesis suggests ease of pollen removal and hence outcrossing potential is comparable across populations with low and high autonomous selfing ability. Whereas, if the longer pollen-collecting hairs retracted slowly, less pollen to be available for outcrossing and male fitness would decrease due to pollen discounting (Holsinger *et al.* 1984). The more rapid retraction we see in *C. americana* may maintain the opportunity for outcrossing.

Traits that facilitate selfing are often the reduction of the spatial and temporal mechanisms that promote outcrossing (i.e., decreases in herkogamy and dichogamy) and are predicted to spread rapidly when selfing is favored (Lande and Schemske 1985, Charlesworth *et al.* 1990, Stone *et al.* 2014). However, this idea is challenged in mixed-mating systems where selfing can occur and yet outcrossing is maintained. The association of pollen-collecting hair length, a key component of secondary pollen presentation thought to have evolved to enhance outcrossing, with autonomous selfing ability suggests selection on the same trait to enhance outcrossing and selfing. In natural populations high- and low-autonomy populations have comparable levels of pollinator visitation and outcrossing rates (Koski *et al*. 2019). Longer hairs may maintain more pollen into female phase and be available for selfing in high autonomy populations. Expedited retraction coupled with the showy floral display that is found across all populations, on the other hand, results in similar hair length six hours after opening suggesting outcross function is the same across low and high autonomy populations. Maintenance of

outcrossing function of the pollen-collecting hairs with modification that allows for increased selfing in cases of pollinator failure, provides the 'best of both worlds' of mixed mating systems (Stebbins 1974, Lloyd 1992). Furthermore, the apparent evolution of pollen-collecting hairs to promote selfing without compromising their outcrossing function, suggests a morphological mechanism for an evolutionarily stable mixed-mating system.

Literature cited

Armbruster, W.S. 1988 Multilevel comparative analysis of the morphology, function and evolution of *Dalechampia* blossoms. *Ecology* 69: 1746–1761.

Barrett, S. C. H. 2002. The evolution of plant sexual diversity. *Nature Reviews Genetics* 3: 274–284.

Barrett, S. C. H. 2008. Major Evolutionary Transitions in Flowering Plant Reproduction: An Overview. *International Journal of Plant Sciences* 169: 1–5.

Brys, R., Geens, B., Beeckman, T., Jacquemyn, H. 2013. Differences in dichogamy and herkogamy contribute to higher selfing in contrasting environments in the annual *Blackstonia perfoliata* (Gentianaceae). *Annals of Botany* 111: 651–661.

Belaoussoff, S., Shore, J.S. 1995. Floral correlates and fitness consequences of mating-system variation in *Turnera ulmifolia. Evolution* 49: 545–556.

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

Charlesworth, D., Morgan, M.T., Charlesworth, B. 1990. Inbreeding depression, genetic load, and the evolution of outcrossing in a multilocus system with no linkage, Evolution 44: 1469-1498.

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.

Fenster, C.B., Martén- Rodríguez, S. 2007. Reproductive assurance and the evolution of pollination specialization. *International Journal of Plant Sciences* 168: 215-228.

Fishman, L., Kelly, A. J., Willis, J. H. 2022. Minor Quantitative Trait Loci Underlie Floral Traits Associated with Mating System Divergence in Mimulus. *Evolution* 56: 2138-2155.

Galloway, L. F., J. R. Etterson, J. L. Hamrick. 2003. Outcrossing rates and inbreeding depression in the herbaceous autotetraploid *Campanula americana*. *Heredity* 90: 308-15.

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

Holsinger, K. E., Feldman, M.W., Christiansen, F.B. 1984. The evolution of self-fertilization in plants: a population genetic model. *American Naturalist* 124: 446- 453.

Howell, G.J., Slater, A.T., Knox, R.B. 1993. Secondary pollen presentation in angiosperms and its biological significance. *Australian Journal of Botany* 41: 417–438.

Jost, L. 1918 Die Griffelhaare der Campanulablüte. *Floral* 111/112: 478-490.

Kalisz, S., Vogler, D.W. 2003. Benefits of autonomous selfing under unpredictable pollinator environments. *Ecology* 82: 2928-2942.

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., L. Kuo, K. M. Niedermaier, 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.

Koski, M. H., Galloway, L. F., Busch, J. W. 2019. Pollen limitation and autonomous selfing ability interact to shape variation in outcrossing rate across a species range. *American Journal of Botany* 106: 1– 8.

Lande, R., Schemske, D.W. 1985. The evolution of self-fertilization and inbreeding depression in plants. 1. Genetic models. *Evolution* 1985: 24-40.

Leibman, L., A. Rowe, M. H. Koski, L. F. Galloway. 2018. Populations with greater flexibility in floral traits modify mating system in response to the pollinator environment. *Functional Ecology* 32: 1457-1466.

Leins, P., Erbar, C. 2006. Secondary pollen presentation syndromes of the Asterales a phylogenetic perspective. *Botanische Jahrbücher Für Systematik, Pflanzengeschichte Und Pflanzengeographie* 127: 83–103.

Lloyd, D. G. 1992. II. The selection of self-fertilization. *International Journal of Plant Sciences* 153: 370–380.

Motten, A.F., Stone, J.L. 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.

Nyman, Y. 1993a. The Pollen-Collecting Hairs of *Campanula* (Campanulaceae). I. Morphological Variation and the Retractive Mechanism. *American Journal of Botany* 80: 1427–1436.

Nyman, Y. 1993b. The Pollen-Collecting Hairs of *Campanula* (Campanulaceae). II. Function and adaptive significance in relation to pollination. *American Journal of Botany* 80: 1437–1443.

Paszke *et al.* 2019. PyTorch: An Imperative Style, High-Performance Deep Learning Library. *arXiv* 1912.01703

Pujol, B., Zhou, S.R., Sahchez-Vilas J., Pannell, J.R. 2009. Reduced inbreeding depression after species range expansion. *Proceeding of the National Academy of Sciences USA* 106: 15379– 15383.

Ren, S., He, K., Girshick, R., Sun, J. 2016. Faster R-CNN: Towards real-time object detection with region proposal networks. *arXiv* 1506.01497v3

Schoen, D. J., Morgan, M.T., Bataillon, T . 1996. How does self-pollination evolve? Inferences from floral ecology and molecular genetic variation. *Philosophical Transactions of the Royal Society of London, Series B* 351: 1281– 1290.

Shetler, S.G. 1979. Pollen-collecting hairs of Campanula (Campanulaceae), I: Historical review. *Taxon* 28: 205–215.

Stebbins, G.L. 1974. *Flowering plants: evolution above the species level*. Cambridge, MA: Harvard University Press.

Stephenson A.G., Good S.V., Vogler D.W. 2000. Interrelationships among inbreeding depression, plasticity in the self-incompatibility system, and the breeding system of *Campanula rapunculoides* L. (Campanulaceae). *Annals of Botany* 85: 211–219.

Stone, J.L., VanWyk, E.J., Hale, J.R. 2014. Transmission advantage favors selfing allele in experimental populations of self-incompatible *Witheringia solanaceae* (Solanaceae). Evolution 68: 1845-1855.

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

Toräng, P., Vikström, L., Wunder, J., Wötzel, S., Coupland, G., Ågren, J. 2017. Evolution of the selfing syndrome: Anther orientation and herkogamy together determine reproductive assurance in a self-compatible plant: evolution of the selfing syndrome. *Evolution* 71: 2206–2218.

Totland, Ø., Schulte-Herbrüggen, B. 2003. Breeding System, Insect Flower Visitation, and Floral Traits of Two Alpine Cerastium Species in Norway. *Arctic, Antarctic, and Alpine Research* 35: 242–247.

Vogler, D.W., Kalisz, S. 2001. Sex among the flowers: the distribution of plant mating systems. *Evolution* 55: 202-204.

Vranken, S., Brys, R., Hoffmann, M., Jacquemyn, H. 2014. Secondary pollen presentation and the temporal dynamics of stylar hair retraction and style elongation in *Campanula trachelium* (Campanulaceae). *Plant Biology* 16: 669–676.

Webb, C. J., Lloyd, D. G. 1986. The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. Herkogamy. *New Zealand Journal of Botany* 24: 163–178.

Westerkamp, C., Weber, A. 1997. Secondary and tertiary pollen presentation in *Polygala myrtifolia* and allies (Polygalaceae, South Africa). *South African Journal of Botany* 63: 254–258.

Yeo, P. F. 1993. *Secondary pollen presentation: form, function, and evolution* (1st ed.). Springer, Vienna.

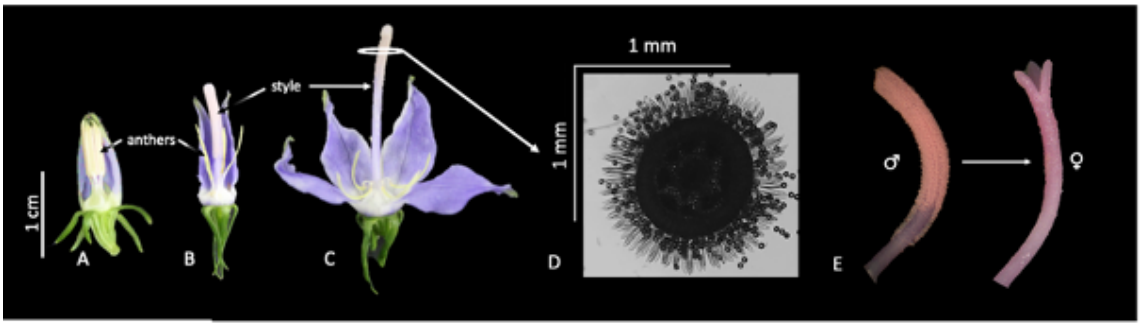


Figure 1. Secondary pollen presentation in *Campanula americana.* First, (A) pollen is deposited from the anthers onto hairs along the style while the flower is in bud. The flower opens and the style begins to elongate (B and C). Pollen is present along a cross section of the style (D). Over time, the hairs retract, the stigmatic lobes open and the flower becomes functionally female (E).

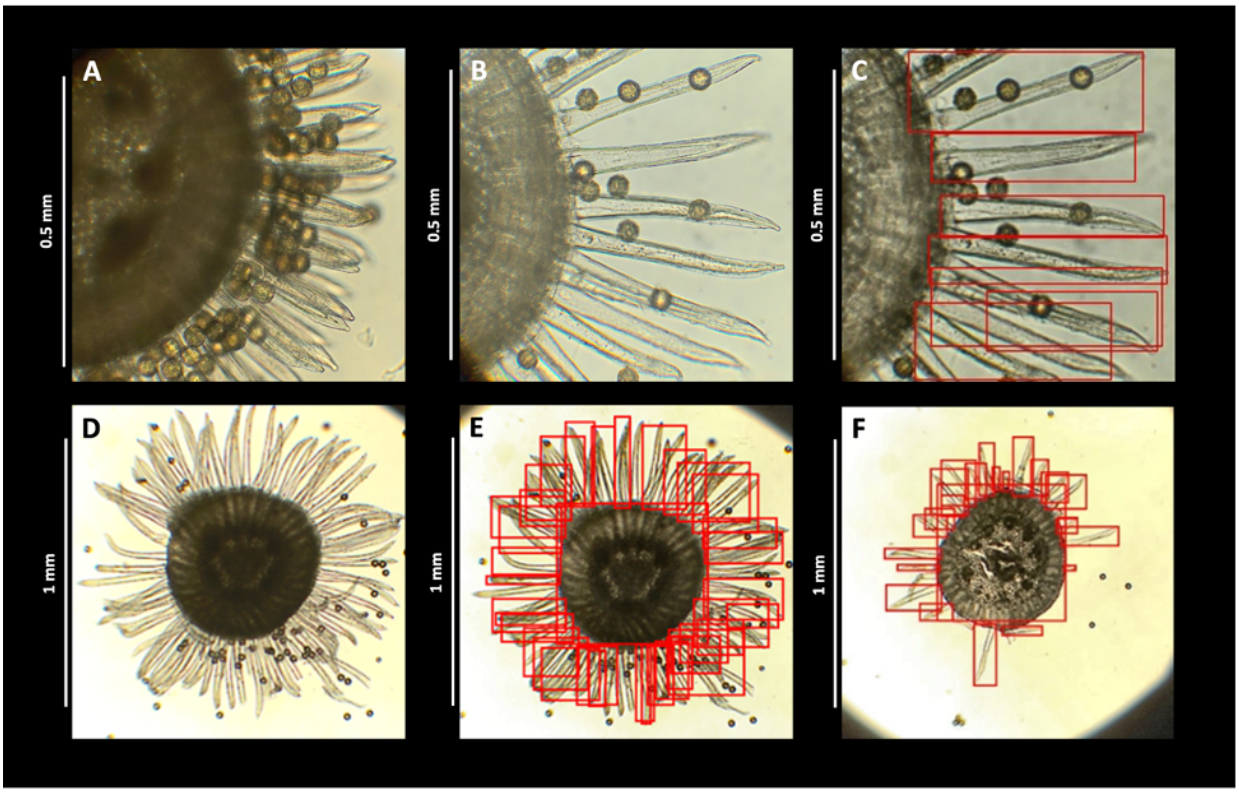


Figure 2. A cross sectional view of the style of *Campanula americana* and its pollen-collecting hairs. A 200x view of a low autonomy population, AL79 (A) and a high autonomy population, KS60 (B), with an example of the AI image detection model output for the 200x images used to get average hair length (C). Hairs do not retract evenly around the style, so we imaged at 40x to get the whole style in view. A high autonomy population, IA18 is shown at time 0 (D) with AI model output at time 0 (E) and 6 hours after floral anthesis (F).

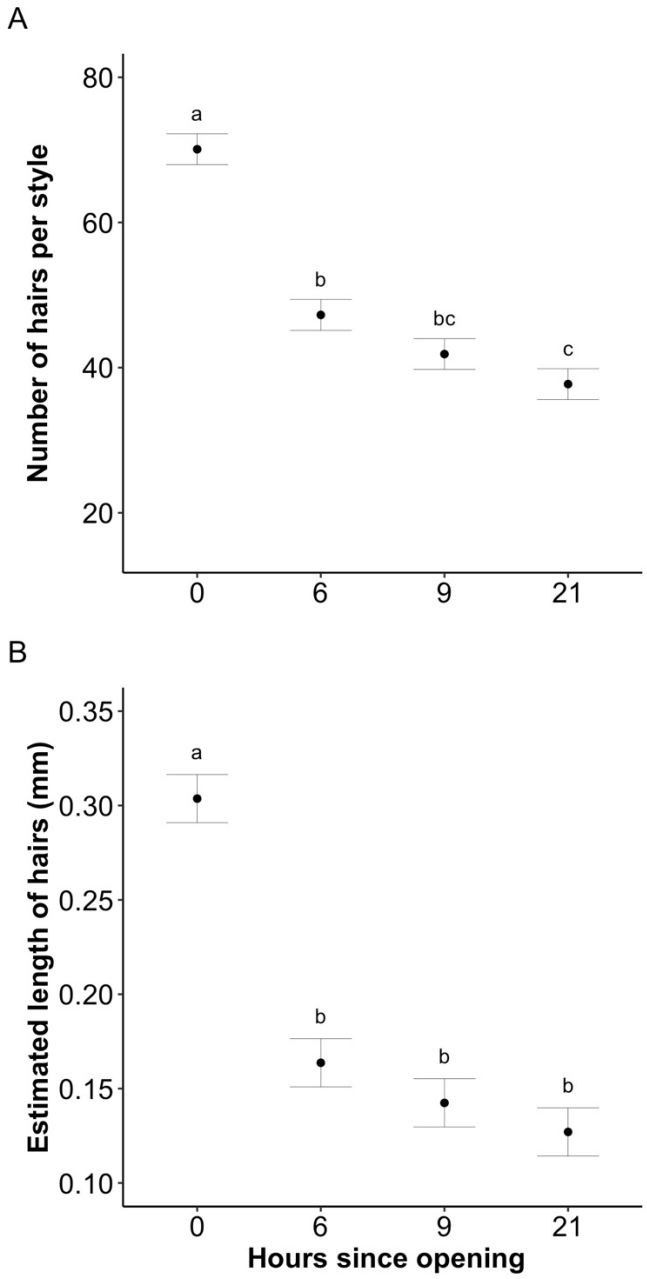


Figure 3. The (A) number and (B) estimated length of *Campanula americana* pollen-collecting hairs over the first day of floral anthesis. Points represent averages across nine populations at each timepoint. Error bars are SE. Letters signify statistical difference from post-hoc Tukey test.

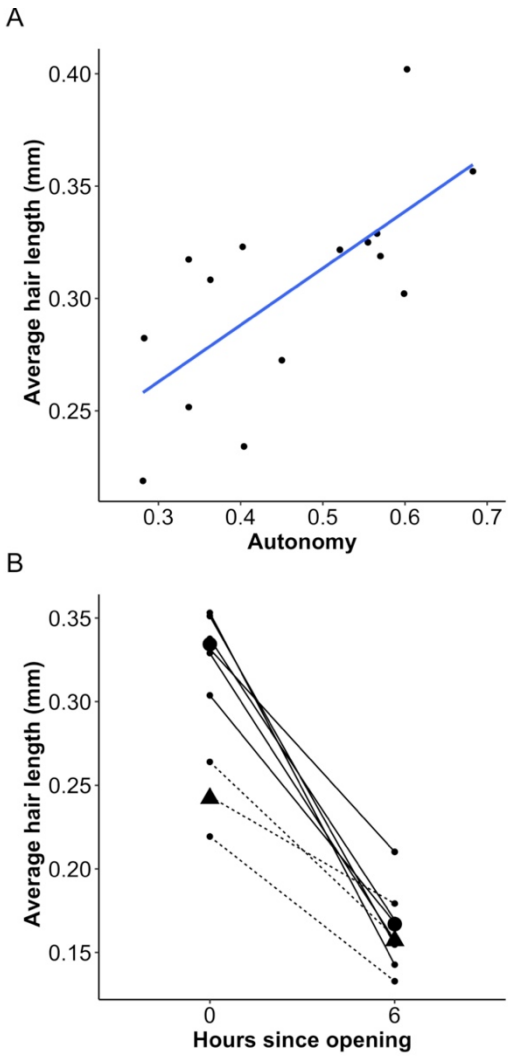


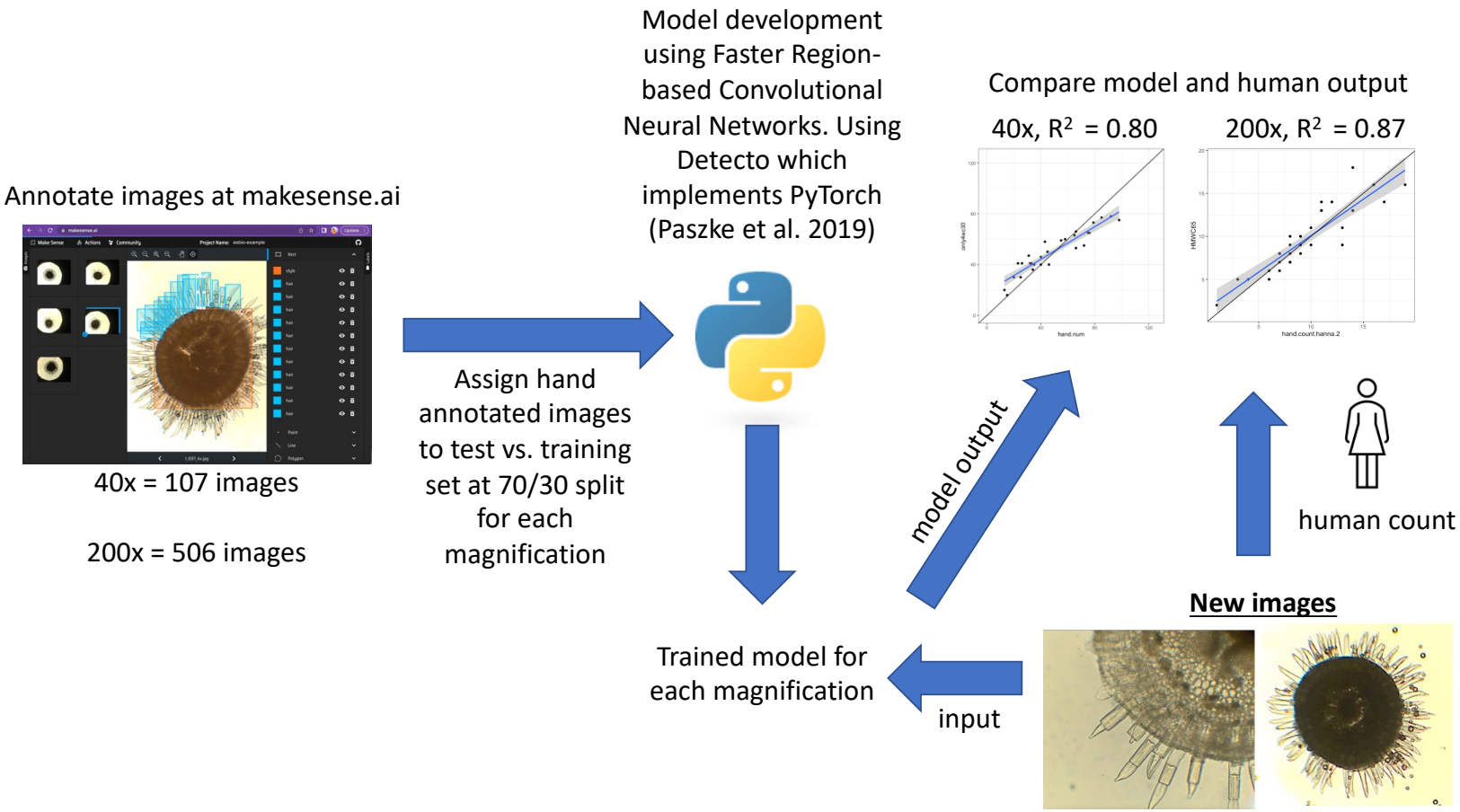
Figure 4. The association of hair traits with autonomous selfing ability in *Campanula americana*. (A) Average pollen-collecting hair length for 15 populations of *C. americana* across a range of autonomy values. (B) Average pollen-collecting hair length at floral opening and 6 hours after floral anthesis. Statistics for (B) were run on continuous values for autonomy but displayed categorically to illustrate patterns, timepoint x autonomy $p < 0.005$. Large shapes in (B) represent the means for categorical high (circle) vs low autonomy (triangle) populations, with populations over 0.50 categorized as high autonomy. Small circles represent individual populations averages, high autonomy populations are solid lines and low autonomy populations are dashed lines.

S1. Autonomous selfing assessment

Autonomous selfing values were assessed and then averaged with previous year's data (Table S1). In brief, after plants were harvested, I scored the number of flowers produced and the number of fruits set on at least two flowering nodes. We calculated autonomous fruit set for each individual as the proportion of fruits to total flowers produced and then averaged individuals in populations.

Table S1. Phylogenetic, geographic, and autonomy data for the 15 *Campanula americana* populations used in this study. Autonomy data was collected from the lab in 2017 and 2020-2022, indicated in the last column. An asterisk in the experiment year column indicates population was only used at time 0 for the 2022 dataset.

Population	Clade	Latitude	Longitude	Autonomy mean	Autonomy median	Autonomy SD	Experiment year	Autonomy data years
AL8	Western	32.26	-86.49	0.45	0.47	0.20	2022*	2022
AL79	Western	32.93	-88.21	0.40	0.40	0.25	both	all
AL1	Western	33.86	-86.55	0.28	0.20	0.26	2022*	2022
ALBG	Western	34.66	-86.52	0.34	0.35	0.19	2022	all
AR2	Western	36.15	-94.30	0.52	0.49	0.21	2022	2021-22
VA73	Appalachian	37.35	-80.55	0.34	0.29	0.26	both	2020-22
MO1	Western	38.96	-94.32	0.36	0.39	0.17	2021	2021
KS60	Western	39.05	-95.68	0.60	0.60	0.20	2022	2017,2022
PA103	Western	40.55	-80.31	0.28	0.24	0.23	both	2020-21
IL6	Western	41.72	-87.97	0.57	0.60	0.26	both	2020-21
IA11	Western	41.80	-91.55	0.60	0.62	0.15	2021	2021
IA18	Western	43.00	-91.17	0.55	0.56	0.20	2022	2022
WI4	Western	43.41	-89.64	0.68	0.70	0.23	both	2020-21
WI9	Western	44.07	-89.49	0.40	0.48	0.22	2021	2021
MN4	Western	44.80	-92.98	0.57	0.59	0.25	both	2020-21



Supplementary Figure 1. Workflow for the AI image detection model. We used makesense.ai to annotate images at each magnification. We then split those images into a training and test set to train the model. Once the model was trained, we exposed the model to new images and compared the output to the output we would get from human counts.

CHAPTER 3:

Pollen-collecting hair length: a phylogenetically constrained trait that scales allometrically across the Campanulaceae family

Abstract

Secondary pollen-presentation, the relocation of pollen from the anthers to elsewhere on the flower, has evolved multiple times across many plant families. While hypotheses suggest it evolved to promote outcrossing, a byproduct of relocation may be protection of pollen from abiotic agents of loss. We tested the protective function of secondary pollen presentation in the Campanulaceae where pollen is relocated to pollen-collecting hairs along the style. The hairs retract over time and release pollen for transfer. Taxa vary in the degree to which pollen is exposed to environmental factors due to variation in the corolla shape and size. We used phylogenetic comparative methods across 39 species to evaluate pollen-collecting hair length. We also measured floral shape and size to estimate exposure of pollen to environmental agents. We anticipated longer pollen-collecting hairs in taxa with more exposed pollen presentation but found there was no relationship between estimates of pollen exposure and pollen-collecting hair length. However, pollen-collecting hair length scaled allometrically with floral size, and variation in pollen-collecting hairs as well as most floral trait was phylogenetically structured. These results suggest that variation in pollen exposure across species does not structure variation in the pollen-collecting hairs, and it is unlikely they serve as protection from environmental pressures.

Introduction

Floral traits primarily evolve to promote effective pollen transfer. Pollinator efficiency and access varies with size (Armbruster 1988, Copete *et al.* 2018) and shape (Nilsson 1988) of the flower, suggesting corollas evolve to avoid wasting gametic resources on inefficient pollinators. An extreme example of this is self-pollinating flowers where corollas remain closed retaining more pollen than flowers open to pollinators (cleistogany; Fargue *et al.* 2006, Kulkarni and Baskaran 2013). Pollen can also be lost or damaged due to environmental factors. For example, the corolla can protect pollen from rain (Huang *et al.* 2002, Sun *et al*. 2008, Mao and Huang 2009), UV radiation (Flint and Caldwell 1983, Zhang et. al 2014), and desiccation (Vaknin *et al.* 2021). In total, the size and shape of the corolla can influence pollen persistence and therefore transfer efficiency. In addition to the corolla, there may be additional floral traits that also affect pollen persistence.

Secondary pollen presentation occurs when pollen is relocated from the anthers to another structure within the flower. This form of pollen presentation is hypothesized to have evolved to promote outcrossing (Westerkamp and Weber 1997) through extending the length of male phase, optimizing the placement of pollen on biotic vectors, prolonging pollen viability, and preventing intrasexual conflict (Howell *et al.* 1993, Nyman 1993a). There are multiple different forms of secondary pollen presentation across plant families, and pollen protection may occur as a byproduct of the relocation and presentation of pollen (Howell *et al.* 1993), in addition to its outcrossing function. For example, in some species a “pollen box” must receive physical pressure from a pollinator to release the pollen for outcrossing (Hildebrans 1870, Brantjes 1983, Leins and Erbar 2006), thus protecting pollen from being lost to climactic factors

or pollen thieves. Secondary pollen presentation in *Asteraceae* is thought to protect the pollen after anther dehiscence, reducing exposure to humidity, UV, and desiccation of the pollen grains (Brantjes 1983). In Fabaceae, the pollen is relocated onto the two most anterior petals known as the keel, which is hypothesized to serve a protective function (Lavin and Delgardo 1990). To understand whether secondary pollen presentation results in a protective function, we need to determine the extent to which it mitigates pollen exposure to environmental stressors.

Secondary pollen presentation is ubiquitous across the Campanulaceae. In members of this largely outcrossing family (Roquet *et al.* 2008), pollen is relocated from the anthers to hairs along the style in the bud. Once the flower opens, the pollen-covered style elongates and hairs start retracting, progressively releasing the pollen. Species in the family differ in the timing of their sexual phases, but most are protandrous with flowers opening in male phase. It is hypothesized that secondary pollen presentation in this family evolved to prominently display pollen to pollinators, compensating for the bell-shaped corollas and inward pointing anthers that shield pollen within the flowers (Yeo 1993). Early work noted morphological differences in the hairs between species (Jost 1918, Shetler 1979, Nyman 1993a). The controlled release of pollen through the retraction of hairs is thought to contribute to pollen transfer efficiency by slowly making pollen available to pollinators, ensuring that the pollen is transferred efficiently and lengthening the male phase to promote outcross opportunities (Yeo, 1993).

Because pollen-collecting hairs hold onto pollen, they may also serve a protective function in addition to promoting outcrossing. Longer hairs may protect pollen from physical loss due to rain and wind. Longer hairs may also protect hairs from desiccation and UV radiation

by creating larger stacks of pollen, where outer layers help shield pollen deeper in the hair structure. Such structure is akin to the UV and desiccation protection provided by larger packages of pollen grouped together by viscous substances or filaments (Eisikovitch *et al*. 1987, Demchick and Day 1996). The length of the pollen-collecting hairs is suggested to influence their ability to hold onto the pollen, with shorter hairs being unable to retain it (Nyman 1993a). This has been evidenced in *Campanula americana*, with populations with longer pollen-collecting hairs able to retain more pollen over time (Chapter 2, Koski *et al.* 2018, Leibman *et al.* 2018). Species in Campanulaceae vary in the openness of their corolla, from bell-like structures (i.e., campanulate, hence the vernacular name of the "bellflower" family) to flat and spreading (i.e., rotate; Roquet 2008). Flower shape is expected to result in different levels of shielding of the style and consequent exposure of pollen to the outside environment. It is not known whether pollen-collecting hairs have evolved to protect pollen when pollen is not enclosed within corollas.

In this study, we survey pollen-collecting hair and floral traits across largely outcrossing species in the Campanulaceae to determine if there is a relationship between the length of pollen-collecting hairs and the corolla shape. Specifically, we ask (1) *Is there phylogenetic structure in pollen-collecting hair length or in other floral traits?* (2) *Does pollen-collecting hair length scale allometrically with flower size?* And finally, we test (3) *Is pollen-collecting hair length related to environmental exposure of pollen ?* We hypothesize that in cases where pollen presentation is enclosed within more bell-shaped flowers, hairs play less of a protective role than hairs holding pollen that is more exposed to the surrounding environment. Therefore, we expect a positive relationship between pollen exposure and hair length. We address these

questions with phylogenetic comparative analyses of pollen-collecting hairs and multiple floral traits across 39 species in the Campanulaceae.

Methods

We collected style samples and measurements of corolla structure from 39 species across the Campanulaceae for use in phylogenetic comparative analyses (Table S1). We made collections from botanical gardens including the Missouri Botanic Garden, St. Louis, MO, USA; The Royal Botanic Garden, Edinburgh, UK; and the Botanic Garden Berlin, Germany from June through July of 2022. Additionally, we sourced seeds from the California Botanic Garden, Claremont, CA, USA as well as from Plant World Seeds, UK and grew plants for floral collection in the greenhouse at the University of Virginia. All species identities were confirmed.

Style sampling, dissection, and measurements of hairs and pollen

We sampled an average of three male-phase styles from early male-phase flowers for each species and fixed them in FAA solution (2:10:1:5 v: v: v: v, 37% formaldehyde: 95% ethanol: 100% acetic acid: H_2O). Samples were dissected and imaged to view the pollen-collecting hairs (Chapter 2). First, style samples were vortexed and rinsed with additional fixative to remove most pollen. Three to four thin (< 0.5mm) cross-sectional slices were made with a 15° stab knife on a dissecting scope. The thinnest dissection with the least amount of pollen in view was imaged on a standard light microscope at 40x. To measure pollen-collecting hair length, we used ImageJ to draw a segmented line, tracing three hairs per style and then averaged hair length for each species. Additionally, we measured the diameter of three pollen grains from a single style sample image per species. One species, *Githopsis diffusa*, lacked

pollen in every sample and was excluded from analyses of pollen size. Pollen diameter and pollen-collecting hair lengths were converted from pixels to metric units using Image-J to assess the ratio of pixels to millimeters (40x = 15.26 pixels/ 0.05 mm). For *Campanula americana*, which had extensive sampling across 15 populations in prior work (Chapter 2), we took the average hair length across all populations (0.3mm), which was also the approximate median of that dataset.

Corolla trait measurement and measurements of openness

We measured six corolla traits and three style traits on five individuals per species. The corolla traits were angle of openness, petal length, diameter of floral opening, base to petal split length, petal width, petal tip to petal tip (Figure 1A, 1C, 1D, 1E, 1F, 1I). The style traits were the total style length, the length of style that had pollen-collecting hairs, and the length of the style that was visible from a side-view of the flower (Figure 1B, 1G, 1H). For the first 12 species we sampled, all measurements were taken with a digital caliper directly from flowers and through ImageJ. Then, we regressed hand and digital measurements against each other to determine which measurements could be reliably obtained through images and which measurements needed to be done by hand. For the remaining species, we measured the diameter of the corolla and the petal tip to tip distance by hand while all other traits were measured from images in ImageJ after collection. A ruler was included in all images to scale flowers and convert pixels to metric units in ImageJ. We used species' means of traits for analysis. For *C. americana*, we took floral measurements from one population with long pollen-

collecting hairs and one population with short pollen-collecting hairs. There was no difference between populations in their corolla traits, so their values were averaged for analysis.

We used factor analysis to synthesize corolla traits into a measure of floral shape. We first relativized all species-level floral trait data, except for angle, to account for differences in flower size. To do this, we divided the average petal length for each species by the mean petal length of the dataset to get a relative petal length and then divided each floral trait by the relative petal length in that species. We then calculated the Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy for each corolla trait to determine whether individual traits should be included in the factor analysis (Kaiser 1974). The KMO indicates the proportion of variance in the variables that is associated with underlying factors. Values closer to 1 indicate the factor analysis represents variation in the data. We used a 0.5 cutoff in KMO (Kaiser 1974) to determine which traits to exclude from the floral factor. We then performed parallel analysis using the fa.parallel function from the psych package in R to determine the appropriate number of variable clusters to assign in the factor analysis. Finally, we conducted a rotational factor analysis using a maximum likelihood approach (psych package in R). Two species, *C. andrewsii* and *C. sabatia*, were included in the factor analysis but not in subsequent analyses due to lack of pollen-collecting hair length measurements.

We calculated the proportion of the style that had hairs and the proportion of the style that was visible by dividing the raw values for those traits by the style length. To calculate the amount of pollen exposure, we subtracted the length of the style that was visible from the length of style that had hairs and then divided that by the length of the style that had hairs. Zero represents full exposure and one represents no exposure, i.e., full protection of the pollen.

Phylogeny construction

We constructed a phylogeny of the species included in this study using previously published data on the PET-D gene. We downloaded FASTA files of the sequences from the NIH GenBank repository, including all entries for a species if there were multiple. We aligned the sequences using MUSCLE and constructed a tree in MEGAX using maximum likelihood methods with out-of-the-box MEGAX settings. We confirmed that individuals from the same species clustered together and pruned the tree to a single representative of each species (Table S1, Figure S1). Prior phylogenetic work in Campanulaceae used the PET-D gene to construct a phylogeny, so we visually compared our tree to the larger published trees to check for consistency in topology (Roquet 2008). We found that major splits and ordering of species were similar and therefore concluded that our tree approximated a pruned version of prior work and appropriately represented relationships for subsequent analyses. Several species pairs had no branch length differences, as their PET-D sequences did not differ, so 0.00000001 was added to all branch lengths to allow analyses to run while keeping relative distances the same. For phylogenetic linear regression, we rescaled branch lengths to be proportional to time (ultrametric) using the chronos function in the R package APE (Paradis *et al.* 2004, Katzer *et al.* 2019). The non-ultrametric tree was used to determine the phylogenetic signal.

Evolutionary associations and phylogenetic distributions of pollen-collecting hair, style, and floral traits

We used phylogenetic linear regression to test for evolutionary associations between traits using the phylolm function in the R package PHYLOLM (Ho and Ane 2014). For each association, we fit four evolutionary models: (1) Brownian motion (BM), where traits evolve randomly according to drift; (2) Early Burst (EB), where the rate of evolution slows over time; (3) Orenstein-Uhlenbeck (OU), where traits evolve toward an optimum; and (4) Pagel's lambda (PL), where the rate of evolution is optimized from the data. The model fits were compared using the Akaike information criterion and the best fit model or models were used to estimate evolutionary associations between traits.

We then tested whether variance in traits is described by phylogenetic relationships, i.e., whether species look alike because they are closely related. We calculated the phylogenetic autocorrelation measure, Moran's I, for floral shape, style, pollen-collecting hair, and pollen traits. A significant Moran's I indicates that the data is not randomly dispersed and is clustered according to the phylogeny.

Results

Campanula flowers vary substantially in shape and size. The average pollen-collecting hair had a six-fold difference in length between the shortest and longest species (0.09 - 0.59 mm). Petal and style length varied 10-fold across populations (petal 5.5 – 52.94 mm, style 2.57-26.31 mm). Pollen diameter varied two-fold, from 0.02 to 0.04 mm. A single floral shape factor best described the data and included four traits with KMO values above 0.5 (Table 1, Figure 2). The factor ranged from -1.60 to 2.35 across species. As the floral shape factor increased, the flower's angle of openness, diameter, and petal tip to tip distance got larger while the distance

from the petal base to the mid-petal split got shorter (Figure 2). The shape factor ranges from bell shaped (smaller values) to more open flowers (larger values, Figure 3). The proportion of the style with pollen-collecting hairs varied eight-fold, from as little as 9% of the style being covered with pollen collecting hairs to as much as 75%. The proportion of the style that was exposed ranged from zero to one (e.g., Figure 3). The proportion of the pollen protected also ranges from fully protected to fully exposed (0-1).

Floral size metrics were more tightly associated than shape. Two size metrics, petal length and style length, were positively correlated (Figure 4). Floral shape was not associated with petal length, style length, or the amount of the style that had hairs on it (SI Table 2), but more bell-like flowers had less style exposure and more hair protection (Figure 5, Table 2). Pollen-collecting hair length increased with style length, petal length, and pollen size (Figure 6, Table 2). The hypothesis that pollen-collecting hairs serve as a mechanism of pollen protection was rejected as pollen-collecting hair length was not associated with floral shape (Figure 7) or style exposure metric (SI Table 2). There was also no support after accounting for variation in pollen-collecting hair length associated with style length (SI Table 2). Most traits showed phylogenetic signal (Table 3), with the exception of the proportion of the style with hair (Moran's I $p = 0.41$) and the pollen size (Moran's I $p = 0.11$).

Discussion

In this study we used comparative techniques to evaluate the potential contribution of secondary pollen presentation to pollen protection. We did this by evaluating the evolutionary associations between multiple floral, style, and pollen-collecting hair traits across 39 species

from the Campanulaceae. We hypothesized that pollen-collecting hairs would serve a protective function and therefore we would find longer pollen-collecting hairs on species with more exposed pollen presentation. We found pollen-collecting hair length had no association with floral shape or amount of pollen exposure. Rather, there was an allometric relationship, with length increasing with all floral size traits. Pollen-collecting hair length also showed significant phylogenetic clustering. These results suggest that pollen exposure does not structure variation in pollen-collecting hair length across species and therefore hairs are unlikely to protect pollen from environmental agents.

Pollen-collecting hairs increased in size with other floral traits (Figure 5). Given the scale of variation – 10-fold in style, petal, and pollen-collecting hairs – this allometric relationship is perhaps not surprising. The relationship is consistent with models of flower development where single genes have large effect on the phenotypes of many floral organs (Coen and Meyerowitz 1991, Weigel and Meyerowitz 1994). However, in other taxa variation in floral traits remains after accounting for size (Robertson *et al*. 1994). Similarly, while the size of pollen-collecting hairs and other floral traits were highly correlated, the traits also showed evidence for phylogenetic signal. This indicates that while substantial variation was explained by size, there also exists patterned variation that is unexplained by size alone. In addition, the floral shape factor was not correlated with petal or style size. Size is often a strong integrating factor constraining morphological variation (Klingenberg 2010). This decoupling of size and shape in Campanulaceae suggests floral shape is evolutionarily labile, with variation in shape from more bell-like to more open flowers perhaps reflecting underlying developmental

patterns or rates from selection by ecological factors (Kilngenberg 2010, Wilson and Sánches-Villagra 2010), such as pollinators (Anderson and Johnson 2007, Smith and Kriebel 2017).

Contrary to our hypothesis, we did not find an association between the amount of pollen exposure and pollen-collecting hair length. This suggests that pollen-protection is not a factor in structuring variation in pollen-collecting hair length across the Campanulaceae. There was, however, significant variation in the amount of pollen exposure across Campanulaceae, with less bell-shaped flowers having more exposed styles and a higher proportion of their hairs unshielded by the corolla (Figure 4). It may be that in cases where pollen is exposed, the pollen itself has evolved hardiness to environmental pressures (e.g., Zhang et. al 2014, Koski and Galloway 2018), rather than the pollen-collecting hairs providing protection. Additionally, in *Campanula americana* there is a clinal increase of hair length across populations independent of floral trait measurements and pollen-exposure (Chapter 2). The increased hair length in *C. americana* is associated with autonomous selfing ability, suggesting that there are alternative agents of selection that structure pollen-collecting hair variation under similar pollen-exposure pressures. Furthermore, the amount of intraspecific variation in pollen-collecting hair length across 15 populations of *C. americana* (0.18 – 0.44 mm, Chapter 2) spans 53% of the interspecific variation characterized in this study (0.09 - 0.59 mm). That range within a species is all the more dramatic when considering that the variation in pollen-collecting hair length in *C. americana* occurs across flowers of the same size, e.g. petal length, whereas in taxa across the Campanulaceae the range of pollen-collecting hair length is found in flowers with a ten-fold range of size. This suggests that selection on mating system can break the allometric size relationships among floral traits found across the Campanulaceae.

Most floral traits, including pollen-collecting hair length, had significant phylogenetic signal, indicating that phylogeny constrains their evolution. However, the amount of the style with pollen-collecting hairs and pollen diameter did not show evidence of phylogenetic clustering (Table 3). This suggests that these traits are evolutionarily labile such that selective agents may lead to divergence from phylogenetic patterns. The amount of the style with pollen-collecting hairs can influence the placement of pollen on pollinators. For example, the size of the abdomen of *Megachilidae campanulaceae* aligns with the length of pollen presentation along the style of *C. americana* (personal observation). While *M. campanulaceae* is not specialized on *C. americana,* most Campanulaceae are bee pollinated, selection associated with pollinator type, size, and abundance may cause divergence from phylogenetic patterns. Pollinators have influenced the evolution of the size of floral traits related to pollination other taxa (Cosacov *et al.* 2014, Katzer *et al.* 2019). To test the role of pollinator traits in size evolution in Campanulaceae, pollinator type and size would need to be known for each species. We did not have the data available to answer this question, but it would be an interesting avenue of exploration for future work.

This study used phylogenetic comparative methods to explore hypotheses for variation in pollen-collecting hair morphology across the Campanulaceae family. We anticipated the pollen-collecting hairs would have a protective function and therefore be longer when pollen was exposed to environmental factors compared to pollen that was protected within a flower. We did not find evidence to support this protective hypothesis. We did find that pollen-collecting hair length was phylogenetically constrained and associated with flower size. Furthermore, the substantial intraspecific variation in pollen-collecting hair length in *C.*

americana relative to that found across the family, suggests mating system is a strong selective agent and can break phylogenetically constrained allometric size relationships.

Literature Cited

Anderson, B., Johnson, S.D. 2007. The geographical mosaic of coevolution in a plant-pollinator mutualism. Evolution 62: 220-225.

Armbruster, W.S. 1988. Multilevel comparative analysis of the morphology, function, and evolution of *Dalechampia* blossoms. *Ecology* 69:1746–61.

Brantjes, N.B.M. 1983. Regulated pollen issue in *Isotoma*, Campanulaceae, and evolution of secondary pollen presentation. *Acta Botanica Neerlandica* 32: 213-222.

Coen, E.S., Meyerowitz, E.M. 1991. The war of the whorls: genetic interactions controlling flower development. *Nature* 353: 31-37.

Cole, F.R., Firmage, D.H. 1984. The floral ecology of *Platanthera blephariglottis*. *American Journal of Botany* 71: 700–710.

Copete, J. C., Flórez, D. M., Núñez-Avellaneda, L. A. 2018. Pollination Ecology of the *Manicaria saccifera* (ARECACEAE): A Rare Case of Pollinator Exclusion. *Pollination in plants.*

Cosacov, A., Cocucci, A.A., Sérsic, A.N. 2014. Geographical differentiation in floral traits across the distribution range of the Patagoian oil-secreting *Calceolaria polyrhiza*: do pollinators matter? *Annals of Botany* 113: 251-266

Demchik, S., Day, T.A. 1996. Effect of enhanced UV-B radiation on pollen quantity, quality, and seed yield in *Brassica rapa* (Brassicaceae). *American Journal of Botany* 83: 573–579.

Eisikovitch, D., Kevan, P.G., Fowle S., Thomas, K. 1987. The significance of pollen longevity in Asclepias syriaca under natural conditions. *Pollen Spore* 29: 121-128.

Fargue, A. *et al.* 2006. Predictive study of the advantages of cleistogamy in oilseed rape in limiting unwanted gene flow. *Euphytica* 151: 1–13.

Flint, S.D., Caldwell, M.M. 1983. Influence of floral optical properties on the ultraviolet radiation environment of pollen. *American Journal of Botany* 70: 1416-1419.

Hildebrand, F. 1870: F. Delpino's weitere Beobachtungen über die Dichogamie im Pflanzenreich. D. Cyphiaceae. *Bot. Zeitung* (Berlin) 40: 638.

Ho, L.S.T., Ane, C. 2014. A linear-time algorithm for Gaussian and non-Gaussian trait evolution models. *Systemic Biology* 63: 397-408.

Howell, G.J., Slater, A.T., Knox, R.B. 1993. Secondary pollen presentation in angiosperms and its biological significance. *Australian Journal of Botany* 41: 417–438.

Huang, Z.-H., Lie, H.-L., Huang, S.-Q. 2015. Interspecific pollen transfer between two coflowering species was minimized by bumblebee fidelity and differential pollen placement on the bumblebee body. *Journal of Plant Ecology* 8: 109–115.

Huang, S.-Q., Shi, X.-Q. 2013. Floral isolation in *Pedicularis*: how do congeners with shared pollinators minimize reproductive interference? *New Phytologist* 199: 858-865.

Huang, S.-Q., Takahashi, Y., Dafni, A. 2002. Why does the flower stalk of *Pulsatilla cernua* (Ranunculaceae) bend during anthesis? American Journal of Botany 89: 1599–1603.

Kaiser, H.F. An index of factorial simplicity. 1974. Psychometrika. 39:31–36.

Katzer, A.M., Wessinger, C.A., Hileman, L.C. 2019. Nectary size is a pollination syndrome trait in *Penstemon*. *New Phytologist* 223: 377-384.

Koski, M.H., Galloway, L.F. 2018. Geographic variation in pollen color is associated with temperature stress. *New Phytologist* 218: 370-379.

Koski, M. H., Kuo, L., Niedermaier, K.M., Galloway, L.F. 2018. Timing is everything: Dichogamy and pollen germinability underlie variation in autonomous selfing among populations. *American Journal of Botany* 105: 241-248.

Klingenberg, C. 2010. Evolution and development of shape: integrating quantitative approaches. *Nature Reviews Genetics* 11: 623-635.

Kulkarni, R.N., Baskaran, K. 2013. From herkogamy to cleistogamy - development of cleistogamy in periwinkle. *Journal of Heredity* 104: 140–14.

Lavin, M., Delgardo, A. 1990. Pollen brush of Papilionoideae (Leguminaceae): morphological variation and systematic utility. *American Journal of Botany* 77: 1294- 312.

Leibman, L., Rowe, A., Koski, M.H., Galloway, L.F. 2018. Populations with greater flexibility in floral traits modify mating system in response to the pollinator environment. *Functional Ecology* 32: 1457-1466.

Leins, P., Erbar, C. 2006. Secondary pollen presentation syndromes of the Asterales a phylogenetic perspective. *Botanische Jahrbücher Für Systematik, Pflanzengeschichte Und Pflanzengeographie* 127: 83–103.

Mao, Y.-Y., Huang, S.-Q. 2009. Pollen resistance to water in 80 angiosperm species: flower structures protect rain-susceptible pollen. *New Phytologist* 183: 892-899.

Nilsson, L. 1988. The evolution of flowers with deep corolla tubes. *Nature* 334: 147–149.

Nyman, Y. 1993a. The pollen-collecting hairs of *Campanula* (Campanulaceae). I. Morphological variation and the retractive mechanism. *American Journal of Botany* 80: 1427–1436.

Nyman, Y. 1993b. The pollen-collecting hairs of *Campanula* (Campanulaceae). II. Function and adaptive significance in relation to pollination. *American Journal of Botany* 80: 1437–1443.

Paradis, E., Claude, J., Stimmer, K. 2004. APE: analyses pf phylogenetics and evolution in R language. *Bioinformatics* 20: 289-290.

Robertson, A., Diaz, A., Macnair, M. 1994. The quantitative genetics of floral characters in *Mimulus guttatus*. *Heredity* 72: 300–311.

Roquet, C., Sáez, L., Aldasoro, J., Susanna, A., Alarcón, M., Garcia-Jacas, N. 2008. Natural delineation, Molecular Phylogeny and Floral Evolution in Campanula. *Systematic Botany* 33: 203-217.

Smith, S.D., Kriebel, R., Convergent evolution of floral shape tied to pollinator shifts in I*ochrominae* (Solanaceae). *Evolution* 72: 688-697.

Stewart, A.B., Dudash, M.R. 2017. Field evidence of strong differential pollen placement by Old World bat-pollinated plants, *Annals of Botany* 119: 73–79

Sun, J.-F., Gong, Y.-B., Renner, S. S., Huang, S.-Q. 2008. Multifunctional bracts in the dove tree *Davidia involucrata* (Nyssaceae: Cornales): Rain protection and pollinator attraction. *American Naturalist* 171: 119–124.

Vaknin, Y., Eisikowitch, D., Mishal, A. 2021. Floral and pollen traits of *Moringa oleifera* Lam. and *Moringa peregrina* (Forssk.) Fiori provide reproductive adaptations for arid conditions. *Agronomy* 11: 1090.

Weigel, D., Meyerowitz, E.M. 1994. The ABCs of floral homeotic genes. *Cell* 78: 203-209.

Westerkamp, C., Weber, A. 1997. Secondary and tertiary pollen presentation in *Polygala myrtifolia* and allies (Polygalaceae, South Africa). *South African Journal of Botany* 63: 254–258.

Wilson, L. A. B., Sánchez-Villagra, M. R. 2010. Diversity trends and their ontogenetic basis: an exploration of allometric disparity in rodents. *Proceedings of the Royal Society* B 277: 1227–1234

Yeo, P. F. 1993. *Secondary pollen presentation: form, function, and evolution* (1st ed.). Springer, Vienna.

Zhang, C., Yang, Y.-P., Duan, Y.-W. 2014. Pollen sensitivity to ultraviolet B (UV-B) suggests floral structure evolution in alpine plants. *Scientific Reports* 4: 4520.

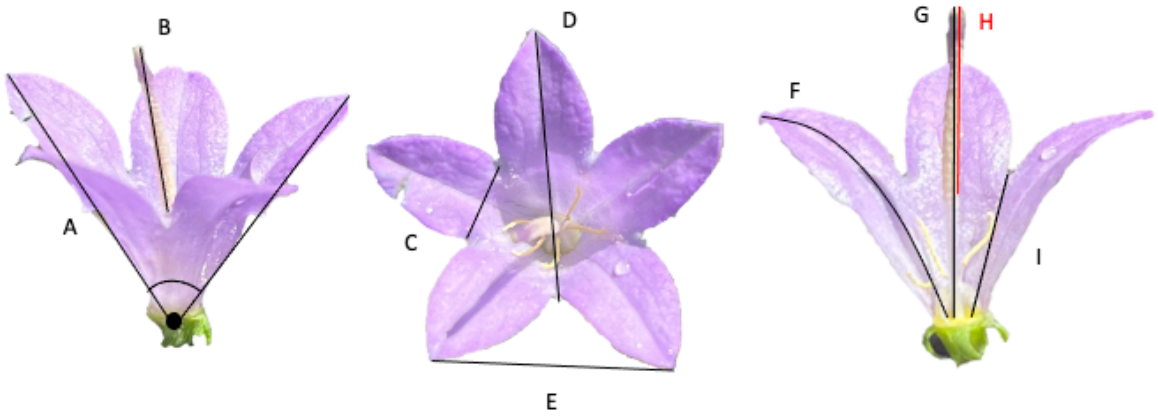


Figure 1: Floral and style measurements taken on Campanulaceae flowers: (A) angle of openness, (B) length of exposed style, (C) width of petal at the split, (D) diameter of the corolla, (E) distance between tips of petals, (F) length of full petal, (G) length of style, (H) length of style with pollen collecting hairs, (I) length of petal from base to split. Example flower is *Adenophora khasiana.*

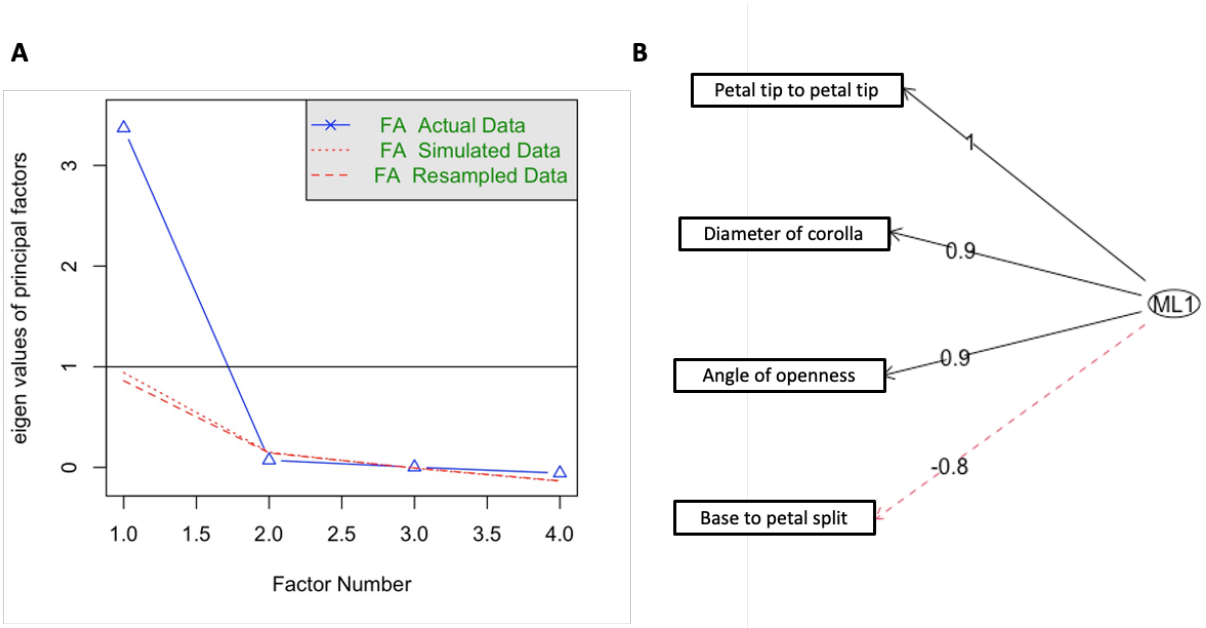


Figure 2. Creating a single measure of floral openness from floral traits measured on species from Campanulaceae. (A) A parallel analysis scree plot used to determine the number of factors represented in the data. The optimal factor number is when the actual data is above the simulated data and above an eigen value of 1. Here one factor represents the data well. (B) A diagram of the loadings of floral traits included in the single floral factor. Values range from -1 to 1. The sign indicates the direction of the relationship while the absolute value indicates the strength of the relationship. Stronger relationships are closer to one. The red line indicates a negative relationship and the dotted line indicates the floral trait is marginally within the factor.

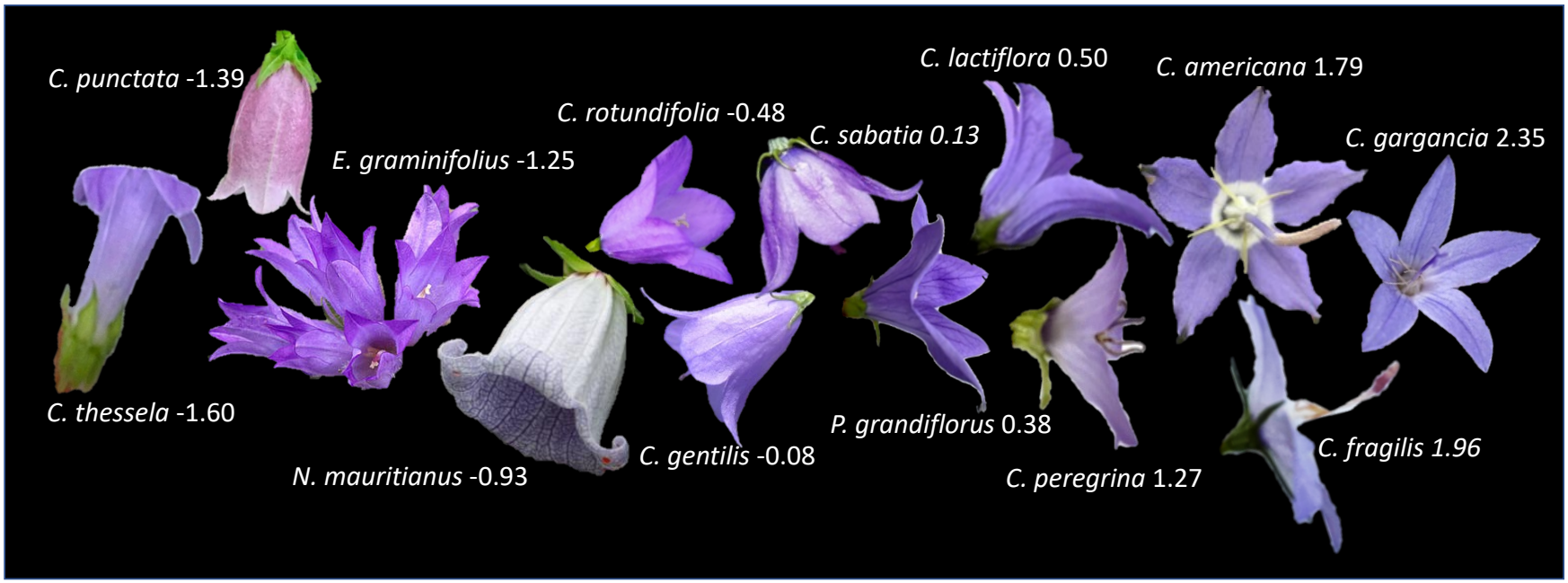


Figure 3. Floral shape variation across Campanulaceae. The floral shape factor combines the petal tip to tip, diameter, angle of openness, and petal base to split distance to describe the floral openness. Flowers range from bell shaped (-1.60) to open (2.35). Images are not to scale, rather are shown to illustrate shape.

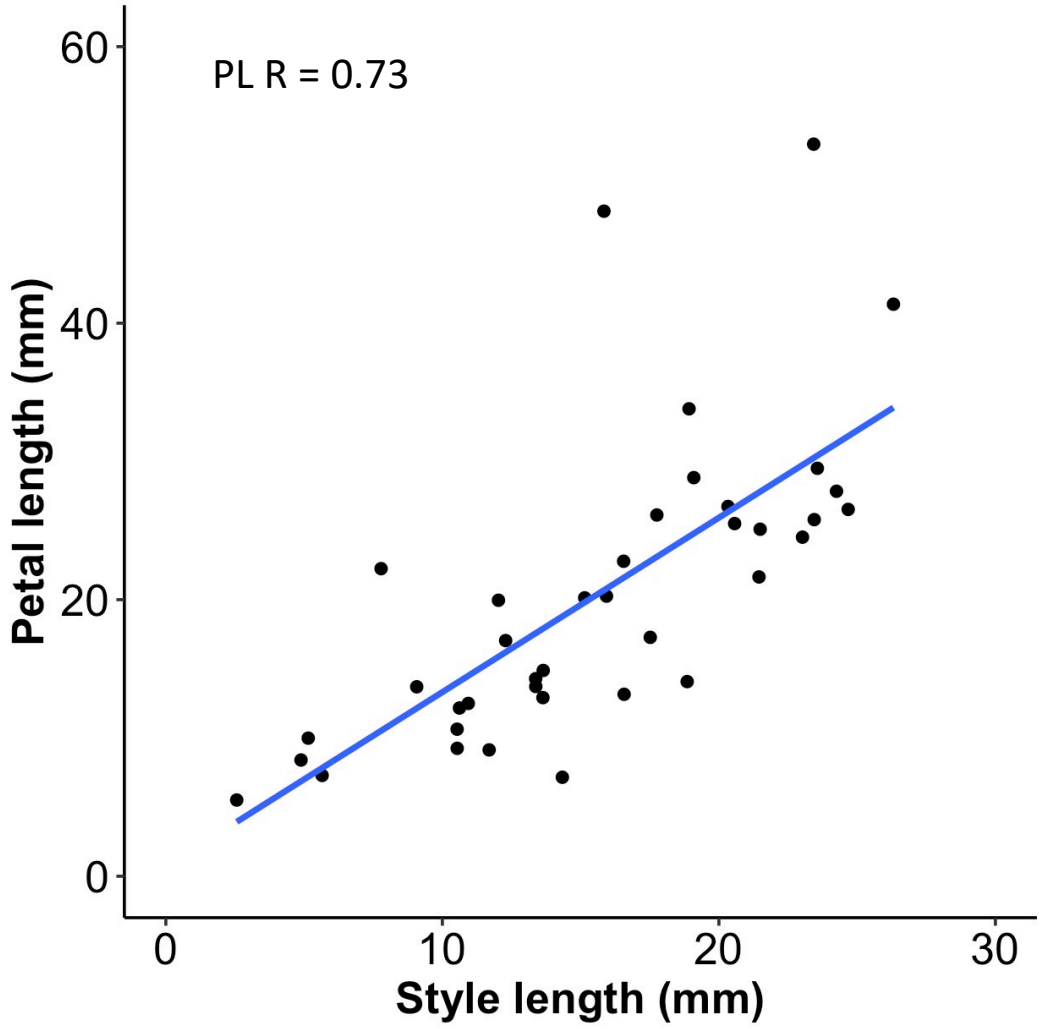


Figure 4. The relationship between style length and petal length across 39 species from Campanulaceae. R value is from Pagel's lambda (PL), the best fit model by AIC.

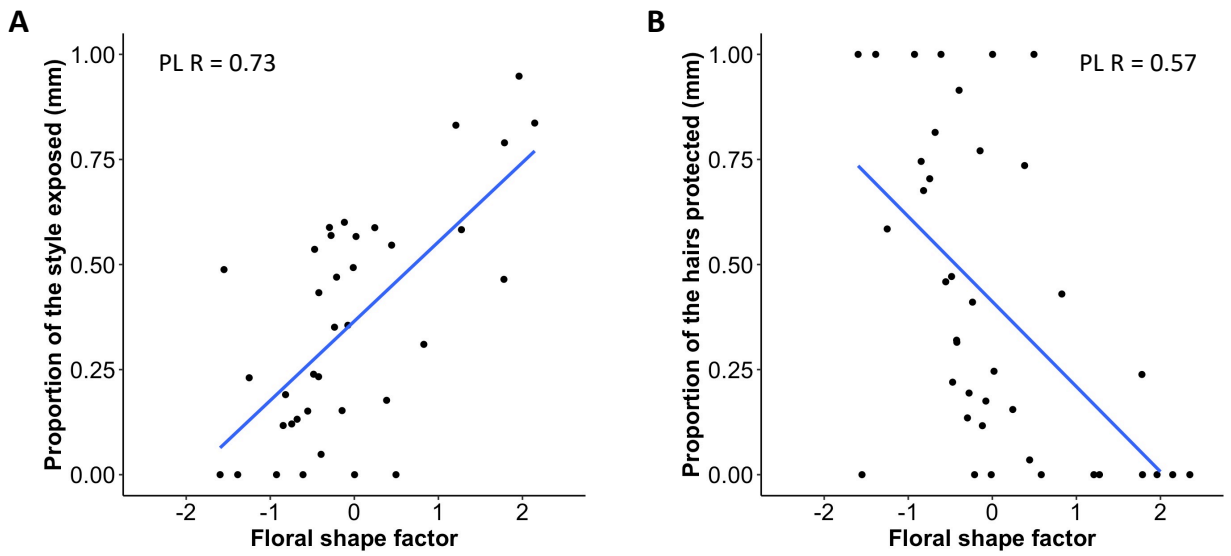


Figure 5. The relationship between floral openness (shape factor) and (A) the proportion of the style that is exposed and (B) the proportion of the pollen-collecting hairs that are protected. Floral openness increases across the x-axis, with more bell-shaped flowers on the left and more open flowers on the right. R values are from the best fit model by AIC, Pagels lambda (PL).

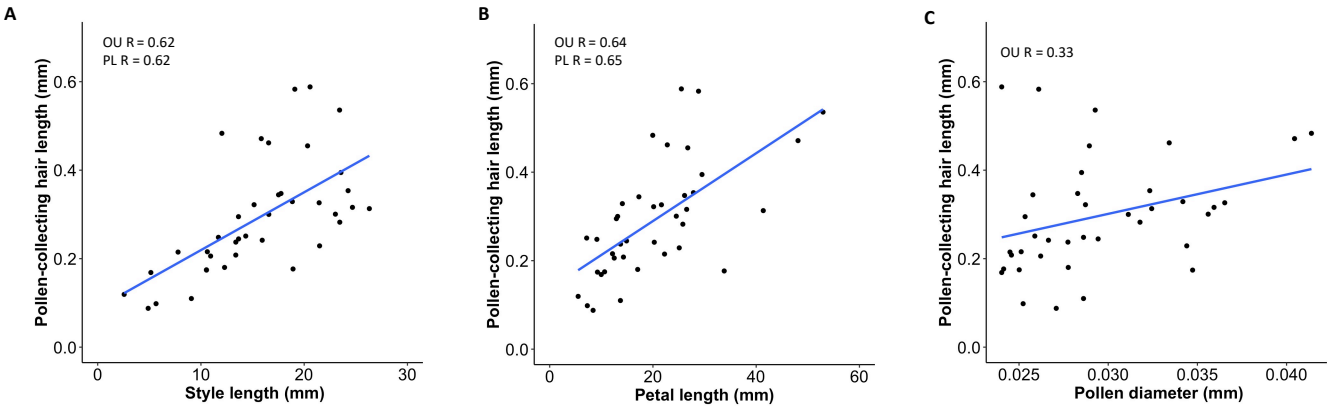


Figure 6. The relationship between (A) style length, (B) petal length, (C) pollen size, and pollen-collecting hair length across 39 species from Campanulaceae. R values are from the best fit model(s) by AIC, Pagals Lambda (PL) and Orenstein-Uhlenbeck (OU).

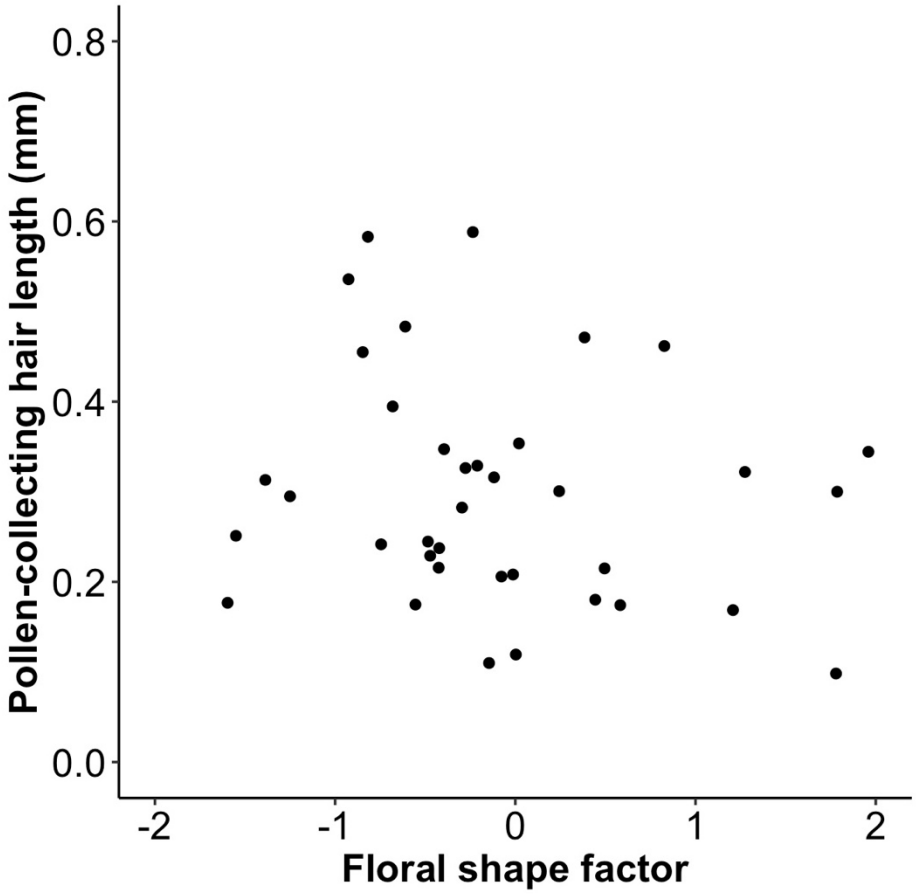


Figure 7. There is no relationship between floral shape factor and length of pollen-collecting hairs across 39 species of Campanulaceae. As floral shape factor increases, the flower gets less bell-shaped and the pollen is more exposed.

Table 1. Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy for floral traits measured on multiple species of Campanulaceae. Values under 0.5 were removed from the factor creation.

Model	Angle	Petal length (mm)	Diameter (mm)	Base to split (mm)	Petal width (mm)	Tip to tip (mm)
all floral traits	0.81	0.47	0.71	0.69	0.38	0.8
removing under 0.5	0.90	removed	0.76	0.81	removed	0.72

Table 2. Floral traits that are correlated across Campanulaceae species after accounting for phylogenetic relationships. The best fit model by AIC for each correlation is shown. Four evolutionary models were tested, and the best fit was either Pagel's lambda (PL) or a tie between Pagel's lambda and Orenstein-Uhlenbeck (OU). UC stands for uncorrected value; see Methods. Full models output listed out in Supplementary Table 2.

Response variable	Independent variable	Best model by AIC	Estimate	P value	R
Petal length (UC)	Style length (UC)	**PL**	1.3	**<0.001**	0.73
Pollen-collecting hair length (UC)	Style length (UC)	**OU**	0.01	**<0.001**	0.62
		PL	0.01	**<0.001**	0.62
Pollen-collecting hair length (UC)	Petal length (UC)	**OU**	0.01	**<0.001**	0.64
		PL	0.01	**<0.001**	0.65
Proportion of the style exposed	Floral shape factor	**PL**	0.26	**<0.001**	0.73
Proportion of the hairs protected	Floral shape factor	**PL**	-0.24	**<0.001**	0.57
Pollen-collecting hair length (UC)	Pollen diameter	**OU**	9.5	**0.04**	0.33
		PL	7.9	0.09	0.28

Table 3. Moran's I and associated p-values for floral shape, style, hair, and pollen traits across 39 Campanulaceae species. Significant values are bolded and indicate a non-random association of traits.

Trait	Moran's I	P value
Pollen-collecting hair length	0.20	**0.02**
Petal length	0.15	**0.03**
Style length	0.21	**0.01**
Floral shape factor	0.17	**0.03**
Proportion of the style exposed	0.14	**0.04**
Proportion of the style with hair	-0.01	0.41
Proportion of the hairs protected	0.20	**0.01**
Pollen diameter	0.08	0.11

Table S1. Genbank ascension information for Campanulaceae species included in analysis of floral traits and pollen-collecting hair traits. Asterisk designates species not included in pollen size analysis.

Genbank ascension	Species
KU983956.1	*Adenophora khasiana*
JX915239.1	*Adenophora liliifolia*
MK556281.1	*Adenophora potaninii*
MK556279.1	*Adenophora remotiflora*
FN396984.1	*Campanula aff. Linifolia*
JX915225.1	*Campanula alliariifolia*
JX915133.1	*Campanula americana*
JX915199.1	*Campanula barbata*
JX914799.1	*Campanula bononiensis*
JX915231.1	*Campanula carpatica*
JX915161.1	*Campanula cochleariifolia*
JX915117.1	*Campanula fragilis*
JX915171.1	*Campanula garganica*
LT674563.1	*Campanula gentilis*
JX915018.1	*Campanula komarovii*
LR701324.1	*Campanula lactiflora*
FN397023.1	*Campanula makaschvilii*
JX914974.1	*Campanula patula*
JX915125.1	*Campanula peregrina*
JX914784.1	*Campanula poscharskyana*
JX915031.1	*Campanula punctata*
JX915233.1	*Campanula rapunculoides*
JX915165.1	*Campanula rotundifolia*
JX915130.1	*Campanula sarmatica*
JX914820.1	*Campanula saxifragoides*
JX914825.1	*Campanula takhtadzhianii*
JX914689.1	*Campanula thessala*
JX914678.1	*Campanula trachelium*
JX914759.1	*Campanula xylocarpa*
MK556217.1	*Codonopsis clematidea*
JX915215.1	*Edraianthus graminifolius*
JX915141.1	*Githopsis diffusa**
FN397069.1	*Jasione laevis*
MK556223.1	*Legousia hybrida*
FN397079.1	*Nesocodon mauritianus*
KF028808.1	*Platycodon grandiflorus*
JX914943.1	*Trachelium caeruleum*
JX915146.1	*Triodanis leptocarpa*
JX915147.1	*Triodanis perfoliata*

Table S2. Phylogenetically corrected association of quantitative floral traits across Campanulaceae species using phylogenetic linear regression. Models tested: Brownian motion (BM), Orenstein-Uhlenbeck (OU), Early Burst unrooted (EB), and Pagels Lambda (PL). Best model was chosen by AIC, bolded in Model column, and highlighted in green in AIC. Significant associations ($p < 0.05$) are bolded.

Response variable	Independent variable	Model	Estimate	p value	R-squared	AIC	Interpretation
Petal length (UC)	Style length (UC)	BM	1.3	**<0.001**	0.63	273.9	Petal length increases with stylar length
		OU	1.3	**<0.001**	0.56	271.3	
		EB	1.3	**<0.001**	0.63	275.9	
		PL	1.3	**<0.001**	0.53	263.4	
Petal length (UC)	Floral shape factor	BM	0.02	0.99	0	312.5	NA
		OU	-4.06	**0.02**	0.14	297.9	
		EB	0.02	0.99	0	314.5	
		PL	-1.6	0.38	0.02	291.9	
Style length	Floral shape factor	BM	2.05	0.08	0.08	268.6	NA
		OU	-2.45	**0.01**	0.16	251.8	
		EB	2.05	0.08	0.08	270.6	
		PL	-0.14	0.89	0	246.5	
Pollen-collecting hair length (UC)	Style length (UC)	BM	0.01	**0.001**	0.18	-16.98	Pollen-collecting hair length increases with stylar length
		OU	0.01	**<0.001**	0.38	-60.63	
		EB	0.01	**0.001**	0.16	-14.98	
		PL	0.01	**<0.001**	0.38	-60.97	
Pollen-collecting hair length (UC)	Petal length (UC)	BM	0	0.15	0.06	-11.69	Pollen-collecting hair length increases with petal length
		OU	0.01	**<0.001**	0.413	-63.05	
		EB	0.01	0.14	0.06	-9.69	
		PL	0.01	**<0.001**	0.42	-63.08	
PCH length residuals (from stylar regression)	Petal length (UC)	BM	0	0.13	0.06	-9.86	NA
		OU	0	0.88	0	-59.68	
		EB	0	0.13	0.06	-7.86	
		PL	0	0.92	0	-60.98	
PCH length residuals (from stylar regression)	Floral shape factor	BM	0.08	**0.01**	0.15	-13.92	NA
		OU	0.02	0.27	0.03	-60.94	
		EB	0.08	**0.01**	0.15	-11.92	
		PL	0.02	0.31	0.03	-62.06	
Pollen-collecting hair length (UC)	Floral shape factor	BM	0.09	**0.001**	0.25	-20.95	NA
		OU	-0.03	0.16	0.05	-44.37	
		EB	0.1	**0.001**	0.25	-18.95	
		PL	0.003	0.91	0	-46.02	
Proportion of the style with hair	Floral shape factor	BM	0.09	**0.04**	0.1	13.91	NA
		OU	0.01	0.78	0	-19.07	
		EB	0.09	**0.04**	0.1	15.91	
		PL	0.01	0.86	0	-18.89	

Proportion of the style exposed	Floral shape factor	BM	0.24	**<0.001**	0.55	-1.46	As the flower gets less bell shaped, the style is more exposed
		OU	0.23	**<0.001**	0.5	-5.48	
		EB	0.24	**<0.001**	0.55	0.55	
		PL	0.26	**<0.001**	0.54	-7.991	
Proportion of the hairs protected	Floral shape factor	BM	-0.27	**<0.001**	0.41	29.68	As the flower gets less belll shaped, more of the hairs are exposed
		OU	-0.23	**<0.001**	0.32	23.52	
		EB	-0.27	**<0.001**	0.41	31.68	
		PL	-0.24	**<0.001**	0.33	21.21	
PCH length residuals (from stylar regression)	Proportion of the style with hair	BM	-0.01	0.95	0	-7.44	NA
		OU	0.07	0.5	0.01	-60.15	
		EB	-0.01	0.95	0	-5.44	
		PL	0.07	0.47	0.01	-61.52	
Pollen-collecting hair length (UC)	Proportion of the style with hair	BM	0.22	**0.05**	0.1	-13.57	NA
		OU	0.13	0.25	0.03	-43.66	
		EB	0.22	**0.05**	0.1	-11.57	
		PL	0.11	0.33	0.03	-47.03	
Pollen-collecting hair length (UC)	Proportion of the style exposed	BM	0.17	0.08	0.08	-12.7	NA
		OU	0	0.15	0.06	-44.53	
		EB	0.17	0.08	0.08	-10.69	
		PL	-0.04	0.57	0.01	-46.28	
PCH length residuals (from stylar regression)	Proportion of the style exposed	BM	0.1	0.34	0.02	-8.4	NA
		OU	0.07	0.17	0.05	-61.62	
		EB	0.09	0.34	0.02	-6.4	
		PL	0.07	0.18	0.05	-62.86	
Pollen-collecting hair length (UC)	Proportion of the hairs protected	BM	-0.14	**0.05**	0.09	-13.43	NA
		OU	0.09	0.1	0.07	-45.13	
		EB	-0.14	**0.05**	0.09	-11.43	
		PL	0.05	0.4	0.02	-46.62	
PCH length residuals (from stylar regression)	Proportion of the hairs protected	BM	-0.12	0.12	0.06	-9.98	NA
		OU	-0.08	0.09	0.07	-62.67	
		EB	-0.12	0.12	0.02	-7.98	
		PL	-0.07	0.1	0.07	-63.97	
Pollen-collecting hair length (UC)	Pollen diameter	BM	-0.58	0.92	0	-5.19	Hair length increases with pollen diameter, OU and PL models equivalent, significant in OU and marginal in PL
		OU	9.5	**0.04**	0.11	-46.5	
		EB	-0.57	0.92	0	-3.19	
		PL	7.9	0.09	0.08	-47.22	

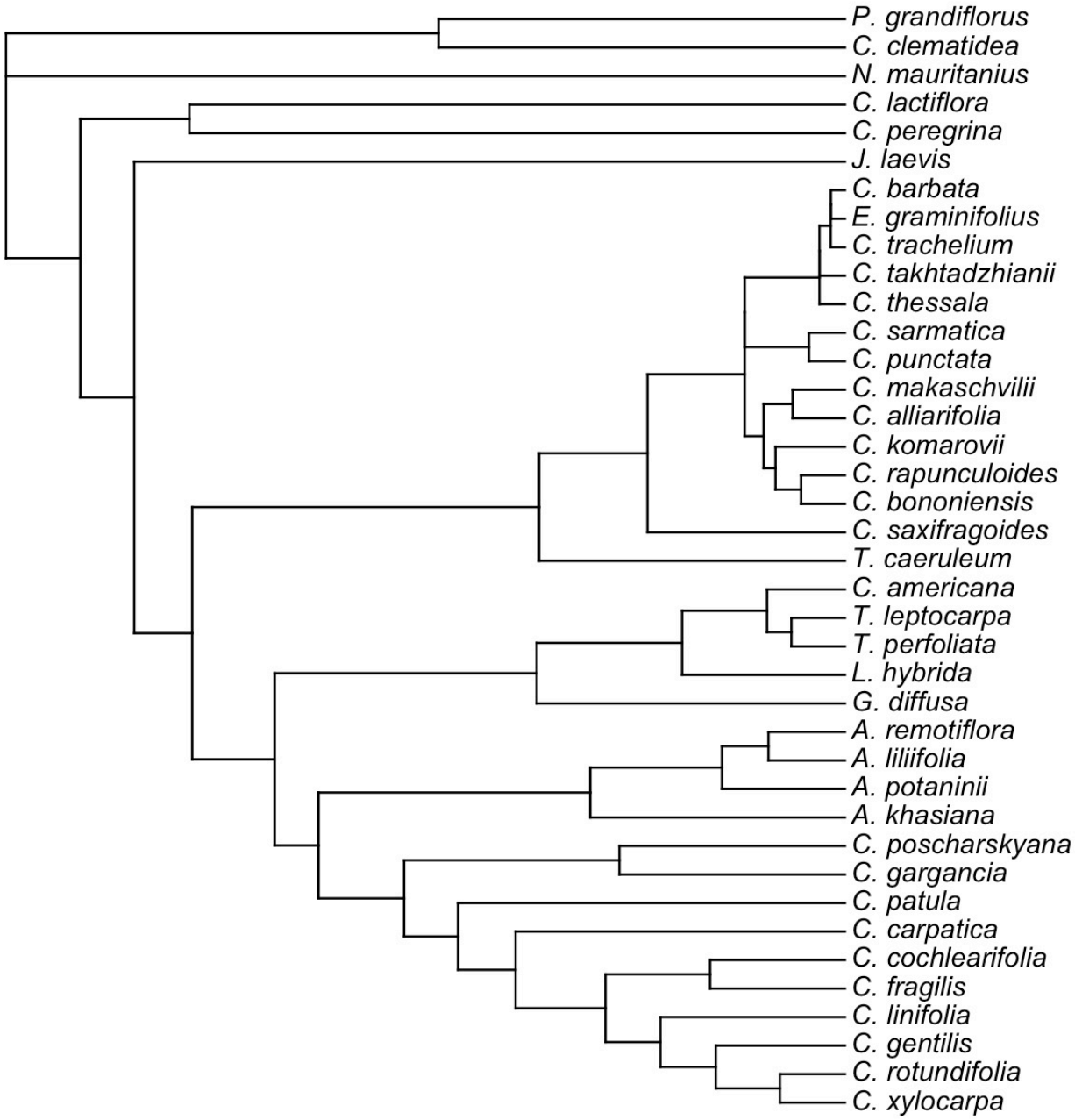


Figure S1. An ultrametric phylogenetic tree of 39 species from the Campanulaceae family using the PET-D gene.