DROUGHT RESPONSE, HYBRIDIZATION, AND ECOLOGICAL ISOLATION IN SYMPATRIC *MIMULUS* SPECIES

by

SAMUEL J. MANTEL

(Under the direction of Andrea L. Sweigart)

ABSTRACT

Understanding how reproductive isolation is maintained in diverged species is integral to understanding species preservation and the evolutionary dynamics of natural sympatric systems. Are partially reproductively isolated species which hybridize at some level destined to merge into a single species, or can isolation be maintained despite the homogenizing effects of geneflow? Ecological isolation, based in divergent selection to variable ecological conditions in partially isolated taxa, has been implicated as an important factor maintaining species boundaries in the face of hybridization. Here we aim to understand the phenotypic and genetic basis of important divergent drought response traits in sympatric sister species, *Mimulus guttatus* and *M. nasutus*, which may play and important role in maintaining species boundaries when the species exist in sympatry. Under water-limited conditions we have identified substantial differentiation in survival and seedset between sympatric *M. guttatus* and *M. nasutus* in the greenhouse. Consistent with observed microhabitat differences in the field, *M. guttatus* was almost completely unable to produce seeds under drought conditions. While *M. nasutus* was able to decrease flower and seed maturation time under drought, *M. guttatus* was unable to do so. This difference may be indicative of differential evolution of plasticity to a water limiting environment in *Mimulus,* which may contribute to immigrant inviability and maladaptation in hybrids. In a custom-made mapping population of admixed individuals made from a *M. nasutus* and three *M. guttatus* parental lines, we

investigate the phenotypic and genetic basis of this divergent drought response in sympatric *Mimulus* which holds considerable promise for identifying genomic variation responsible for reproductive isolation in this system.

INDEX WORDS: drought, sympatry, speciation, ecological isolation, QTL, hybridization

DROUGHT RESPONSE, HYBRIDIZATION, AND ECOLOGICAL ISOLATION IN SYMPATRIC *MIMULUS* SPECIES

by

SAMUEL J. MANTEL

BS, University of Wisconsin – Green Bay, 2016

A Dissertation Submitted to Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2022

DROUGHT RESPONSE, HYBRIDIZATION, AND ECOLOGICAL ISOLATION IN SYMPATRIC *MIMULUS* SPECIES

by

SAMUEL J. MANTEL

Major Professor:	Andrea L. Sweigart
Committee:	Jill T. Anderson
	Lisa A. Donovan
	Robert J. Schmitz
	Michael A. White

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
May 2022

ACKNOWLEDGEMENTS

Over my time at the University of Georgia I have been incredibly fortunate to have the support, guidance, and help of many wonderful people. Firstly, I want to thank my advisor Andrea Sweigart, who's patience, unwavering support, enthusiasm for science, and positivity made it possible for me to complete this work. Andrea, I thank you for teaching me to be an effective scientist, for the engaging and stimulating environment you create in the lab, and for your ability to leave me feeling optimistic and capable no matter my current struggles. I am incredibly lucky to have you as a mentor and friend.

I am also grateful to my committee, Jill Anderson, Lisa Donovan, Bob Schmitz, and Mike White, who have helped me immensely to develop projects, offered invaluable feedback, and committed their time and effort to making my project the best it could be.

I also thank all present and former Sweigart Lab members who I had the pleasure of working with, Rachel Kerwin, Matt Zuellig, Taylor Harrel, Gabbie Sandstedt, Makenzie Whitener, Matt Farnitano, Lucy Bell, and Alex Sotola. You have not only been amazing collogues, but great friends.

This work could not have been completed without the help of many fabulous undergraduate researchers, Aaiyah Stoudemire, Violet Iyahne, Suni Thakur, Brennen Anderson, Selma Kajtazovic, Andy Austin, Irina Matuzava, Sydney Rowell, Nolan Stubbs, Duncan Lindsey, Kristen Reynolds, Drea Sotelo, Laurel Fore, and Michaela Yarbough, who collected and analyzed data with me. Thank you as well to the botany greenhouse staff, Greg Cousins, Kevin Tanner, Noah Hentherinton, and Mike Boyd who kept my plants alive and helped immensely with experiment logistics.

I want to thank the friends I've made at UGA particularly Gabbie Sandstedt, Matt Zuellig, Inam Jameel, Paul Ginsburg, Nick Arthur, and Nick Batora who have supported me and made graduate school an amazing experience. And finally, I thank my parents, who's hard work and support is the reason I've accomplished anything. I love you.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Understanding the forces responsible for the origin and maintenance of biodiversity is a fundamental goal of evolutionary biology. The species, the unit by which such biodiversity is identified and measured, is commonly understood, yet no definition appears adequate in all situations and scenarios. One of the most frequently used definitions, the biological species concept, indicates that speciation has occurred when previously interfertile populations become genetically isolated through the evolution of reproductive barriers (Dobzhansky, 1937; Mayr, 1942). The most straightforward, and most commonly invoked model of the speciation process, is that of allopatric speciation, in which a single, panmictic population is broken up into subpopulations by a physical barrier, preventing gene flow between them. This separation, in physically preventing reproduction between subpopulations, allows for the accumulation of genetic differences (Jordan, 1905; Dobzhansky, 1937, 1940; Mayr, 1942, 1970), through stochastic processes, such as genetic drift acting on standing variation or new mutation, as well as through more directed processes, such as local adaptation to divergent ecological conditions. As a by-product of this divergence, reproductive isolation arises between emerging species via a variety of potential mechanisms, but this isolation is of no consequence unless/until the physical barriers separating populations are removed, allowing for secondary contact. Reproductive barriers are frequently broken down into two categories, based on when they act to prevent species homogenization in sympatry. First, prezygotic barriers prevent the successful formation of hybrid zygotes, either by precluding mating (e.g., differences in mating system, reproductive timing, behavior, etc.), or by impeding fertilization (e.g., gamete killing, gametic incompatibility, etc.). Second, postzygotic barriers cause decreased fitness in any hybrid individuals produced, acting extrinsically if hybrids are maladapted to parental habitats, or intrinsically if hybrids are in some way inviable and/or sterile (Coyne & Orr, 2004). These distinct

categories are by no means mutually exclusive however, with multiple reproductive barriers frequently contributing to reproductive isolation between diverging populations (Coyne & Orr, 2004).

To fully understand the speciation process and its effects on biodiversity, it is necessary to identify the traits and genetic loci underlying reproductive barriers, and investigate the mechanisms driving their evolution in a sympatric scenario. Ultimately, following secondary contact, one of two outcomes will occur. Populations which are sufficiently genetically similar, with few reproductive barriers separating them, may fuse back into a single interbreeding population. Alternatively, if sufficient genetic differentiation has accumulated, complete reproductive isolation will allow species boundaries to be stably maintained in sympatry (Harrison & Larson, 2014). However, newly formed species may arrive in secondary sympatry with an intermediate level of divergence, allowing for hybrids to be formed relatively frequently but preventing complete homogenization, at least in the short term. Indeed, hybridization is widespread across the tree of life (Teeter *et al.*, 2008; Payseur & Rieseberg, 2016; Schumer *et al.*, 2016; Turissini & Matute, 2017) yet hybridizing species remain distinct. As such, the many examples of species pairs that remain genetically and phenotypically divergent despite considerable ongoing gene flow has led to considerable argument against the use of a strict biological species concept. Rather, many species boundaries are most accurately described as semipermeable (Harrison & Larson, 2014). In these systems, certain regions of the genome appear to be exchanged unimpeded between species, while others resist introgression (Barton, 1979; Harrison, 1990), and still others reflect the process of adaptive introgression (Grant & Grant, 2010; Whitney *et al.*, 2010; Pardo-Diaz *et al.*, 2012; Hedrick, 2013; Marques *et al.*, 2019). Additionally, these scenarios are generally occurring against the backdrop of genome-wide selection against hybridization (Orr, 1995; Arnegard *et al.*, 2014; Kenney & Sweigart, 2016; Christie & Strauss, 2018; Calfee *et al.*, 2021), which would seem to conflict with the pervasive nature of hybridization. However, these observations could be better explained by understanding the processes through which populations resist ongoing introgression, or remain stable despite it, as well as the consequences of hybridization on adaptation, speciation, and species maintenance.

Adaptation to divergent ecological conditions can be a major contributor to reproductive isolation (Dillon, 1970; Schluter, 2000; Coyne & Orr, 2004; Rundle & Nosil, 2005; Sobel *et al.*, 2010). Such ecological selection, however, is still a poorly understood source of both prezygotic and postzygotic isolation, with little known about the ecological, genetic, physiological, and molecular processes involved when organisms respond to changing conditions. Furthermore, reciprocal transplant experiments have frequently demonstrated strong local adaptation to variable environmental conditions (Leimu and Fischer, 2008; Hereford, 2009). Plants, being unable to track favorable conditions in real time, are uniquely influenced by environmental conditions, making the understanding of mechanisms of phenotypic plasticity and adaptation in the context of dynamic, shifting environments, a major goal. As in diverse taxa, the locations and interconnectedness of plant populations oftentimes vary dramatically over space and time, meaning ecological isolation may be of special importance in maintaining species fidelity in such lineages.

What factors, then, might we expect to play a role in the permeability of species boundaries in the context of local adaptation? The genetic architecture of adaptive traits, the number of alleles underlying important traits, their density across the genome, their relative effect sizes, under what conditions they are advantageous, and their dominance and epistatic relationships all surely contribute. If multiple traits and genetic loci contribute to local adaptation, ecological isolation may be a potent barrier between species, even with considerable gene flow; however, clusters of linked adaptive alleles or few large effect alleles, may more quickly and easily introgress (Fry, 2003; Thibert-Plante & Hendry, 2009). But, it should also be noted that chromosomal rearrangements can suppress recombination when heterozygous, and facilitate this accumulation of linked loci that contribute to isolation, even in the face of hybridization (Rieseberg, 2001; Noor *et al.*, 2001; Navarro & Barton, 2003).

On top of this, conditionally neutral (CN) alleles, which are favored in one environment, but neutral in another, are predicted to easily spread through hybridizing populations, even when interspecific gene flow is rare (Sambatti *et al.*, 2012). Alleles involved in adaptive introgression, are also, unsurprisingly, expected to spread rapidly though hybridizing populations (Hedrick, 2013; Marques *et al.*,

2019). Antagonistically pleiotropic (AP) alleles, on the other hand, which are favored in one environment but selected against in another, are expected to resist introgression (Nosil *et al.*, 2005). AP loci, that show tradeoffs in alternative habitats, produce extrinsic postzygotic isolation, with maladapted hybrids disfavored due to the combination of native and foreign alleles. With these three potential outcomes, hybridizing populations balance the continued introduction of adaptive or neutral foreign alleles with selection against hybrids carrying incompatibilities or maladapted alleles (Barton & Hewitt, 1985). Beyond the individual fitness effects of introgressing alleles, dominance and epistatic relationships between alleles, including 'new' allele pairs which only exist in hybrid individuals (*i.e.* which did not previously share a genetic background), will also likely be important for the effects of ecological isolation. Entirely dominant loci may allow hybrids to recapitulate the locally adapted phenotype of one of their parent species, but at loci involving mostly additive effects, hybrids will display phenotypes that are intermediate to those of their parent populations (Hermansen *et al.*, 2011) or in multigenic scenarios, express phenotypically mismatched traits which do not integrate well together (Thompson *et al.*, 2021). In such cases, unless environmental conditions intermediate to parental habitats exist, ecological selection will disfavor hybrids. Similarly, if transgressive segregation occurs, where hybrid phenotypes fall outside of the range of their parent taxa, hybrids are likely to be maladapted to parental habitats (Rieseberg *et al.*, 1999).

While expected to be common, environmentally-dependent fitness tradeoffs at a genetic level have seen mixed empirical support. Ascertainment bias and lack of power likely contributes to these disparities, as studies are required to demonstrate positive selection on native alleles across two environments rather than only one to establish AP (Anderson *et al.*, 2011). Regardless, some studies measuring fitness effects of individual loci in divergent natural habitats find evidence for AP loci (Hawthorne & Via, 2001; Barrett *et al.*, 2008; Lowry & Willis, 2010) while others find evidence of only CN loci (Weinig *et al.*, 2003; Verhoeven *et al.*, 2004, 2008; Gardner & Latta, 2006; Lowry *et al.*, 2009) and still others find evidence of both (Hall *et al.*, 2010; Anderson *et al.*, 2013).

Water availability is a crucial factor influencing species distributions in plants, (Cornwell & Grubb, 2003; Engelbrecht *et al.*, 2007), and is therefore frequently associated with local adaptation within and between species and is one of the most important axes of evolutionary divergence in land plants (Hall & Willis, 2006; Lasky *et al.*, 2012, 2014; Lee & Mitchell-Olds, 2013; Kooyers *et al.*, 2015). Despite the potential importance of water availability as an axis of plant divergence, there are few detailed characterizations of drought adaptation between closely related species that grow sympatrically (Eckhart *et al.*, 2004; Geber & Eckhart, 2005; Dunning *et al.*, 2016; Mantel & Sweigart, 2019). Research into the phenotypic and genetic basis of adaptation to water limitation has often shown it to be complex (Passioura, 1996; Bartels & Sunkar, 2005; Maggio *et al.*, 2006; Tardieu, 2012; Kooyers, 2015; Gilbert & Medina, 2016; Volaire, 2018), with plants evolving a range of physiological, developmental, and life history adaptations to overcome it. These traits are generally categorized into three strategies; drought escape, avoidance, and tolerance (Ludlow, 1989; Kooyers, 2015). When employing a drought escape strategy, plants typically grow and develop quickly, allowing them to reproduce before drought-induced senescence (Bazzaz, 1979; Geber & Dawson, 1990; Arntz & Delph, 2001; Volis *et al.*, 2002; Sherrard & Maherali, 2006). In contrast, a drought avoidance strategy involves increasing water-use efficiency (WUE) to prevent drought-induced senescence (Cohen, 1970; Mooney *et al.*, 1976; Geber & Dawson, 1990, 1997; Mckay *et al.*, 2003; Kooyers, 2015). This may involve decreased stomatal conductance, increased leaf succulence, increased root growth, or other similar changes. Finally, a drought tolerance strategy aims to prevent drought-induced senescence despite unfavorable conditions through physiological changes such as osmotic adjustment, or sugar accumulation (Schulze, 1986; Ehleringer, 1993; Dawson, 1993; Kooyers, 2015). Because these strategies involve diverse mechanisms and suites of traits, adaptation to dry soils is often accompanied by dramatic phenotypic changes, which can have important consequences for reproductive isolation between closely related sympatric species.

Drought escape, via a shift in the timing of the initiation of reproduction (flowering time), has been frequently invoked as a key trait for drought escape (Franks, 2007; Berger and Ludwig, 2014; Paccard *et al.*, 2014; Lowry *et al.*, 2014; Brouillette *et al.*, 2014; Kooyers *et al.*, 2015). Because faster

flowering directly allows for more rapid reproduction prior to the onset of drought, flowering time is a major determinant of plant fitness under drought stressed conditions (Franks *et al.*, 2007; Munguía-Rosas *et al.*, 2011; Berger & Ludwig, 2014), and an important divergent trait among many populations and species which can quickly lead to phenological isolation (Martin *et al.*, 2005; Hall & Willis, 2006; Martin & Willis, 2007; Franks & Weis, 2009; Nakazato *et al.*, 2013; Fishman *et al.*, 2014a). In plants, critical photoperiod, or the minimum daylength required for the initiation of flowering, is a key determining factor in a plant's decision to begin reproduction (Kobayashi & Weigel, 2007; Golembeski *et al.*, 2014). However, the initiation of flowering is a complex trait that is highly dependent on climatic conditions, with the decision to flower regulated by an integrated genetic network responding to many environmental cues such as vernalization, temperature, and nutrient levels, as well as photoperiod (Simpson & Dean, 2002; Cho *et al.*, 2017). Although these strategies can, and frequently are, employed simultaneously, drought escape (at the expense of avoidance and tolerance) may be most beneficial in annual plants which have short growing seasons followed by terminal drought (Ludlow, 1989; Ackerly *et al.*, 2000), maximizing fitness by increasing stomatal conductance (low WUE) allowing them to grow, flower, and set seed prior to the onset of substantial soil drying (Cohen, 1970; Mooney *et al.*, 1976; Geber & Dawson, 1990, 1997; Mckay *et al.*, 2003).

Here, we focus on the genetics of ecological isolation between two closely related species of yellow monkeyflower: *Mimulus guttatus* and *M. nasutus* (Phrymaceae), which are ~200-500 kya diverged (Brandvain *et al.*, 2014). Note that taxonomists have reclassified several *Mimulus* species, including those of the yellow clade, as *Erythranthe* (Nesom, 2012, 2014), but because debate still exists on the optimal status of these species (Lowry *et al.*, 2019; Nesom *et al.*, 2019) we refer to them as *Mimulus*. These species, and the other yellow monkeyflowers of the *M. guttatus* species complex, are an ideal system for the study ecological isolation, with abundant genetic and phenotypic diversity among and between populations which are frequently locally adapted to abiotic conditions (Wu *et al.*, 2008).

Natural populations of *M. guttatus,* an outcrosser with large, bee-pollinated flowers, are abundant throughout much of western North America. The distribution of *M. nasutus*. a selfer with reduced, often

cleistogamous flowers, overlaps broadly with that of *M. guttatus*, but its range is more restricted. The two species are reproductively isolated by numerous barriers including floral morphology, flowering phenology, and pollen-pistil interactions (Kiang & Hamrick, 1978; Martin & Willis, 2007; Fishman *et al.*, 2014a; Kenney & Sweigart, 2016), as well as hybrid incompatibilities (Vickery, 1964, 1978; Sweigart *et al.*, 2006; Martin & Willis, 2010; Sweigart & Flagel, 2015; Case *et al.*, 2016; Zuellig & Sweigart, 2018b) Despite these barriers, evidence of substantial unidirectional (from *M. nasutus* into and *M. guttatus* background) introgression is observed in sympatry (Sweigart & Willis, 2003; Brandvain *et al.*, 2014; Kenney & Sweigart, 2016) and contemporary hybrids can be observed (Vickery, 1964; Kenney & Sweigart, 2016). This system of incomplete isolation with persistent hybridization is ideal for investigating the evolutionary forces that drive the evolution isolation within populations and the factors that affect their eventual maintenance or degradation.

This work focuses on a sympatric site located at Catherine Creek (CAC) within the Columbia River Gorge in southern Washington, where plants grow on a hillside system of ephemeral seeps and streams fed by spring rain and snowmelt out of the Cascades. Large populations of both *M. guttatus* and *M. nasutus* occur at this site, but relative abundance varies both temporally and spatially, as edaphic conditions, including water availability, are highly heterogeneous across the site. Although the two *Mimulus* species often grow within a meter of each other, they are found in distinct microhabitats. The small-flowered *M. nasutus* begins flowering earlier, and occurs in shallow soils or patches of moss in and around flowing streams that dry up quickly in late-spring. The large-flowered *M. guttatus* grows in microsites that stay wet through spring and into summer (Kiang & Hamrick, 1978; Kenney & Sweigart, 2016). This asynchronous flowering is caused, at least in part, by *M. nasutus'* ability to flower under much shorter day lengths (<12 hours, occurring in early spring) than *M. guttatus* (Friedman & Willis, 2013; Fishman *et al.*, 2014a), which requires 14+ hours of daylight (not occurring until summer) to flower. Early-season flowering is likely a key drought escape strategy for CAC *M. nasutus*, allowing it to complete reproduction before transient water sources are depleted.

Previous studies have shown that allopatric populations of *M. guttatus* and *M. nasutus*, are divergent in a variety of drought response related traits (Kiang & Hamrick, 1978; Wu *et al.*, 2010; Ivey & Carr, 2012; Kooyers *et al.*, 2015), however, little is known about variation in these phenotypes within and between sympatric populations. This issue is of fundamental importance for understanding species maintenance: if multiple traits with complex genetic architecture are necessary confer drought adaptation, ecological isolation might be a potent barrier between species, even amid considerable gene flow.

Here we investigate the phenotypic and genetic basis of divergence in drought response traits between sympatric *M. guttatus* and *M. nasutus* at CAC, seeking to understand how local adaptation to the unique moisture conditions of their microsites interacts with ongoing hybridization and introgression between the species and the long-term effects these factors are likely to have on species boundaries. Our investigation provides an in-depth characterization of ecological isolation between these species, adding to our understanding of both species' maintenance as well as the speciation process.

References

Ackerly DD, Dudley SA, Sultan SE, Schmitt J, Coleman JS, Linder CR, Sandquist DR, Geber MA, Evans AS, Dawson TE, *et al.* 2000. The Evolution of Plant Ecophysiological Traits: Recent Advances and Future Directions: New research addresses natural selection, genetic constraints, and the adaptive evolution of plant ecophysiological traits. *BioScience* **50**: 979–995.

Anderson JT, LEE C-R, MITCHELL-OLDS T. 2011. LIFE-HISTORY QTLS AND NATURAL SELECTION ON FLOWERING TIME IN BOECHERA STRICTA, A PERENNIAL RELATIVE OF ARABIDOPSIS. *Evolution* **65**: 771–787.

Anderson JT, Lee C-R, Rushworth CA, Colautti RI, Mitchell-Olds T. 2013. Genetic trade-offs and conditional neutrality contribute to local adaptation. *Molecular Ecology* **22**: 699–708.

Arnegard ME, McGee MD, Matthews B, Marchinko KB, Conte GL, Kabir S, Bedford N, Bergek S, Chan YF, Jones FC, *et al.* 2014. Genetics of ecological divergence during speciation. *Nature* **511**: 307–311.

Arntz AM, Delph LF. 2001. Pattern and Process: Evidence for the Evolution of Photosynthetic Traits in Natural Populations. *Oecologia* **127**: 455–467.

Barrett SCH, Colautti RI, Eckert CG. 2008. Plant reproductive systems and evolution during biological invasion. *Molecular Ecology* **17**: 373–383.

Bartels D, Sunkar R. 2005. Drought and Salt Tolerance in Plants. *Critical Reviews in Plant Sciences* **24**: 23–58.

Barton NH. 1979. The dynamics of hybrid zones. *Heredity* **43**: 341–359.

Barton NH, Hewitt GM. 1985. Analysis of Hybrid Zones. *Annual Review of Ecology and Systematics* **16**: 113–148.

Bazzaz FA. 1979. The Physiological Ecology of Plant Succession. *Annual Review of Ecology and Systematics* **10**: 351–371.

Berger JD, Ludwig C. 2014. Contrasting adaptive strategies to terminal drought-stress gradients in Mediterranean legumes: phenology, productivity, and water relations in wild and domesticated Lupinus luteus L. *Journal of Experimental Botany* **65**: 6219–6229.

Brandvain Y, Kenney AM, Flagel L, Coop G, Sweigart AL. 2014. Speciation and Introgression between Mimulus nasutus and Mimulus guttatus. *PLOS Genetics* **10**: e1004410.

Calfee E, Gates D, Lorant A, Perkins MT, Coop G, Ross-Ibarra J. 2021. Selective sorting of ancestral introgression in maize and teosinte along an elevational cline. *PLOS Genetics* **17**: e1009810.

Case AL, Finseth FR, Barr CM, Fishman L. 2016. Selfish evolution of cytonuclear hybrid incompatibility in Mimulus. *Proceedings of the Royal Society B: Biological Sciences* **283**: 20161493.

Cho L-H, Yoon J, An G. 2017. The control of flowering time by environmental factors. *The Plant Journal* **90**: 708–719.

Christie K, Strauss SY. **2018**. Along the speciation continuum: Quantifying intrinsic and extrinsic isolating barriers across five million years of evolutionary divergence in California jewelflowers. *Evolution* **72**: 1063–1079.

Clausen J, Keck DD, Hiesey WM. **1940**. *Experimental studies on the nature of species. 1, Effect of varied environments on Western North American plants ; Jens Clausen, David D. Keck, William M. Hiesey.* Washington, D.C: Carnegie Institution of Washington.

Cohen D. **1970**. The expected efficiency of water utilization in plants under different competition and selection regimes. *Israel Journal of Botany* **19**: 50–4.

Cornwell WK, Grubb PJ. **2003**. Regional and local patterns in plant species richness with respect to resource availability. *Oikos* **100**: 417–428.

Coyne JA, Orr HA. **2004**. *Speciation*. Oxford, New York: Oxford University Press.

Dawson TE. **1993**. Hydraulic lift and water use by plants: implications for water balance, performance and plant-plant interactions. *Oecologia* **95**: 565–574.

Dillon LS. **1970**. Speciation and Changing Environment. *American Zoologist* **10**: 27–39.

Dobzhansky T. 1937. Genetics and the Origin of Species. *Columbia University Press.*

Dobzhansky T. 1940. Speciation as a stage in evolutionary divergence. *The American Naturalist* **74**: 312-321.

Dunning LT, Hipperson H, Baker WJ, Butlin RK, Devaux C, Hutton I, Igea J, Papadopulos AST, Quan X, Smadja CM, *et al.* 2016. Ecological speciation in sympatric palms: 1. Gene expression, selection and pleiotropy. *Journal of Evolutionary Biology* **29**: 1472–1487.

Eckhart VM, Geber MA, McGuire CM. **2004**. EXPERIMENTAL STUDIES OF ADAPTATION IN CLARKIA XANTIANA. I. SOURCES OF TRAIT VARIATION ACROSS A SUBSPECIES BORDER. *Evolution* **58**: 59–70.

Ehleringer JR. **1993**. Variation in leaf carbon isotope discrimination in Encelia farinosa: implications for growth, competition, and drought survival. *Oecologia* **95**: 340–346.

Engelbrecht BMJ, Comita LS, Condit R, Kursar TA, Tyree MT, Turner BL, Hubbell SP. **2007**. Drought sensitivity shapes species distribution patterns in tropical forests. *Nature* **447**: 80–82.

Fishman L, Sweigart AL, Kenney AM, Campbell S. **2014**. Major quantitative trait loci control divergence in critical photoperiod for flowering between selfing and outcrossing species of monkeyflower (Mimulus). *The New Phytologist* **201**: 1498–1507.

Franks SJ, Sim S, Weis AE. **2007**. Rapid Evolution of Flowering Time by an Annual Plant in Response to a Climate Fluctuation. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 1278–1282.

Franks SJ, Weis AE. **2009**. Climate change alters reproductive isolation and potential gene flow in an annual plant. *Evolutionary Applications* **2**: 481–488.

Friedman J, Willis JH. **2013**. Major QTLs for critical photoperiod and vernalization underlie extensive variation in flowering in the Mimulus guttatus species complex. *The New Phytologist* **199**: 571–583.

Fry JD. **2003**. MULTILOCUS MODELS OF SYMPATRIC SPECIATION: BUSH VERSUS RICE VERSUS FELSENSTEIN. *Evolution* **57**: 1735–1746.

Gardner KM, Latta RG. **2006**. Identifying loci under selection across contrasting environments in Avena barbata using quantitative trait locus mapping. *Molecular Ecology* **15**: 1321–1333.

Geber MA, Dawson TE. **1990**. Genetic Variation in and Covariation between Leaf Gas Exchange, Morphology, and Development in Polygonum arenastrum, an Annual Plant. *Oecologia* **85**: 153–158.

Geber MA, Dawson TE. **1997**. Genetic Variation in Stomatal and Biochemical Limitations to Photosynthesis in the Annual Plant, Polygonum arenastrum. *Oecologia* **109**: 535–546.

Geber MA, Eckhart VM. **2005**. EXPERIMENTAL STUDIES OF ADAPTATION IN CLARKIA XANTIANA. II. FITNESS VARIATION ACROSS A SUBSPECIES BORDER. *Evolution* **59**: 521–531.

Gilbert ME, Medina V. **2016**. Drought Adaptation Mechanisms Should Guide Experimental Design. *Trends in plant science* **21**: 639–647.

Golembeski GS, Kinmonth-Schultz HA, Song YH, Imaizumi T. **2014**. Photoperiodic flowering regulation in Arabidopsis thaliana. *Advances in botanical research* **72**: 1–28.

Grant PR, Grant BR. **2010**. Conspecific versus heterospecific gene exchange between populations of Darwin's finches. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365**: 1065–1076.

Hall MC, Lowry DB, Willis JH. **2010**. Is local adaptation in Mimulus guttatus caused by trade-offs at individual loci? *Molecular Ecology* **19**: 2739–2753.

Hall MC, Willis JH. **2006**. Divergent Selection on Flowering Time Contributes to Local Adaptation in Mimulus Guttatus Populations. *Evolution* **60**: 2466–2477.

Harrison RG. **1990**. Hybrid zones: windows on evolutionary process. *Oxford Surveys in Evolutionary Biology* **7**: 69–128.

Harrison RG, Larson EL. **2014**. Hybridization, Introgression, and the Nature of Species Boundaries. *Journal of Heredity* **105**: 795–809.

Hawthorne DJ, Via S. **2001**. Genetic linkage of ecological specialization and reproductive isolation in pea aphids. *Nature* **412**: 904–907.

Hedrick PW. **2013**. Adaptive introgression in animals: examples and comparison to new mutation and standing variation as sources of adaptive variation. *Molecular Ecology* **22**: 4606–4618.

Hermansen JS, Sæther SA, Elgvin TO, Borge T, Hjelle E, Sætre G-P. **2011**. Hybrid speciation in sparrows I: phenotypic intermediacy, genetic admixture and barriers to gene flow. *Molecular Ecology* **20**: 3812–3822.

Ivey CT, Carr DE. **2012**. Tests for the joint evolution of mating system and drought escape in Mimulus. *Annals of Botany* **109**: 583–598.

Jordan DS. 1905. The origin of species through isolation. *Science* **22**: 545-562.

Kenney AM, Sweigart AL. **2016**. Reproductive isolation and introgression between sympatric Mimulus species. *Molecular Ecology* **25**: 2499–2517.

Kiang YT, Hamrick JL. **1978**. Reproductive Isolation in the Mimulus guttatus M. nasutus Complex. *The American Midland Naturalist* **100**: 269–276.

Kobayashi Y, Weigel D. **2007**. Move on up, it's time for change—mobile signals controlling photoperiod-dependent flowering. *Genes & Development* **21**: 2371–2384.

Kooyers NJ. **2015**. The evolution of drought escape and avoidance in natural herbaceous populations. *Plant Science* **234**: 155–162.

Kooyers NJ, Greenlee AB, Colicchio JM, Oh M, Blackman BK. **2015**. Replicate altitudinal clines reveal that evolutionary flexibility underlies adaptation to drought stress in annual Mimulus guttatus. *New Phytologist* **206**: 152–165.

Lasky JR, Des Marais DL, Lowry DB, Povolotskaya I, McKay JK, Richards JH, Keitt TH, Juenger TE. **2014**. Natural Variation in Abiotic Stress Responsive Gene Expression and Local Adaptation to Climate in Arabidopsis thaliana. *Molecular Biology and Evolution* **31**: 2283–2296.

Lasky JR, Des Marais DL, McKAY JK, Richards JH, Juenger TE, Keitt TH. **2012**. Characterizing genomic variation of Arabidopsis thaliana: the roles of geography and climate. *Molecular Ecology* **21**: 5512–5529.

Lee C-R, Mitchell-Olds T. **2013**. Complex trait divergence contributes to environmental niche differentiation in ecological speciation of Boechera stricta. *Molecular Ecology* **22**: 2204–2217.

Lowry DB, Hall MC, Salt DE, Willis JH. **2009**. Genetic and Physiological Basis of Adaptive Salt Tolerance Divergence between Coastal and Inland Mimulus guttatus. *The New Phytologist* **183**: 776–788.

Lowry DB, Sobel JM, Angert AL, Ashman T-L, Baker RL, Blackman BK, Brandvain Y, Byers KJRP, Cooley AM, Coughlan JM, *et al.* 2019. The case for the continued use of the genus name Mimulus for all monkeyflowers. *TAXON* **68**: 617–623.

Lowry DB, Willis JH. **2010**. A Widespread Chromosomal Inversion Polymorphism Contributes to a Major Life-History Transition, Local Adaptation, and Reproductive Isolation. *PLOS Biology* **8**: e1000500.

Ludlow MM. **1989**. Strategies of response to water stress.

Maggio A, Zhu J-K, Hasegawa PM, Bressan RA. **2006**. Osmogenetics: Aristotle to Arabidopsis. *The Plant Cell* **18**: 1542–1557.

Mantel SJ, Sweigart AL. **2019**. Divergence in drought-response traits between sympatric species of Mimulus. *Ecology and Evolution* **9**: 10291–10304.

Marques DA, Meier JI, Seehausen O. **2019**. A Combinatorial View on Speciation and Adaptive Radiation. *Trends in Ecology & Evolution* **34**: 531–544.

Martin NH, Bouck AC, Arnold ML. **2005**. Loci affecting long-term hybrid survivorship in Louisiana irises: implications for reproductive isolation and introgression. *Evolution; International Journal of Organic Evolution* **59**: 2116–2124.

Martin NH, Willis JH. **2007**. Ecological Divergence Associated with Mating System Causes Nearly Complete Reproductive Isolation between Sympatric Mimulus Species. *Evolution* **61**: 68–82.

Martin NH, Willis JH. **2010**. Geographical variation in postzygotic isolation and its genetic basis within and between two Mimulus species. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365**: 2469–2478.

Mayr E. 1942. Systematics and the origin of species, from the viewpoint of a zoologist. *Harvard University Press.*

Mayr E. 1970. Populations, species, and evolution: an abridgment of animal species and evolution. *Harvard University Press.*

Mckay JK, Richards JH, Mitchell-Olds T. **2003**. Genetics of drought adaptation in Arabidopsis thaliana: I. Pleiotropy contributes to genetic correlations among ecological traits. *Molecular Ecology* **12**: 1137–1151.

Mooney HA, Ehleringer J, Berry JA. **1976**. High photosynthetic capacity of a winter annual in death valley. *Science (New York, N.Y.)* **194**: 322–324.

Munguía-Rosas MA, Ollerton J, Parra-Tabla V, De-Nova JA. **2011**. Meta-analysis of phenotypic selection on flowering phenology suggests that early flowering plants are favoured. *Ecology Letters* **14**: 511–521.

Nakazato T, Rieseberg LH, Wood TE. **2013**. The genetic basis of speciation in the Giliopsis lineage of Ipomopsis (Polemoniaceae). *Heredity* **111**: 227–237.

Navarro A, Barton NH. **2003**. Accumulating Postzygotic Isolation Genes in Parapatry: A New Twist on Chromosomal Speciation. *Evolution* **57**: 447–459.

Nesom GL. **2012**. TAXONOMY OF ERYTHRANTHE SECT. ERYTHRANTHE (PHRYMACEAE). *Phytoneuron* **40**: 41.

Nesom GL. **2014**. UPDATED CLASSIFICATION AND HYPOTHETICAL PHYLOGENY OF ERYTHRANTHE SECT. SIMIOLA (PHRYMACEAE). *Phytoneuron*: 6.

Nesom GL, Fraga NS, Barker WR, Beardsley PM, Tank DC, Baldwin BG, Olmstead RG. **2019**. Response to "The case for the continued use of the genus name Mimulus for all monkeyflowers". *TAXON* **68**: 624–627.

Noor MAF, Grams KL, Bertucci LA, Reiland J. **2001**. Chromosomal inversions and the reproductive isolation of species. *Proceedings of the National Academy of Sciences* **98**: 12084–12088.

Nosil P, Vines TH, Funk DJ. **2005**. Reproductive Isolation Caused by Natural Selection Against Immigrants from Divergent Habitats. *Evolution* **59**: 705–719.

Orr HA. **1995**. The Population Genetics of Speciation: The Evolution of Hybrid Incompatibilities. *Genetics* **139**: 1805–1813.

Pardo-Diaz C, Salazar C, Baxter SW, Merot C, Figueiredo-Ready W, Joron M, McMillan WO, Jiggins CD. **2012**. Adaptive Introgression across Species Boundaries in Heliconius Butterflies. *PLOS Genetics* **8**: e1002752.

Passioura JB. **1996**. Drought and drought tolerance. *Plant Growth Regulation* **20**: 79–83.

Payseur BA, Rieseberg LH. **2016**. A genomic perspective on hybridization and speciation. *Molecular Ecology* **25**: 2337–2360.

Rieseberg LH. **2001**. Chromosomal rearrangements and speciation. *Trends in Ecology & Evolution* **16**: 351–358.

Rieseberg LH, Archer MA, Wayne RK. **1999**. Transgressive segregation, adaptation and speciation. *Heredity* **83**: 363–372.

Rundle HD, Nosil P. **2005**. Ecological speciation. *Ecology Letters* **8**: 336–352.

Schluter D. **2000**. *The Ecology of Adaptive Radiation*. OUP Oxford.

Schulze ED. **1986**. Carbon Dioxide and Water Vapor Exchange in Response to Drought in the Atmosphere and in the Soil. *Annual Review of Plant Physiology* **37**: 247–274.

Schumer M, Cui R, Powell DL, Rosenthal GG, Andolfatto P. **2016**. Ancient hybridization and genomic stabilization in a swordtail fish. *Molecular Ecology* **25**: 2661–2679.

Sherrard ME, Maherali H. **2006**. The Adaptive Significance of Drought Escape in Avena barbata, an Annual Grass. *Evolution* **60**: 2478–2489.

Simpson GG, Dean C. **2002**. Arabidopsis, the Rosetta Stone of Flowering Time? *Science* **296**: 285–289.

Sobel JM, Chen GF, Watt LR, Schemske DW. **2010**. The Biology of Speciation. *Evolution* **64**: 295–315.

Sweigart AL, Fishman L, Willis JH. **2006**. A Simple Genetic Incompatibility Causes Hybrid Male Sterility in Mimulus. *Genetics* **172**: 2465–2479.

Sweigart AL, Flagel LE. **2015**. Evidence of Natural Selection Acting on a Polymorphic Hybrid Incompatibility Locus in Mimulus. *Genetics* **199**: 543–554.

Sweigart AL, Willis JH. **2003**. Patterns of Nucleotide Diversity in Two Species of Mimulus Are Affected by Mating System and Asymmetric Introgression. *Evolution* **57**: 2490–2506.

Tardieu F. **2012**. Any trait or trait-related allele can confer drought tolerance: just design the right drought scenario. *Journal of Experimental Botany* **63**: 25–31.

Teeter KC, Payseur BA, Harris LW, Bakewell MA, Thibodeau LM, O'Brien JE, Krenz JG, Sans-Fuentes MA, Nachman MW, Tucker PK. **2008**. Genome-wide patterns of gene flow across a house mouse hybrid zone. *Genome Research* **18**: 67–76.

Thibert-Plante X, Hendry AP. **2009**. Five questions on ecological speciation addressed with individual-based simulations. *Journal of Evolutionary Biology* **22**: 109–123.

Thompson KA, Urquhart-Cronish M, Whitney KD, Rieseberg LH, Schluter D. **2021**. Patterns, Predictors, and Consequences of Dominance in Hybrids. *The American Naturalist* **197**: E72–E88.

Turissini DA, Matute DR. **2017**. Fine scale mapping of genomic introgressions within the Drosophila yakuba clade. *PLOS Genetics* **13**: e1006971.

Verhoeven KJF, Poorter H, Nevo E, Biere A. **2008**. Habitat-specific natural selection at a flowering-time QTL is a main driver of local adaptation in two wild barley populations. *Molecular Ecology* **17**: 3416–3424.

Verhoeven KJF, Vanhala TK, Biere A, Nevo E, Damme JMM van. **2004**. The Genetic Basis of Adaptive Population Differentiation: A Quantitative Trait Locus Analysis of Fitness Traits in Two Wild Barley Populations from Contrasting Habitats. *Evolution* **58**: 270–283.

Vickery RK. **1964**. Barriers to Gene Exchange Between Members of the Mimulus guttatus Complex (Scrophulariaceae). *Evolution* **18**: 52–69.

Vickery RK. **1978**. Case Studies in the Evolution of Species Complexes in Mimulus. In: Hecht MK, Steere WC, Wallace B, eds. Evolutionary Biology. Evolutionary Biology. Boston, MA: Springer US, 405–507.

Volaire F. **2018**. A unified framework of plant adaptive strategies to drought: Crossing scales and disciplines. *Global Change Biology* **24**: 2929–2938.

Volis S, MENDLINGER S, WARD D. **2002**. Differentiation in populations of Hordeum spontaneum Koch along a gradient of environmental productivity and predictability: plasticity in response to water and nutrient stress. *Biological Journal of the Linnean Society* **75**: 301–312.

Weinig C, Dorn LA, Kane NC, German ZM, Halldorsdottir SS, Ungerer MC, Toyonaga Y, Mackay TFC, Purugganan MD, Schmitt J. **2003**. Heterogeneous Selection at Specific Loci in Natural Environments in Arabidopsis thaliana. *Genetics* **165**: 321–329.

Whitney KD, Ahern JR, Campbell LG, Albert LP, King MS. **2010**. Patterns of hybridization in plants. *Perspectives in Plant Ecology, Evolution and Systematics* **12**: 175–182.

Wu CA, Lowry DB, Cooley AM, Wright KM, Lee YW, Willis JH. **2008**. Mimulus is an emerging model system for the integration of ecological and genomic studies. *Heredity* **100**: 220–230.

Wu CA, Lowry DB, Nutter LI, Willis JH. **2010**. Natural variation for drought-response traits in the Mimulus guttatus species complex. *Oecologia* **162**: 23–33.

Zuellig MP, Sweigart AL. **2018**. Gene duplicates cause hybrid lethality between sympatric species of Mimulus. *PLOS Genetics* **14**: e1007130.

CHAPTER II

DIVERGENCE IN DROUGHT-RESPONSE TRAITS BETWEEN SYMPATRIC SPECIES OF *MIMULUS*[1]

[1] Mantel S.J. and A.L. Sweigart. 2019. Divergence in drought response traits between sympatric species of *Mimulus*. Ecology & Evolution 9: 10291-10304. Reprinted here with permission.

Abstract

Differential adaptation to local environmental conditions is thought to be an important driver of speciation. Plants, whose sedentary lifestyle necessitates fine-tuned adaptation to edaphic conditions such as water availability, are often distributed based on these conditions. Populations occupying water-limited habitats may employ a variety of strategies, involving numerous phenotypes, to prevent and withstand desiccation. In sympatry, two closely related *Mimulus* species – *M. guttatus* and *M. nasutus* – occupy distinct microhabitats that differ in seasonal water availability. In a common garden experiment, we characterized natural variation within and between sympatric *M. guttatus* and *M. nasutus* in the ability to successfully set seed under well-watered and drought conditions. We also measured key phenotypes for drought adaptation, including developmental timing, plant size, flower size, and stomatal density. Consistent with their microhabitat associations in nature, *M. nasutus* set seed much more successfully than *M. guttatus* under water-limited conditions. This divergence in reproductive output under drought was due to differences in mortality after the onset of flowering, with *M. nasutus* surviving at a much higher rate than *M. guttatus*. Higher seed set in *M. nasutus* was mediated, at least in part, by a plastic increase in the rate of late-stage development (*i.e.*, fruit maturation), consistent with the ability of this species to inhabit more ephemeral habitats in the field. Our results suggest adaptation to water availability may be an important factor in species maintenance of these *Mimulus* taxa in sympatry.

Introduction

Reciprocal transplant experiments in diverse taxa have often shown that organisms are strongly adapted to their local environments (Leimu and Fischer, 2008; Hereford, 2009). Over time, divergent selection among these distinct environments can lead to reproductive isolation (Schluter, 2000; Rundle and Nosil, 2005), either by preventing closely related species from coming together at all (*i.e.*, geographic isolation) or by limiting opportunities for interspecific gene flow if they do. In the latter case, when closely related species occur sympatrically, adaptation to different microhabitats can promote premating isolation if species are spatially or temporally separated, and/or extrinsic postzygotic isolation if hybrid progeny suffer a fitness disadvantage in parental habitats (Coyne and Orr, 2004). Although adaptation to

different habitats is thought to be one of the most important drivers of speciation (Sobel *et al.*, 2010), in most cases, little is known about the ecological factors involved or the particular phenotypes that contribute to divergence.

In plants, water availability is a key determinant of species distributions (Cornwell and Grubb, 2003; Engelbrecht *et al.*, 2007) and heterogeneity in soil moisture is often associated with local adaptation within species (Clausen *et al.*, 1940; Hall and Willis, 2006; Lasky *et al.*, 2012; Lasky *et al.*, 2014; Lee and Mitchell-Olds, 2013; Kooyers *et al.*, 2015). To succeed in water-limited environments, plants have evolved a diverse array of physiological, developmental, and life history adaptations (Bartels and Sunkar, 2005; Maggio *et al.*, 2006; Kooyers, 2015). These traits are often categorized into three strategies – those that enable plants to escape, avoid, or tolerate drought conditions (Ludlow, 1989; Kooyers, 2015). In an escape strategy, plants typically develop rapidly and reproduce prior to drought-induced senescence. In contrast, with avoidance and tolerance strategies, plants prevent drought induced senescence by increasing water-use-efficiency (*e.g.*, via a decrease in stomatal conductance) or though physiological changes (*e.g.*, osmotic adjustment, root growth). Because these strategies involve diverse mechanisms and suites of traits, adaptation to dry soils is often accompanied by dramatic phenotypic changes, which can have important consequences for reproductive isolation between closely related sympatric species. For example, a shift to earlier flowering – a hallmark of drought escape – can lead to phenological isolation (Martin *et al.*, 2005; Franks and Weis, 2009; Fishman *et al.*, 2014). Despite the potential importance of water availability as an axis of plant divergence, there are few detailed characterizations of drought adaptation between closely related species that grow sympatrically (Eckhart *et al.*, 2004; Geber and Eckhart, 2005; Dunning *et al.*, 2016).

Here, we focus on divergence in drought response traits between the yellow monkeyflowers *Mimulus guttatus* and *M. nasutus*. *Mimulus guttatus* is a phenotypically and genetically diverse, primarily outcrossing species that occupies wet soils across western North America (Wu *et al.*, 2008). *Mimulus nasutus* is a highly selfing species that diverged recently (~200KYA) from *M. guttatus* (Brandvain *et al.*, 2014). The two species are largely allopatric, but sympatric populations of *M. nasutus* and annual

ecotypes of *M. guttatus* are not uncommon throughout their shared range. In addition to their divergent mating systems and associated floral traits (Fishman *et al.*, 2002), the two species show clear ecological differentiation, with *M. nasutus* flowering earlier and tending to occupy microhabitats that dry out sooner than *M. guttatus* (Kiang and Hamrick, 1978). This shift to earlier flowering in *M. nasutus* is caused, at least in part, by an ability to flower under much shorter day lengths (<10 hrs) than *M. guttatus*, which often requires at least 14 hours of daylight to initiate reproduction (Friedman and Willis, 2013; Kooyers *et al.*, 2015). When the two species co-occur, divergence in critical photoperiod (Fishman *et al.*, 2014), and in flowering phenology more generally, is a major barrier to interspecific mating (Kiang and Hamrick, 1978; Martin and Willis, 2007; Kenney and Sweigart, 2016). Despite this strong barrier, hybridization between sympatric populations of *M. guttatus* and *M. nasutus* can be substantial (Kenney and Sweigart, 2016) and there is clear evidence of ongoing interspecific introgression (Sweigart and Willis, 2003; Brandvain *et al.*, 2014; Kenney and Sweigart, 2016).

How, then, are these two *Mimulus* species maintained in the face of considerable gene flow? In a previous study (Kenney and Sweigart, 2016), we began to address this question by focusing on populations of *M. guttatus* and *M. nasutus* that have come into secondary contact at Catherine Creek (CAC), a gradually sloping, rocky meadow with streams and seeps that flow down into the Columbia River Gorge (Figure 2.1). Edaphic conditions, including water availability, are highly heterogeneous at this site, and although the two *Mimulus* species often grow within a meter of each other, they are found in somewhat distinct microhabitats: *M. nasutus* occurs in patches of moss in and around flowing streams that dry up in late-spring, whereas *M. guttatus* grows in deeper seeps that stay wet through spring and into summer. The two species also flower asynchronously at the CAC site (Kenney and Sweigart, 2016) due, in part, to divergence at two major genetic loci for critical photoperiod (Fishman *et al.*, 2014). The ability to flower under short days is likely a key drought escape strategy for CAC *M. nasutus*, allowing most individuals to complete reproduction by late May before ephemeral sources of water (snow melt and rain) are depleted. Nevertheless, even with species divergence in critical photoperiod, there is substantial overlap in the flowering phenologies of CAC *M. nasutus* and *M. guttatus*, as well as a large number of

genetically admixed individuals that flower at intermediate times (Kenney and Sweigart, 2016). There is also ongoing introgression at CAC, mostly from *M. nasutus* into *M. guttatus* (Brandvain *et al.*, 2014), including at one of the two mapped critical photoperiod loci (Kenney and Sweigart, 2016).

Given these observations at CAC, a key question is whether introgression from *M. nasutus* might facilitate drought escape in *M. guttatus*. The answer depends to some degree on whether drought adaptation is mediated by a relatively simple shift to earlier flowering (via a decrease in critical photoperiod) or requires a more complex, coordinated set of traits. Do the latest-flowering *M. nasutus* and hybrids have additional mechanisms to deal with the onset of terminal drought? If an *M. guttatus* individual happens to germinate in a dry microsite, does it have any adaptations that might allow it to survive and reproduce? If an *M. guttatus* seedling carries an introgressed, photoperiod-response allele from *M. nasutus*, would it have high fitness in a dry site, or are additional traits needed? Previous work has shown that closely related *Mimulus* taxa, including populations of *M. guttatus* and *M. nasutus*, are differentiated by a variety of drought escape and avoidance traits (Kiang and Hamrick, 1978; Wu *et al.*, 2010; Ivey and Carr, 2012; Kooyers *et al.*, 2015), but little is known about variation in these phenotypes within and between sympatric populations. This issue is of fundamental importance for understanding species maintenance: if multiple traits (and genetic loci) are needed for drought adaptation, microhabitat isolation might be a potent barrier between species, even with considerable gene flow.

In this study, we performed a common garden experiment to investigate the phenotypic basis of microhabitat isolation between sympatric *M. guttatus* and *M. nasutus*. Using inbred lines derived from the sympatric CAC site and other natural populations, we grew plants under water-limited conditions to simulate the onset of summer drought experienced by both *Mimulus* species across their native ranges. Because we were interested in exploring traits related to drought response beyond critical photoperiod, we grew all plants under inductive light conditions (16-hour days). First, we examined the overall impact of drought on growth and fitness within and between species. Next, we dissected the phenotypic basis of dramatic differences in seed set between *M. guttatus* and *M. nasutus* under drought, exploring vegetative and reproductive traits, developmental rates, and survival across the life cycle. Is the larger flowered *M.*

guttatus more vulnerable to desiccation (*e.g.*, Galen *et al.*, 1999; Dudley *et al.*, 2018) due to increased floral input? Do *Mimulus* taxa show variation in leaf traits related to water use efficiency? Do developmental rates vary, allowing for different levels of drought escape/avoidance? Strikingly, we discovered that *M. nasutus* alone is capable of accelerating its developmental rate in response to drought. This developmental shift, which occurs late in the life cycle (*i.e.*, after flowering), has likely played a key role in adaptation within *M. nasutus* and contributed to divergence between species.

Materials and Methods

Plant Lines and Growth Conditions

To characterize natural variation in drought response within and between sympatric *M. guttatus* and *M. nasutus* we generated a collection of inbred lines derived from CAC (Table 2.1). We produced 10 *M. guttatus* lines from wild collected CAC seed through at least four generations of self-fertilization with single seed descent. To minimize maternal effects, we also propagated four CAC *M. nasutus* lines, from already naturally inbred wild collected seed, in the greenhouse for at least two generations. In addition to these lines from CAC, we included another five well-characterized inbred lines (SWB38A, DUN10, IM767, DPRG102, and SF5) that have been involved in previous studies of ecological adaptation including drought response (Wu *et al.*, 2010; Mojica *et al.*, 2012; Friedman *et al.*, 2015, Table 2.1). These previously studied lines include coastal perennial and annual inland ecotypes of *M. guttatus*, which have been recognized as distinct taxonomic groups (Pennell, 1947; Lowry *et al.*, 2008), as well as one population of *M. nasutus*. In total we grew 19 inbred lines: five *M. nasutus* lines (four from CAC), 12 annual *M. guttatus* lines (10 from CAC), and two perennial *M. guttatus* lines.

Seeds were planted into 2.5" pots filled with moist Fafard 3-B potting mix (Sun Gro Horticulture, Agawam, MA, USA), chilled for seven days at 4°C to promote germination, and moved to a Conviron (Winnipeg, Canada) growth chamber with lights set to 16-hour days and temperatures set to 23°C days/16°C nights. For all temporal measurements, we set this day, when pots were moved to the growth chamber, as Day 0. Roughly a week after being moved to the growth chamber, seeds began to germinate,

but exact germination dates were not recorded. Two to three days following germination (Days 8 and 9), we transplanted seedlings into 54 x 28 cm flats (Kord, HC Companies Canada), with holes for drainage, filled with moist Fafard 3-B potting mix and moved them into the UGA greenhouses under 16-hour supplemental light, 23°C days/16°C nights. Midway through the experiment, we discovered that the plants were likely experiencing low levels of 24-hour light from an adjacent greenhouse, which might have contributed to overall faster flowering times (once discovered, we set these adjacent lights to 16-hour days coordinated with our experiment). However, because plants were randomized within their blocks and all plants experienced the same conditions, no systematic bias was introduced. After transplanting, flats were bottom-watered to saturation for five days to allow seedlings to recover from transplant and acclimate within their experimental blocks.

Experimental Design

We grew plants under two distinct watering treatments to examine plant responses to variation in soil moisture conditions. Following transplant on Day 8 or 9 and the five-day establishment period (Days 10-14), we initiated two treatments on Day 15: 1) well-watered, in which we bottom-watered flats daily to maintain soil saturation, and 2) dry-down, in which we simulated the onset of seasonal drought by withholding all additional water and allowing flats to progressively dry from saturation.

To ensure that all plants within each treatment experienced similar levels of soil moisture, we grew plants together in large experimental flats, rather than in individual pots. With this design (modified from Wu *et al.*, 2010), our intention was to minimize variation in soil drying rates due to plant size differences (*i.e.*, larger plants may use water more quickly, reducing soil moisture). In each of the 76 blocks (38 per treatment), nine focal experimental plants were evenly spaced into a 2x5 grid and surrounded by "edge plants" from the IM767 inbred line of *M. guttatus* (9 experimental plants x 38 flats = 342 total plants per treatment; note that one position in each 2x5 grid was left vacant to monitor soil drying rate). With this design, each focal experimental plant was surrounded by eight or (when situated adjacent to the vacant position) seven neighboring plants. Within the two treatments, we randomized the positions of replicates from each of the 19 inbred lines (comprising three groups: *M. nasutus*, annual *M.*

guttatus, and perennial *M. guttatus*) across the 38 blocks (average number of replicates per inbred line in each treatment = 18, range = 9-32). We began experimental treatments on Day 15 when we detected buds on 32 plants (15 in the well-watered and 17 in the dry-down treatment; 72% of these were from the *M. nasutus* line CAC32).

For each experimental flat, we measured dry basis soil moisture content (θ_d) on Days 17, 24, and 32 (Day 0 was when seeds were removed from stratification, watering treatments were initiated on Day 15). To perform this measurement, we took soil samples from the vacant positions and recorded wet soil mass (WM). After drying these soil samples in a 60°C drying oven until their weights were stable (24-48 hrs), we recorded dry soil mass (DM). We then calculated θ_d as (WM – DM)/DM (Figure 2.2). For each flat, we calculated drying rate as the change in θ_d per day between Days 17 and 24. θ_d of flats from the well-watered treatment remained constant or increased over the course of the experiment with a drying rate between 0.010 and 0.305 (mean = 0.164 ± 0.012). Flats from the dry-down treatment decreased over the course of the experiment, with a drying rate between -0.492 and -0.120 (mean: -0.344 ± 0.013). Measurements were discontinued after Day 32 because the standard deviation of θ_d for the dry-down flats overlapped with zero. (Figure 2.2).

Plant Trait Measurements

To investigate variation within and between *Mimulus* species for response to water limitation, we quantified a number of drought-related traits under each watering regime. All temporal values were numbered relative to Day 0, when seeds were transferred into growth chambers following stratification.

Developmental timing: We recorded the dates when plants reached each of three life stages: 1) the production of a bud, 2) the production of an open flower, and 3) the production of a mature fruit. We scored budding date as the first day when the first bud on the primary inflorescence was visible. We scored flowering date as the day when we observed a fully emerged flower displaying a receptive stigma from either the first or second flower pair (*M. guttatus* and *M. nasutus* produce pairs of flowers in sequential progression up flowering stems). Finally, we scored fruiting date as the day when at least one

brown, dehiscent fruit containing visible, mobile seeds was produced. The experiment was terminated on Day 63, the day on which the last pollinated flower set seed (see below for pollination details).

Lifetime maternal fitness: We obtained survival and maturation rates by daily inspection of plants. For most *M. guttatus* plants that survived to flowering, we marked and hand-pollinated one flower, on its first day of stigma receptivity, from the first or second flower pair with pollen donated from the IM767 edge plants (in some cases, we were unable to perform pollinations before plants dropped their corollas; these individuals were dropped from our analyses). IM767, an inbred line derived from the allopatric Iron Mountain population, was used as the common pollen donor as it is likely to be roughly equally differentiated from all CAC samples (pairwise nucleotide diversity, π_s, is ~5% between IM and CAC plants, see Brandvain *et al.,* 2014). Following initial pollinations, the few flowers that remained receptive were hand-pollinated a second time to ensure pollen was not limiting. For most *M. nasutus* plants that survived to flowering, we marked one or two flowers from the first or second flower pair, and allowed them to self-fertilize (in some cases, we marked flowers from later pairs; these individuals were dropped from our analyses). From these marked flowers, we measured an individual's seed production on a per fruit basis. We note that hand-pollination in *M. guttatus* versus self-pollination in *M. nasutus* might contribute to species differences in seed production. Nevertheless, variation in seed set due to treatment or species x treatment will be readily detectable.

Rosette diameter: Using calipers, we measured the rosette diameter of plants at their widest points on Day 25.

Floral traits: For most plants that survived to flowering, we measured the corolla length and width of one marked flower on the first or second flower pair (in some cases, plants dropped their corollas before measurements could be taken) on the day it was recorded as flowering. We measured corolla length as the distance from the base of the calyx to the end of the longest petal when hand straightened and corolla width as the distance between the widest point of the bottom petal lobes.

Stomatal density: For plants that survived to Day 52 with healthy green tissue (those in the well-watered treatment) we made a pressing of the abaxial surface of the largest, fully expanded leaf using GE

Clear 100% Silicone Caulk (General Electric, Boston, MA). We taped these pressings to slides and examined them under a light microscope. For each leaf, we randomly selected four fields of view at 1000x magnification and counted the number of stomata; we took the average of these four values to compute stomatal density (number of stomata per field of view).

Data Analysis

To determine if drying rate of the dry-down flats varied among locations in the greenhouse, we recorded the position of each flat within an 8 x 13 grid (in north-to-south and east-to-west directions, respectively; not all positions contained a flat) and performed a multiple regression analysis. The model had "north-to-south position" (fixed effect) and east-to-west position (fixed effect) as main effects, as well as the two-way interaction effect. We found that flat position was indeed a significant predictor of drying rate (Multiple regression, $F_{3,320} = 29.60$, $P < 0.0001$; north-south position: $F = 33.76$, $P < 0.0001$; east-west position: $F = 33.68$, $P < 0.0001$; north-south position x east-west position: $F = 5.96$, $P = 0.0151$). However, we found no significant differences in the drying rate (change per day in θ_d between Days 17 and 24) experienced by any particular *Mimulus* line or group under dry-down conditions (ANOVA with "group" as a fixed effect with "line" nested within it: $F_{18,305} = 1.0351$, $P = 0.42$). These tests were performed in JMP 13.0 (SAS Institute, Cary, N.C.).

For all drought- and fitness-related traits measured, we used hierarchical ANOVAs to calculate least square means (LSMs) for the three *Mimulus* groups (*M. nasutus*, annual *M. guttatus*, and perennial *M. guttatus*) under each watering treatment. For rosette diameter and seed set, models included "group" (fixed effect with "line" nested within it) and "treatment" (fixed effect) as main effects, "group x treatment" as an interaction effect, and "block" as a random effect. Models were identical for all flower measurements (corolla length, corolla width, days to bud, days bud to flower), but because no perennial *M. guttatus* flowered under dry-down conditions, only annual *M. nasutus* and annual *M. guttatus* were included in effect tests. The model estimating the number of days from flower to fruit included "group" (fixed effect) and "treatment" (fixed effect) as main effects, "group x treatment" as an interaction effect, and "block" as a random effect (LSMs for annual *M. guttatus* in the dry-down treatment could not be

estimated from models including a nested "line" term due to small sample size of this group). Because it was measured only under well-watered conditions, the model estimating stomatal density included only "group" (fixed effect, "line" nested within it) and "block" as a random effect. These ANOVAs were run using the lmerTest package in R v. 3.2.3 using a Satterthwaite approximation to account for different variances among groups. We determined significance using a Bonferroni correction of $\alpha = 0.006$ (to correct for multiple comparisons) and performed post hoc Tukey-Kramer HSD tests ($P < 0.05$) on all significant effects.

To investigate variation in seed set within groups, we used JMP 13.0 (SAS Institute, Cary, N.C.) to perform a two-way ANOVA to calculate LSMs for each plant line; the model included "line" (fixed effect) and "treatment" (fixed effect) as main effects and a "line x treatment" interaction effect. Post hoc *t*-tests were used to compare treatments within each line.

To examine the effect of drought across the entire plant life cycle, we calculated the relative decrease in survival for each plant line at each life stage using the following formula: [(proportion individuals surviving under dry-down) – (proportion surviving under well-watered)] / (proportion surviving under well-watered). Additionally, using JMP 13.0 (SAS Institute, Cary, N.C.), we visualized survivorship to each life stage in CAC *M. nasutus* and *M. guttatus* with Kaplan-Meier Plots and used Cox Proportional Hazards to test for significant differences in maturation rate of each species between treatments. Significant hazards ratios indicate shifts in developmental timing a species exhibited when drought stressed as compared to well-watered individuals.

To investigate the potential for tradeoffs between floral investment and fitness under drought, we conducted linear regression analyses for *M. nasutus* and annual *M. guttatus.* These models tested whether flower size (*i.e.*, average corolla width of each line under well-watered conditions) affected seed production in the dry-down treatment. To test for selection on flowering time under drought conditions, we performed a multiple linear regression examining seed set in CAC *M. nasutus* and *M. guttatus* with "days to bud" (fixed effect) and "line" (fixed effect) as main effects, as well as "days to bud x line" as an interaction effect. These analyses were performed using JMP 13.0 (SAS Institute, Cary, N.C.).

Results

Our simulated drought treatment had clear and consistently negative impacts on *Mimulus* growth and fitness, but the effects were not uniform across the three groups (perennial *M. guttatus*, annual *M. guttatus*, and annual *M. nasutus*). Rosette diameter, flower size (corolla width and length), and seed production were all strongly reduced under dry drown conditions (Table 2.2), but the extent of the reduction in flower size and seed production varied dramatically among groups (*i.e.*, we observed significant "group x treatment" interactions in Table 2.3). As previously documented (Wu *et al.*, 2010), perennial *M. guttatus* performed particularly poorly: none of the 36 plants exposed to drought-like conditions survived to produce any flowers (Table 2.2, Figure 2.3). Similarly, all annual *M. guttatus* lines (including those derived from the sympatric CAC site), were severely impacted by drought, showing an average reduction in seeds per fruit of 97% under the dry-down treatment (Table 2.2, Figure 2.3). In contrast, *M. nasutus* lines showed only a 42% reduction in seeds per fruit under dry-down conditions. Taken together, these results demonstrate a dramatic divergence in drought response between *M. guttatus* and *M. nasutus* that persists even in sympatry.

To explore the phenotypic basis of interspecific differences in seed production under simulated drought, we determined rates of mortality in the two watering regimes during each of three developmental intervals: 1) germination to bud, 2) bud to open flower, and 3) open flower to mature fruit. At each of these life stages, mortality was higher in *M. guttatus* than in *M. nasutus* (Table 2.2, Figure 2.4). In perennial *M. guttatus*, which flowers much more slowly than annual *M. guttatus* or *M. nasutus* (*i.e.*, nearly twice as long under well-watered conditions, Table 2.2; Wu *et al.*, 2010; Twyford and Friedman, 2015), mortality was complete and occurred early; not a single plant survived long enough to produce a bud. Overall survival rates of annual *M. guttatus* under simulated drought were also low. However, in contrast to perennial lines, annual *M. guttatus* budded relatively quickly under the dry-down treatment (roughly three days earlier than under well-watered conditions, Table 2.2) and most deaths occurred *after* bud initiation (only 9% of plants that produced buds survived to produce fruits, $N = 179$, Table 2.2). Indeed, for several lines of *M. guttatus* with high rates of mortality under the dry-down treatment nearly

all plants died only after having initiating reproduction (CAC6, CAC110, CAC415, DPR102, Figure 2.4). In contrast, very few *M. nasutus* plants died after they had produced a mature flower, suggesting this species has diverged for traits that promote fruit maturation even under severe water limitation.

One key question is which phenotypes might explain this difference in late-stage survival between annual *M. guttatu*s and *M. nasutus.* One possibility is that larger flowers in *M. guttatus* make it more vulnerable to drought. However, we found no evidence for tradeoffs between flower size and fitness under drought in either species; lines with larger flowers (*i.e.*, wider corollas) under well-watered conditions showed no deficit in seed set under dry-down conditions (Linear regression, *M. guttatus:* R^2 = 0.03, slope = 0.35, *M. nasutus:* R^2 = 0.06, slope = -7.18). It is also possible that key vegetative traits might differ between the two species. Indeed, we found that annual *M. nasutus* had significantly lower stomatal density (a trait often associated with higher water use efficiency) than leaves from *M. guttatus* (Table 2.2).

Restricting our focus to just the sympatric taxa at CAC, it is clear that interspecific differences in drought response become more pronounced later in the life cycle. Under the long days of our experiment, the two species' flowering phenologies were almost entirely overlapping, regardless of treatment (Figure 2.5). The one exception to this pattern is that CAC *M. guttatus* budded slightly earlier (*i.e.*, less than a day on average) under dry-down than under well-watered conditions (hazards ratio = 0.19, P = 0.0011; Figure 2.5). However, this very small head start in *M. guttatus* seems to have made little difference in terms of fitness: even the earliest flowering CAC *M. guttatus* usually died before making mature fruits or producing seeds (Figure 2.4, Figure 2.6). In CAC *M. nasutus*, on the other hand, dry-down seed production was negatively correlated with flowering time (F = 6.14, P = 0.0166, Figure 2.6), showing that this species experiences selection for early flowering in water-limited environments. In contrast to flowering time, we observed striking differences between CAC *M. guttatus* and *M. nasutus* in fruit maturation rates under dry-down conditions (Figure 2.4). Remarkably, *M. nasutus* fruits matured more than 12 days earlier under simulated drought than under well-watered conditions (hazards ratio = 12.50, P < 0.0001, Figure 2.5). The late-stage drought response in CAC *M. guttatus* was very different: among the

few plants that survived to produce fruit, maturation occurred only one day earlier than among their well-watered counterparts (hazards ratio = 1.00, $P < 0.0001$, Figure 2.5). Taken together, these results suggest the large differences between CAC *M. guttatus* and *M. nasutus* in dry-down survival (Figure 2.4) and seed set (Figure 2.3) are driven by divergence in post-flowering developmental rate.

Discussion

Previous work has shown that divergence in seasonal flowering behavior is a major component of premating isolation between sympatric *M. nasutus* and *M. guttatus* (Martin & Willis, 2007; Kenney & Sweigart, 2016). At CAC, this phenological shift – with *M. nasutus* flowering earlier in the season than *M. guttatus* – is caused, in part, by species divergence in photoperiod response (Fishman *et al.,* 2014), a change that has undoubtedly facilitated drought escape in *M. nasutus* and allowed it to occupy drier microsites. In this study, we explore phenotypes beyond critical photoperiod that might contribute to microhabitat divergence between sympatric *Mimulus* species. Under inductive conditions, typical of mid- to late-season day lengths, when there is substantial phenological overlap between species at CAC (Kenney and Sweigart, 2016), we find dramatic differences between *M. nasutus* and *M. guttatus* in drought response. Consistent with the natural microhabitats they occupy, CAC *M. nasutus* had much higher fitness than CAC *M. guttatus* under water-limited conditions, indicating divergence in drought response traits other than the critical photoperiod requirement. Surprisingly, this fitness difference was not due to flowering time; under 16-hour days, the two species initiated reproduction at roughly the same time in both well-watered and dry-down conditions. Instead, differential fitness under drought was largely caused by differences in mortality *after* the onset of flowering, with *M. guttatus* dying at a much higher rate. Higher survival of *M. nasutus* was mediated, at least in part, by a plastic increase in the speed of late-stage development, particularly during fruit maturation. Discovering the mechanistic basis of this plastic drought response will require additional investigation, but it is likely a key component of species divergence in microhabitat adaptation at CAC.

Although an increase in developmental rate is a hallmark of the drought escape strategy (Ludlow, 1989), few studies investigate time points after bud/flower initiation. Here, if we had restricted our

measurements to flowering time, we would not have detected any difference between *Mimulus* species in drought-induced developmental rate, which arose only after anthesis. Under our long-day experimental conditions, which effectively removed critical photoperiod as a signal for flowering, CAC *M. guttatus* and *M. nasutus* initiated reproduction at similar rates. Additionally, we found little evidence for plasticity in flowering time as an adaptive response to drought. In fact, only *M. guttatus* – the less drought-adapted species – mounted a weak plastic response, flowering slightly earlier (less than a day on average) under dry-down conditions. These results are largely consistent with previous greenhouse studies of drought response using similar inductive conditions (Wu *et al.*, 2010; Ivey and Carr, 2012). Both studies found only modest differences in the intrinsic rate of flowering between *M. nasutus* and *M. guttatus* and little evidence for plastic shifts in flowering time under drought (but see Ivey and Carr, 2012, which found a slight decrease in flowering time for *M. nasutus* under drought conditions). In contrast to these greenhouse studies, plasticity in flowering time has been observed in field transplant experiments involving *M. guttatus* and a closely related selfing species, *M. lacinatus*, which specializes on dry, granite outcrops (Ferris and Willis, 2018).

An important question is whether differences among studies in flowering time and plasticity are due to genetic/phenotypic variability among *Mimulus* populations/species or due to experimental differences. Compared to Wu *et al*. (2010), plants in our study flowered more rapidly (mean days to flower in perennial *M. guttatus*, annual *M. guttatus*, and annual *M. nasutus* is shifted earlier by ~10 days), potentially due to modest levels of light contamination (see Methods) and/or additional environmental variables (*e.g*., greenhouse temperature, light intensity). Because of this earlier flowering, we also began our dry-down treatment nine days sooner than in Wu *et al*. (2010). Thus, timing of the treatments in the two studies was similar relative to flowering (*i.e.*, dry-down treatments started ~10 days before the average date of first flowering in *M. nasutus*), suggesting plants experienced drought at similar developmental stages. Of course, in any of these studies, differences in the timing or intensity of drought relative to plant development, or in other environmental variables (*e.g*., temperature), might affect a plant's ability to mount a plastic response.

Despite the negligible contribution of flowering time to CAC *Mimulus* species differences in drought response, we did find evidence that water limitation imposes selection for early flowering in *M. nasutus*. This result mirrors what has been seen in annual *M. guttatus* subjected to drought in greenhouse experiments (Wu *et al.*, 2010; Ivey and Carr, 2012) and under natural conditions in the field (Hall and Willis, 2006; Mojica *et al.*, 2012; Ferris and Willis, 2018). Indeed, in the alpine Iron Mountain population of annual *M. guttatus*, selection for rapid flowering to escape summer drought trades off with selection for larger flowers, which produce more seeds, but make plants more vulnerable to desiccation (Mojica *et al.*, 2012; Troth *et al.*, 2018). In our experiment, because of extremely high mortality in water-limited *M. guttatus*, we had little power to detect selection for early flowering (very few individuals survived to produce seeds). This level of drought-induced mortality was much higher than what has been observed for annual *M. guttatus* in previous studies (Wu *et al.*, 2010; Ivey and Carr, 2012), which might be due to CAC-specific traits. Alternatively, the difference might be explained by variation among experimental conditions: our dry-down treatment was applied earlier than that of Wu *et al.*, 2010 and was likely more severe than the simulated drought used by Ivey and Carr, 2012.

Given our finding that CAC *Mimulus* species differ dramatically in post-flowering mortality under drought, a key question is which specific phenotypes are involved in this divergent response. At least part of the answer is that *M. nasutus* alone responded plastically to the dry-down conditions, increasing its rate of fruit maturation and setting seed prior to senescence. However, it is not yet clear whether this late-stage drought response was due to particular traits expressed only later in the life cycle or to some threshold requirement for severe water limitation (which, in our experiment, just happened to coincide with late stages of development). Although traits that promote rapid development to escape drought often show tradeoffs with traits for avoidance (*e.g.*, WUE; Geber and Dawson, 1990; McKay *et al.*, 2003; Kenney *et al.*, 2014), previous work in *M. nasutus* and annual *M. guttatus* suggests that the two strategies are not mutually exclusive (Wu *et al.*, 2010; Ivey and Carr, 2012; Kooyers *et al.*, 2015). Thus, it is possible that drought adaptation in *M. nasutus* involves both faster development and traits for avoidance like lower stomatal density that may lead to decreased water loss under drought conditions

(Masle *et al.*, 2005; Franks *et al.*, 2016). Furthermore, while CAC *M. guttatus* generally wilted under drought conditions, *M. nasutus* remained erect and turgid, and seemed to hasten senescence. The ability to undergo osmotic adjustment to maintain turgor is normally associated with drought tolerance (Chaves *et al.*, 2003), but plants might also be able to avoid the negative consequences of drought by accumulating stores of nutrients when water is plentiful and/or reallocating carbohydrate resources during initial water deficits (Kooyers, 2015). This adaptive response to drought has been well documented in cereal crops (Schnyder, 1993; Palta *et al.*, 1994; Yang *et al.*, 2000), and has also been observed in the Mediterranean annual *Lupinus albus*, which diverts resources from stems to seed pods as soon as it senses drought (Rodrigues *et al.*, 1995). Going forward, if we are to achieve a more mechanistic understanding of divergence in drought response between CAC *M. nasutus* and *M. gutattus*, future experiments should investigate a more comprehensive set of physiological, leaf, and whole-plant traits.

In addition to elucidating the mechanisms of drought response within *Mimulus* species, our study provides important insight into the role of differential habitat adaptation in species divergence. Our results suggest that a simple shift in critical photoperiod (from long- to short-day flowering) would be insufficient for CAC *Mimulus* to succeed in microhabitats that dry out sooner in the season. Soil moisture is highly heterogeneous at CAC and although short-day flowering might enable some plants to complete reproduction before they experience any water limitation, other individuals are likely to occupy patches that impose significant drought stress. As we have seen, CAC *M. nasutus* alone is able to cope with such conditions, surviving longer and speeding up its development to produce many more seeds than CAC *M. guttatus*. The picture emerging from this and previous studies (Wu *et al.*, 2010, Ivey and Carr, 2012) is that habitat divergence between *M. nasutus* and *M. guttatus* is complex, involving many traits, both constitutive and plastic. Although some of the key traits involved might be genetically simple (*e.g.*, critical photoperiod: Fishman *et al.*, 2014), the microhabitat isolation we observe at CAC is likely to involve changes at many loci.

These findings have important implications for species maintenance in sympatry. At CAC and other sympatric sites, introgression is ongoing and asymmetric, with most interspecific gene flow

occurring from *M. nasutus* into *M. guttatus* (Sweigart and Willis, 2003; Brandvain *et al.*, 2014; Kenney and Sweigart, 2016). Thus, it is conceivable that in drier years or microsites, introgression of drought response alleles from *M. nasutus* (*e.g.*, for faster fruit maturation) might prove adaptive in *M. guttatus*, allowing it to survive in environments beyond its normal limits. In hybridizing sunflowers, for example, adaptive introgression of drought escape traits from *Helianthus debilis* seem to have facilitated range expansion of *H. annuus* into drier areas (Whitney *et al.*, 2010). On the other hand, our results suggest that introgression of early-flowering *M. nasutus* alleles at the two major photoperiod loci (Fishman *et al.*, 2014) might not allow *M. guttatus* to invade drier microsites at CAC; even when *M. guttatus* flowers early, it is unable to overcome water deficits to set seed. This result might help explain why one of the two photoperiod loci remains highly divergent between species (Kenney and Sweigart, 2016), even in the face of considerable interspecific gene flow. Consistent with the idea that differentially adapted loci contribute to reproductive isolation between species, we find evidence of selection against *M. nasutus* ancestry across the *M. guttatus* genome at CAC (Brandvain *et al.*, 2014; Kenney and Sweigart, 2016). Our work here sets the stage for future experiments to map the genetic basis of key ecological traits and fitness across the complex and variable environments of CAC, an approach that holds great promise for understanding how the process of abiotic adaptation can contribute to speciation.

References

Bartels D, Sunkar R. 2005. Drought and salt tolerance in plants. *Critical Reviews in Plant Sciences* **1:** 23-58.

Brandvain Y, Kenney A, Flagel L, Coop G, Sweigart AL. 2014. Speciation and introgression between *Mimulus nasutus* and *Mimulus guttatus*. *PLoS Genetics* **10:** e1004410.

Chaves MM, Maroco JP, Pereira JS. 2003. Understanding plant responses to drought-from genes to the whole plant. *Functional Plant Biology* **30:** 239–264.

Clausen J, Keck DD, Hiesey WM. 1940. Experimental studies of the nature of species. I. Effects of varied environments on western North American plants. Carnegie Institute of Washington publication no. 520 Carnegie Institute, Washington.

Cornwell WK, Grubb PJ. 2003. Regional and local patterns in plant species richness with respect to resource availability. *Oikos* **100:** 417-428.

Coyne JA, Orr HA. 2004. Speciation. Sunderland, MA: Sinauer Associates, Inc.

Dudley LS, Arroyo MTK, Fernández-Murillo MP. 2018. Physiological and fitness response of flowers to temperature and water augmentation in a high Andean geophyte. *Environmental and Experimental Botany* **150:** 1-8.

Dunning LT, Hipperson H, Baker WJ, Butlin RK, Devaux C, Hutton I, Igea J, Papadopulos AST, Quan X, Smadja CM, Turnbull CGN, Savolainen V. 2016. Ecological speciation in sympatric palms: 1. Gene expression, selection and pleiotropy. *Journal of Evolutionary Biology* **29(8):** 1472-1487.

Eckhart VM, Geber MA, McGuire CM. 2004. Experimental studies of adaptation in Clarkia xantiana. I. Sources of trait variation across a subspecies border. *Evolution* **58:** 59–70.

Engelbrecht BMJ, Comita LS, Condit R, Kursar TA, Tyree MT, Turner BL, Hubbell SP. 2007. Drought sensitivity shapes species distribution patterns in tropical forests. *Nature* **447:** 80-82.

Ferris KG, Willis JH. 2018. Differential adaptation to a harsh granite outcrop habitat between sympatric *Mimulus* species. *Evolution* **72(6):** 1225-1241.

Fishman L, Kelly AJ, Willis JH. 2002. Minor quantitative trait loci underlie floral traits associated with mating system divergence in *Mimulus. Evolution* **56(11):** 2138-2155.

Fishman L, Sweigart AL, Kenney AM, Campbell S. 2014. Major QTLs control divergence in critical photoperiod for flowering between selfing and outcrossing species of monkeyflower (*Mimulus*). *New Phytologist* **201:** 1498-1507.

Franks SJ, Weis AE. 2009. Climate change alters reproductive isolation and potential gene flow in an annual plant. *Evolutionary Applications* **2(4):** 481-488.

Franks SJ, Kane NC, O'Hara NB, Tittes S, Rest JS. 2016. Rapid genome-wide evolution in *Brassica rapa* populations following drought revealed by sequencing of ancestral and descendant gene pools. *Molecular Ecology* **25(15):** 3622-3631.

Friedman J, Willis, JH. 2013. Major QTLs for critical photoperiod and vernalization underlie extensive variation in flowering in the *Mimulus guttatus* species complex. *New Phytologist* **199(2):** 571-583.

Friedman J, Twyford AD, Willis JH, Blackman BK. 2015. The extent and genetic basis of phenotypic divergence in life history traits in *Mimulus guttatus. Molecular Ecology* **24(1):** 111-122.

Galen C, Sherry RA, Carroll AB. 1999. Are flowers physiological sinks or faucets? Costs and correlates of water use by flowers of *Polemonium viscosum*. *Oecologia* **118:** 461–470.

Geber MA, Dawson TE. 1990. Genetic variation in and covariation between leaf gas exchange, morphology, and development in *Polygonum areastrum,* an annual plant. *Oecologia* **85:** 153-158.

Geber MA, Eckhart VM. 2005. Experimental studies of adaptation in Clarkia xantiana: II. Fitness variation across a subspecies border. *Evolution* **59:** 521–531.

Hall MC, Willis JH. 2006. Divergent selection on flowering time contributes to local adaptation in *Mimulus guttatus* populations. *Evolution* **60:** 2466-2477.

Hereford J. 2009. A quantitative survey of local adaptation and fitness trade-offs. *The American Naturalist* **173(5):** 579-588.

Ivey CT, Carr DE. 2012. Tests for the joint evolution of mating system and drought escape in *Mimulus*. *Annals of Botany* **109:** 583–598.

Kenney AM, McKay JK, Richards JH, Juenger TE. 2014. Direct and indirect selection on flowering time, water-use efficiency (WUE, δ^{13}C), and WUE plasticity to drought in *Arabidopsis thaliana. Ecology and Evolution* **4(23):** 4505-4521.

Kenney AM, Sweigart AL. 2016. Reproductive isolation and introgression between sympatric *Mimulus* species. *Molecular Ecology* **25:** 2499-2517.

Kiang YT, Hamrick JL. 1978. Reproductive isolation in the *Mimulus guttatus – M. nasutus* complex. *American Midland Naturalist* **100:** 269-276.

Kooyers NJ. 2015. The evolution of drought escape and avoidance in natural herbaceous populations. *Plant Science* **234:** 155-162.

Kooyers NJ, Greenlee AB, Colicchio JM, Oh M, Blackman BK. 2015. Replicate altitudinal clines reveal that evolutionary flexibility underlies adaptation to drought stress in annual *Mimulus guttatus*. *New Phytologist*, **206(1):** 152-165.

Lasky JR, Des Marais DL, McKay JK, Richards JH, Juenger TE, Keitt TH. 2012. Characterizing genomic variation of *Arabidopsis thaliana*: the roles of geography and climate. *Molecular Ecology* **(22):** 5512-5529.

Lasky JR, Des Marais DL, Lowry DB, Povolotskaya I, McKay JK, Richards JH, Keitt TH, Juenger TE. 2014. Natural variation in abiotic stress responsive gene expression and local adaptation to climate in *Arabidopsis thaliana. Molecular Biology and Evolution* **31(9):** 2283-2296

Lee CR, Mitchell-Olds T. 2013. Complex trait divergence contributes to environmental niche differentiation in ecological speciation of *Boechera stricta. Molecular Ecology* **22(8):** 2204-2217.

Leimu R, Fischer M. 2008. A meta-analysis of local adaptation in plants. *PLoS ONE* **3(12)**: e4010.

Lowry DB, Rockwood RC, Willis JH. 2008. Ecological reproductive isolation of coast and Inland races of *Mimulus guttatus*. *Evolution* **62**: 2196-2214.

Ludlow MM. 1989. Strategies of response to water stress. In: **Kreeb KH, Richter H, Mickley TM.** (eds) Structural and functional responses to environmental stress. SPB Academic Amsterdam

Maggio A, Zhu JK, Hasegawa PM, Bressan RA. 2006. Osmogenetics: Aristotle to *Arabidopsis. The Plant Cell* **18:** 1542-1557.

Martin NH, Bouck AC, Arnold ML. 2005. Loci affecting long-term hybrid survivorship in Louisiana irises: implications for reproductive isolation and introgression. *Evolution* **59(10):** 2116-2124.

Martin NH, Willis JH. 2007. Barriers to geneflow between the monkeyflowers *Mimulus guttatus, M. nasutus* and their hybrids. *Evolution* **61:** 68-82.

Masle J, Gilmore SR, Farquhar GD. 2005. The ERECTA gene regulates plant transpiration efficiency in *Arabidopsis. Nature* **(436):** 866-870.

McKay JK, Richards JH, Mitchell-Olds T. 2003. Genetics of drought adaptation in Arabidopsis thaliana: I. Pleiotropy contributes to genetic correlations among ecological traits. *Molecular Ecology* **12:** 1137–1151.

Mojica JP, Lee YW, Willis JH, Kelly JK. 2012. Spatially and temporally varying selection on intrapopulation quantitative trait loci for a life history trade-off in *Mimulus guttatus*. *Molecular Ecology* **21:** 3718–3728.

Palta JA, Kobata T, Turner NC, Fillery IR. 1994. Remobilization of carbon and nitrogen in wheat as influenced by postanthesis water deficits. *Crop Science* **34:** 118-124.

Pennell FW. 1947. Some hitherto undescribed Scrophulariaceae of the Pacific states. *Proceedings of the Academy of Natural Sciences*. **99:** 151-171.

Rodrigues ML, Pacheco CMA, Chaves MM. 1995. Soil-plant water relations, root distribution and biomass partitioning in *Lupinus albus* L. under drought conditions. *Journal of Experimental Botany.* **46(8):** 947-956.

Rundle HD, Nosil P. 2005. Ecological speciation. *Ecology Letters* **8(3):** 336-352.

Schluter D. 2000. Ecological character displacement in adaptive radiation. *The American Naturalist* **156:** S4-S16.

Schnyder H. 1993. The role of carbohydrate storage and redistribution in the source-sink relations of wheat and barley during grain filling - a review. *New Phytologist* **123:** 233-245.

Sobel JM, Chen GF, Watt LR, Schemske DW. 2010. The biology of speciation. *Evolution* **64(2):** 295–315.

Sweigart AL, Willis JH. 2003. Patterns of nucleotide diversity are affected by mating system and asymmetric introgression in two species of *Mimulus. Evolution* **57:** 2490-2506.

Troth A, Puzey JR, Kim RS, Willis JH, Kelly JK. 2018. Selective trade-offs maintain alleles underpinning complex trait variation in plants. *Science* **361(6401):** 475-478.

Twyford AD, Friedman J. 2015. Adaptive divergence in the monkey flower *Mimulus guttatus* is maintained by a chromosomal inversion. *Evolution* **69(6):** 1476-1486.

Whitney KD, Randell RA, Rieseberg LH. 2010. Adaptive introgression of abiotic tolerance traits in the sunflower *Helianthus annuus. New Phytologist* **187:** 230–239.

Wu CA, Lowry DB, Cooley AM, Wright KM, Lee YW, Willis JH. 2008. *Mimulus* is an emerging model system for the integration of ecological and genomic studies. *Heredity* **100:** 220–230.

Wu CA, Lowry DB, Nutter LI, Willis JH. 2010. Natural variation for drought-response traits in the *Mimulus guttatus* species complex. *Oecologia* **162:** 23–33.

Yang J, Zhang J, Huang Z, Zhu Q, Wang L. 2000. Remobilization of carbon reserves is improved by controlled soil-drying during grain filling of wheat. *Crop Sciences* **40:** 1645-1655.

Table 2.1: Geographic locations of *Mimulus* populations used.

Species/Ecotype	Line Abbreviation	Population	Latitude (N)	Longitude (W)
perennial *M. guttatus*	SWB38A	Sperm Whale Beach, Mendocino County, California	39° 02' 09"	123° 41' 25"
	DUN10	Oregon Dunes National Recreation Area, Lane County, Oregon	43° 53' 35"	124° 08' 16"
annual *M. guttatus*	IM767	Iron Mountain, Highway 20, Linn County, Oregon	44° 24' 03"	122° 08' 16"
	DPR102	Stanislaus National Forest Junction of Highway 120 and Jacksonville Road, Tuolumne County, California	37° 49' 45"	120° 20' 41"
	CAC6	Catherine Creek, Washington side of the Columbia River Gorge off of Hwy. 14	45° 42' 42"	121° 21' 55"
	CAC110			
	CAC112			
	CAC134			
	CAC141			
	CAC162			
	CAC171			
	CAC262			
	CAC277			
	CAC415			
M. nasutus	SF5	Sherar's Falls, Tygh Valley, Wasco County, Oregon	45° 15' 52"	121° 01' 21"
	CAC9	Catherine Creek, Washington side of the Columbia River Gorge off of Hwy. 14	45° 42' 43"	121° 21' 55"
	CAC22			
	CAC27			
	CAC32			

Table 2.2: *Mimulus* species/ecotype least squares means for drought- and fitness-related traits when grown under different watering regimes.

Group	Treatment	Rosette Diameter (mm)	Stomatal Density*	Corolla Length (mm)	Corolla Width (mm)	Days to Bud	Days Bud to Flower	Days Flower to Fruit	% Survival to Bud	% Survival to Flower	% Survival to Fruit	Seeds per Fruit
perennial *M. guttatus*	WW	63.62 b (2.63, 36)	18.98 a (0.64, 24)	40.39 a (0.55, 36)	33.26 a (0.49, 36)	34.37 a (0.55, 36)	12.74 b (0.41, 36)	14.94 b (0.81, 32)	100	100	92	299.15 a (15.13, 36)
	DD	34.68 d (2.65, 36)	-	-	-	-	-	-	0	0	0	1.36 d (15.18, 36)
annual *M. guttatus*	WW	58.45 bc (1.43, 216)	7.66 b (0.37, 111)	23.74 b (0.30, 180)	18.56 b (0.26, 180)	20.67 b (0.34, 208)	14.05 a (0.22, 196)	23.98 a (0.39, 158)	96	94	77	96.48 c (7.94, 181)
	DD	35.22 d (2.83, 215)	-	14.24 c (0.61, 70)	9.01 c (0.54, 70)	17.49 c (0.61, 179)	9.23 c (0.44, 77)	24.06 a (1.18, 17)	83	37	8	2.78 d (16.22, 72)
M. nasutus	WW	88.97 a (2.15, 90)	5.93 c (0.49, 47)	14.84 c (0.45, 59)	7.11 d (0.40, 59)	21.11 b (0.46, 90)	8.96 c (0.33, 90)	25.03 a (0.60, 90)	100	100	100	180.07 b (12.29, 59)
	DD	52.73 c (2.61, 91)	-	11.00 d (0.55, 46)	2.30 e (0.49, 46)	19.52 b (0.56, 82)	8.60 c (0.41, 70)	13.38 b (0.69, 69)	91	79	76	104.17 c (14.98, 46)

Standard error and sample size given in parentheses.

Models: Rosette diameter, corolla length, corolla width, days to bud, days bud to flower, and seeds per fruit: "group" (fixed effect with "line" nested within it) and "treatment" (fixed effect), main effects, "group x treatment", interaction effect "block", random effect. Days flower to fruit: "group" (fixed effect) and "treatment" (fixed effect), main effects, "group x treatment", interaction effect, "block", random effect. Stomatal density: "group" (fixed effect with "line" nested within it) and "block", random effect.

Letters indicate Tukey-Kramer grouping for each trait following ANOVA

*Stomatal Density was only measured on a subset of plants grown under well-watered conditions

Table 2.3: Hierarchical ANOVA results for rosette diameter, corolla width, and seed set using a Satterthwaite approximation including "group" (fixed effect with "line" nested within it) and "treatment" (fixed effect) as main effects, "group x treatment" (interaction effect), "block", random effect. Significance determined using a Bonferroni correction of $\alpha = 0.006$.

		SS	*df*	*MS*	*F*	*p*
Rosette diameter	Group	29,481	2	14,740	72.28	<0.0001
	Treatment	35,633	1	35,633	174.73	<0.0001
	Group*Treatment	1,889	2	944.41	4.63	0.010
Corolla width	Group	3,600	1	3,600	497.61	<0.0001
	Treatment	1,804	1	1,804	249.38	<0.0001
	Group*Treatment	244.93	1	244.93	33.86	<0.0001
Seed set	Group	552,066	2	276,033	39.33	<0.0001
	Treatment	946,886	1	946,886	134.90	<0.0001
	Group*Treatment	552,575	2	276,287	39.36	<0.0001

SS – "Sum-of-Squares", *df* – "degrees of freedom", *MS* – "Mean-Squares

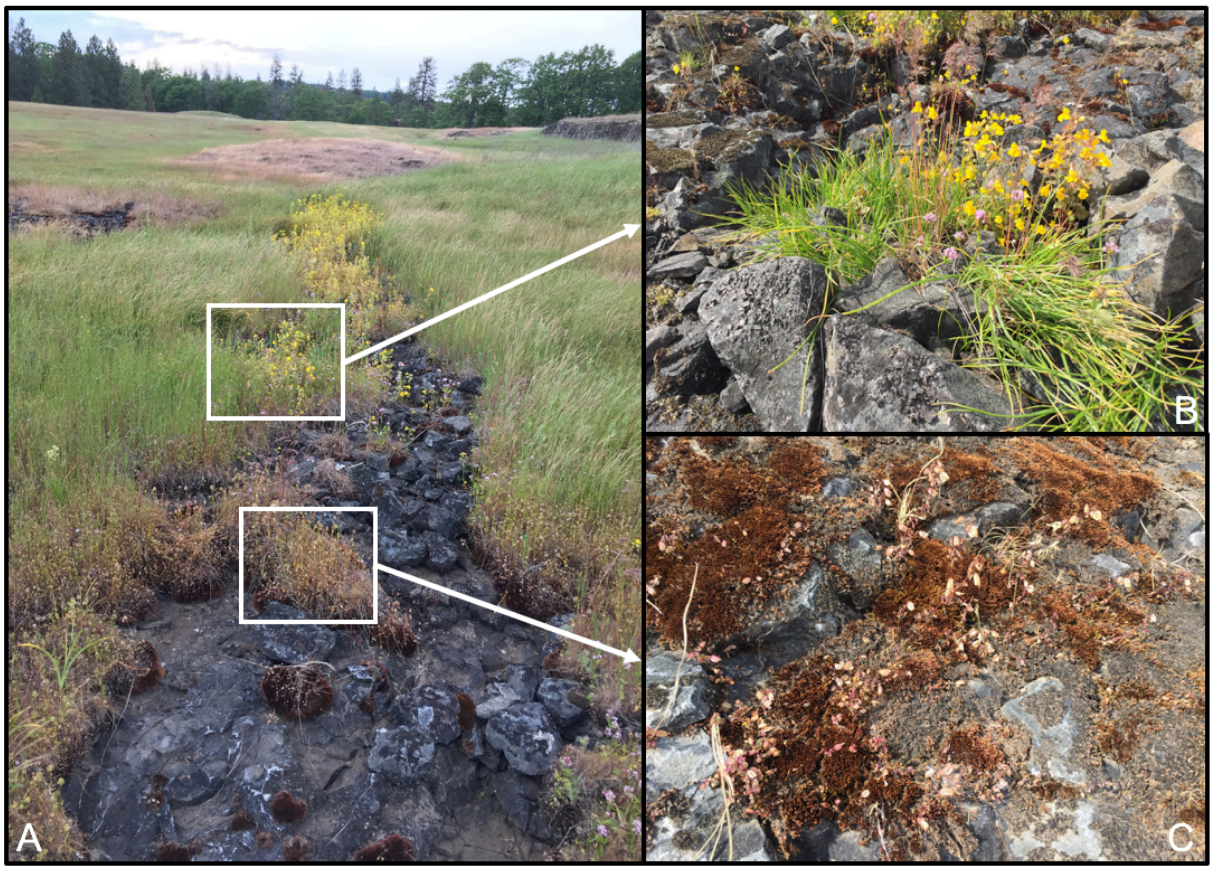


Figure 2.1: One of the streambeds of Catherine Creek (A) in May, with hybrid individuals and putative *Mimulus guttatus* (B) and *M. nasutus* (C) growing in close proximity, often within one meter.

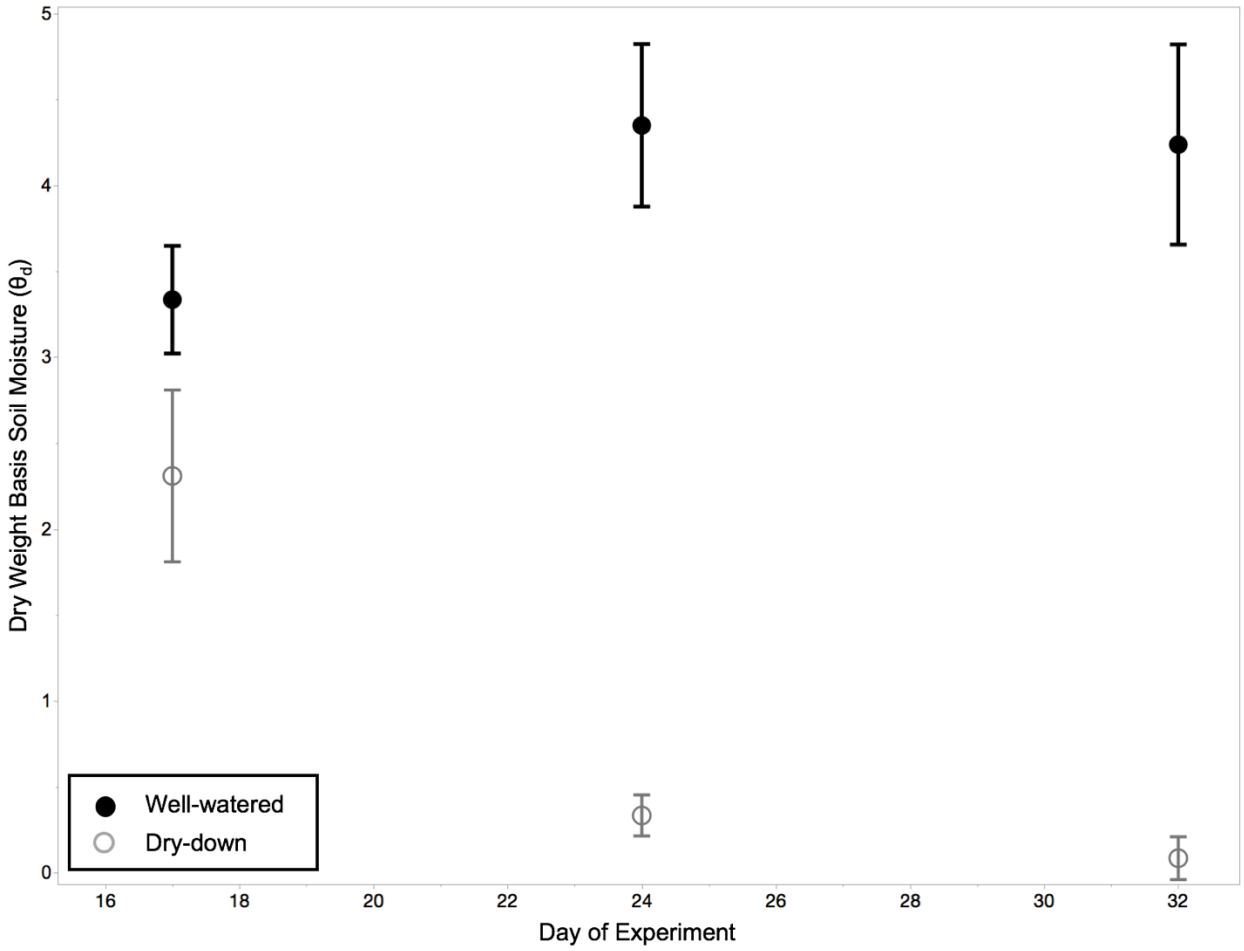


Figure 2.2: Dry weight basis soil moisture (θ_d) in each watering treatment over the course of the experiment. Average soil moisture (error bars, SE) in well-watered flats remained constant or increased over the course of the experiment, while dry-down flats experienced continually decreasing soil moisture as the experiment progressed.

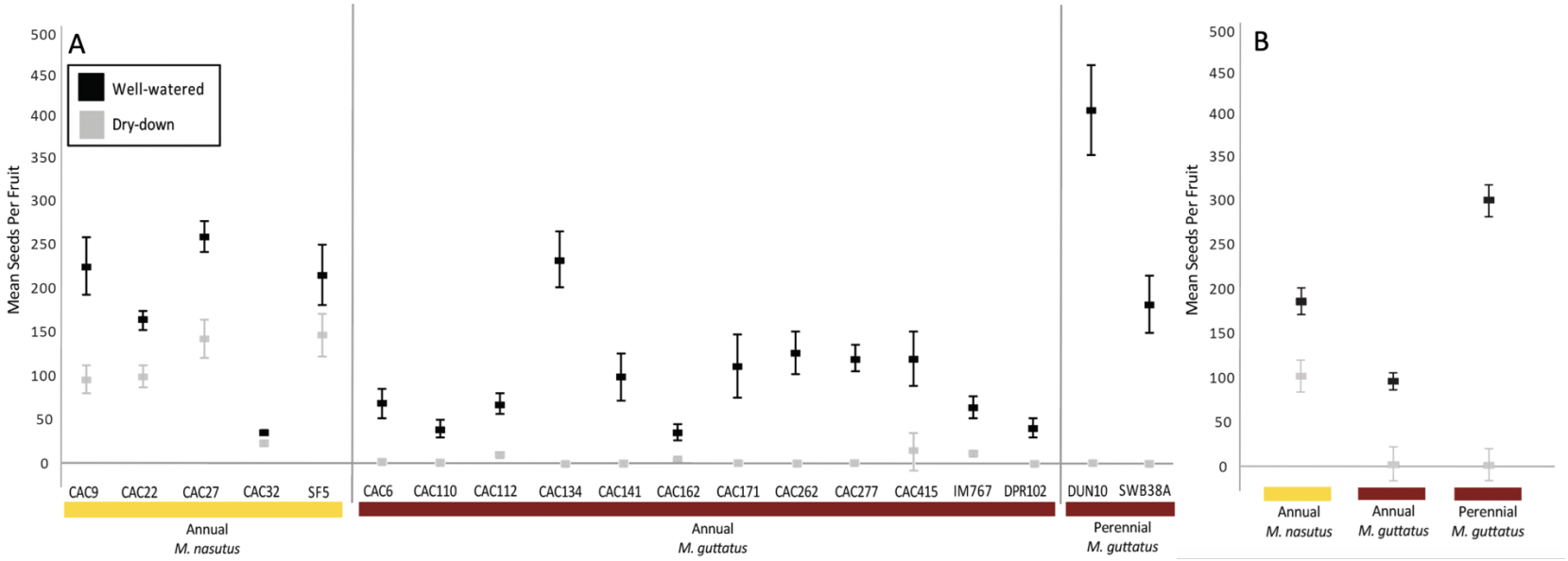


Figure 2.3: The impact of drought treatment on seed production varies among *Mimulus* line and species/ecotypes. (A) Least squares means, seeds per fruit (SE), of experimental lines and **(B)** least squares means, seeds per fruit (SE) of species/ecotypes under each watering regime. Seed production for all lines was significantly lower ($P < 0.05$, except SF5, $P < 0.1$, post-hoc pairwise *t*-tests) and for all species/ecotypes ($P < 0.05$, post-hoc Tukey-Kramer HSD) under simulated drought (grey) than under well-watered conditions (black), but the reduction was much more severe in *M. guttatus*.

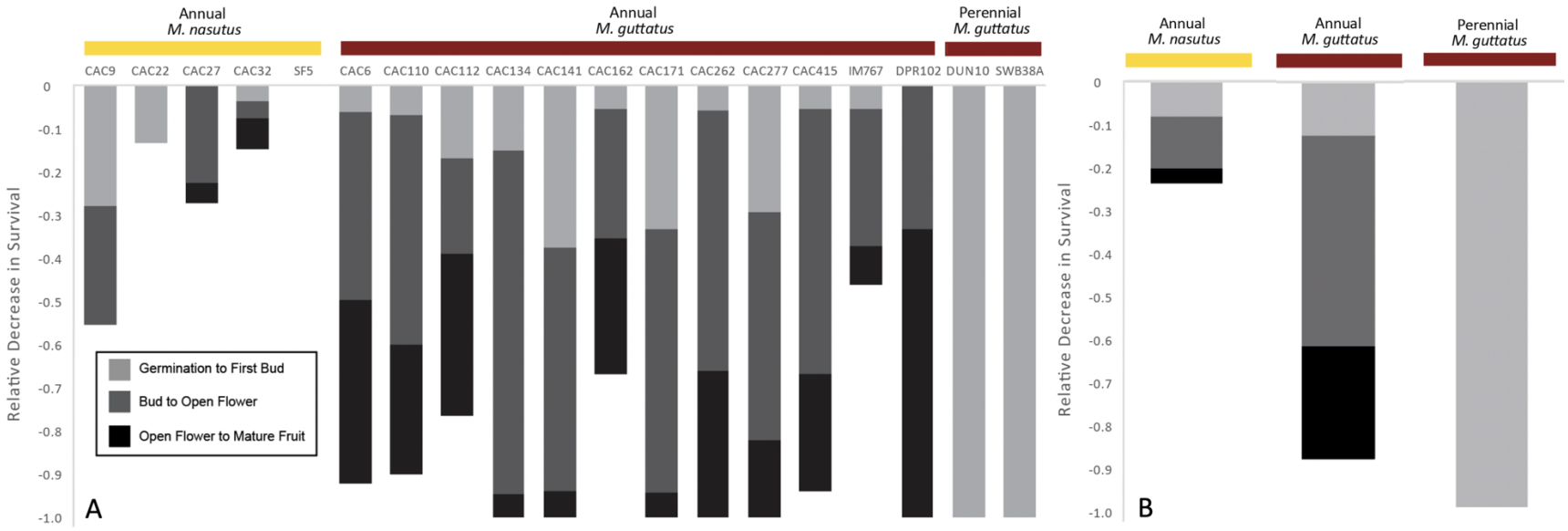


Figure 2.4: Variation among (A) *Mimulus* lines and (B) species/ecotypes in drought response across the life cycle. Reduction in survival rate in dry-down versus well-watered conditions during each of three life stages (germination to first bud, bud to open flower, and open flower to mature fruit).

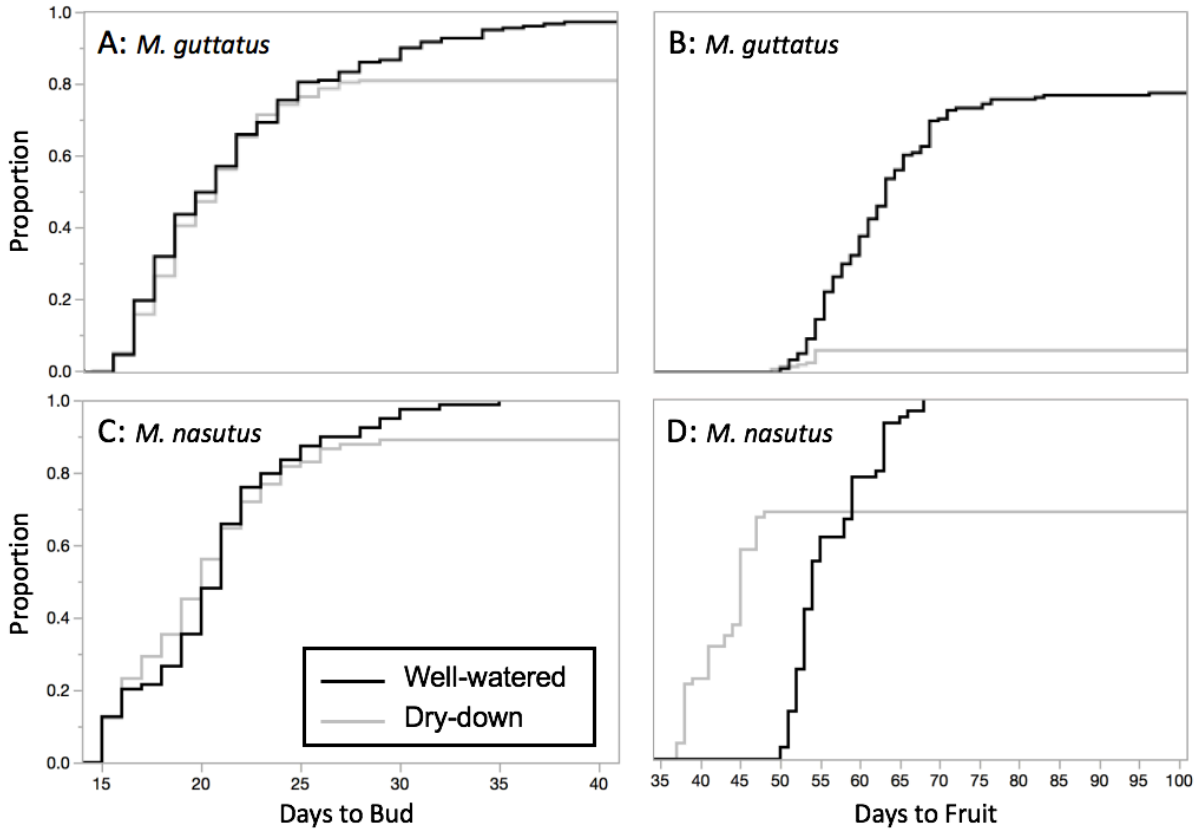


Figure 2.5: Divergence in response to experimental drought between sympatric *M. guttatus* and *M. nasutus* was more pronounced later in the life cycle. Kaplan-Meier Plots showing progression from germination to bud (A, C) and to mature fruit (B, D) of Catherine Creek *M. guttatus* and *M. nasutus* under well-watered (black) and dry-down (grey) conditions. Days are numbered relative to Day 0, when seeds were transferred into growth chambers following stratification.

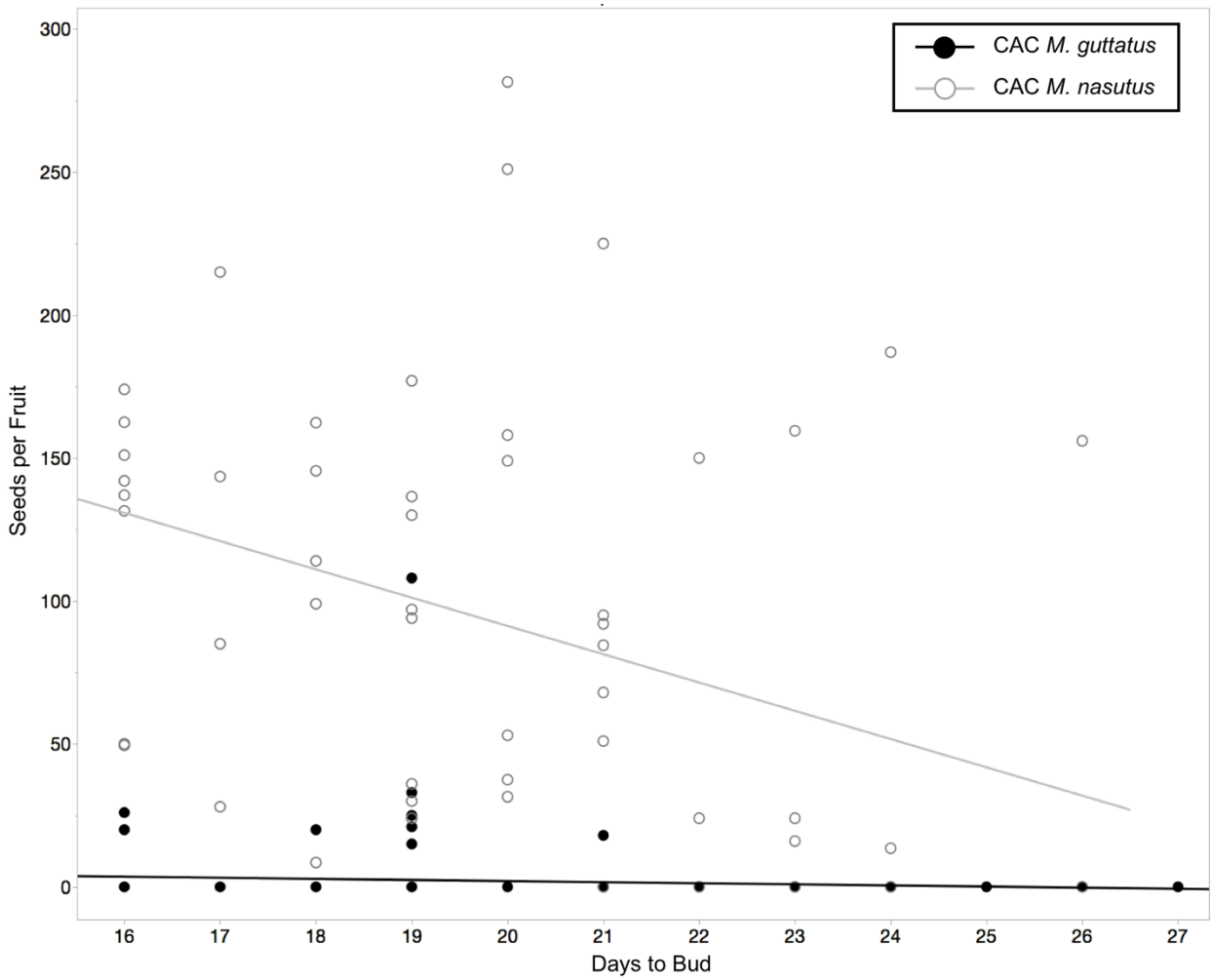


Figure 2.6: Under simulated drought, the effect of flowering time on seed production varies between sympatric *Mimulus* species. In a multiple linear regression (quadratic and cubic regression showed lower support), days to bud and plant line were significant predictors of seeds production in *M. nasutus* ("days to bud": $F = 6.14$, $P = 0.0166$; "line": $F = 7.18$, $P = 0.0004$; "days to bud x line": $F = 0.61$, $P = 0.6145$; $F_{7,49} = 4.95$, $P = 0.0003$, $R^2 = 0.41$), but not in *M. guttatus* ($F_{19,129} = 0.64$, $P = 0.8708$, $R^2 = 0.09$).

CHAPTER III

MULTIVARIATE SELECTION ON DIVERSE PHENOTYPES LEADS TO DIVERGENT ADAPTATION TO DROUGHT BETWEEN NATURALLY HYBRIDIZING SYMPATRIC *MIMULUS* SPECIES[2]

[2]Mantel, S.J. and A.L. Sweigart, To be submitted to *New Phytologist*.

Abstract

Little is known about the phenotypic and genetic basis of ecological isolation between closely related species that have diverged via differential adaptation. Here, in recombinant inbred intercrossed (RIX) individuals we investigate drought response in admixed individuals produced from sympatric *Mimulus guttatus* and *M. nasutus*, sister species which largely grow in distinct microhabitats distinguished by seasonal water availability when growing in sympatry. We find that plasticity, genetic effects, and genetic by environment interactions influence admixed individuals' ability to survive and reproduce successfully under drought conditions. Drought response appears phenotypically and genetically complex, involving many interconnected traits which are frequently genetically correlated across a plant's life. These trait correlations also appear to both facilitate and impede adaptation to drought in these hybrids. Furthermore, tradeoffs across water environments may lead to extrinsic postzygotic isolation via selection against maladapted hybrid genotypes, making differential adaptation to water stress a potentially important factor maintaining reproductive isolation between these species.

Introduction

As sessile organisms, plants are particularly influenced by their abiotic environments, which frequently vary across species ranges. Divergent adaptation to different habitats is likely one of the most important drivers of speciation (Sobel *et al.*, 2010), as it often contributes to ecological isolation between lineages (Dillon, 1970; Schluter, 2000, 2009; Rundle & Nosil, 2005; Lee & Mitchell-Olds, 2011; Nosil, 2012; Safran *et al.*, 2013; Lin *et al.*, 2020). Such ecologically-based reproductive isolation can limit species' ranges and prevent geographic overlap entirely, *i.e.* geographic isolation (Coyne & Orr, 2004). It can also be important for preventing interspecific gene flow upon secondary contact – either by limiting mating opportunities between species in ecologically distinct microsites or by causing extrinsic postzygotic isolation in hybrids carrying maladaptive alleles (Coyne & Orr, 2004; Arnegard *et al.*, 2014; Coughlan *et al.*, 2021; Thompson *et al.*, 2022). Nevertheless, because ecological barriers can sometimes breakdown in secondary sympatry (Taylor *et al.*, 2006; Vonlanthen *et al.*, 2012; Kleindorfer *et al.*, 2014),

it is important to understand the conditions and mechanisms that allow ecologically differentiated species to persist in the face of sustained interspecific gene flow.

The effectiveness of ecological isolation in maintaining species boundaries upon secondary contact depends, in large part, on its genetic basis. Extrinsic postzygotic isolation between species might be quite strong if adaptation to different habitats is due to genetic tradeoffs at individual loci (i.e., antagonistic pleiotropy), with alleles conferring a fitness advantage in their native parental microsites and a disadvantage in foreign ones (Nosil *et al.*, 2005). If, instead, ecological isolation is caused by conditionally neutral alleles that are favored in parental microsites and selectively neutral in foreign ones, hybrids might not suffer a pronounced fitness disadvantage. Under this second scenario, introgression might occur readily between ecologically divergent species in secondary sympatry, especially if effective population sizes are large (Sambatti *et al.*, 2012). The fitness consequences of hybridization likely depend upon the genetic architecture of adaptation to abiotic environments, including the strength of genetic correlations among adaptive traits. If these traits are highly integrated due to extensive pleiotropy, perhaps reconstituting parental phenotypes will be common in hybrid genetic backgrounds (Rieseberg *et al.*, 2003). On the other hand, if ecological adaptation is highly polygenic, introgression between species could result in hybrids with mismatched ecological traits that compromise their fitness (Thompson *et al.*, 2022). Finally, an additional possibility is that differences between species are largely mediated by plastic changes rather than by genetically-based differences, in which case species fusion might be virtually inevitable in the face of high rates of gene flow.

To understand the maintenance of ecological isolation, we examine the phenotypic and genetic basis of drought response differences, which track closely with habitat preferences, between *Mimulus guttatus* and *M. nasutus*, two closely related species of yellow monkeyflowers that often occur in secondary sympatry. Despite their recent divergence (~200 KYA: Brandvain *et al.*, 2014), the two species are morphologically distinct: *M. guttatus* is large-flowered and primarily outcrossing, whereas *M. nasutus* is small-flowered (often cleistogamous) and almost exclusively self-fertilizing. The two species, which range across much of western North America, have also diverged ecologically, with *M. guttatus* tending

to grow along mostly permanent streams or seeps, and *M. nasutus* occupying more marginal habitats that dry out earlier in the season (Kiang & Hamrick, 1978). Across the mosaic landscape of their shared range from California to Washington state, *M. guttatus* and *M. nasutus* are largely allopatric, but sympatric sites where the two species occur within meters are also common. In such areas of secondary contact, there is substantial premating reproductive isolation due to mating system divergence and flowering time differences (Kiang & Hamrick, 1978; Martin & Willis, 2007; Kenney & Sweigart, 2016). Nevertheless, there is also evidence of extensive hybridization at such sites (Kenney & Sweigart, 2016) and genomic signatures of introgression, largely in one direction from *M. nasutus* into *M. guttatus* (Sweigart & Willis, 2003; Brandvain *et al.*, 2014).

In this study, we focus on sympatric *M. guttatus* and *M. nasutus* that co-occur at Catherine Creek, a sloping meadow of intertwining creeks and seeps that empty into the Columbia River. The two species exhibit asynchronous flowering phenologies at this site due, at least in part, to divergence in critical photoperiod, which allows *M. nasutus* to initiate flowering under the short days of early spring while *M. guttatus* must wait for longer days typical of mid-late spring (Kenney and Sweigart 2016). This ability to flower earlier in the season is a hallmark of drought escape (Franks & Weis, 2009; Franks, 2011; Berger & Ludwig, 2014) and likely determines the local distribution of *M. nasutus*, which is most abundant in shallow soil sites that dry out completely by mid-spring. *M. guttatus* is absent from such sites at Catherine Creek and, instead, is restricted to more permanent seeps that maintain moisture until later in the season. Species divergence in photoperiod response is genetically simple – caused primarily by two large-effect loci – and is likely a major contributor to ecological isolation between these species (Fishman *et al.*, 2014a; Kenney & Sweigart, 2016). In addition to critical photoperiod, the two Catherine Creek species are divergent for other important drought-response traits , including the ability to increase the rate of fruit maturation under drought conditions (Mantel & Sweigart, 2019).

Given these divergent ecological adaptations, a key question is whether ongoing hybridization at Catherine Creek will break down species barriers. Water availability at this site is highly heterogeneous – both spatially, across microsites, and temporally, across years, – and intermediate microsites might

alleviate extrinsic postzygotic isolation (Jiggins & Mallet, 2000), serving as a conduit for interspecific gene flow. Additionally, introgression from *M. nasutus* to *M. guttatus* might be neutral or even adaptive if it increases fitness in drier sites or years. Indeed, within *M. guttatus* populations, year-to-year variation in the onset of terminal drought appears to maintain variation for flowering time, with plants that flower rapidly more likely to escape the potentially lethal effects of drought, and plants that delay reproduction producing larger, more fecund flowers (Mojica & Kelly, 2010; Mojica *et al.*, 2012; Troth *et al.*, 2018). Under this type of fluctuating environment, introgression of drought response alleles from *M. nasutus* might be favored in certain years, particularly with a warming climate. On the other hand, genomic variation at Catherine Creek shows a signature of selection against *M. nasutus* ancestry in *M. guttatus* (i.e., increased divergence in low recombination regions, Kenney & Sweigart, 2016), consistent with extrinsic postzygotic isolation, potentially due to hybrids having mismatched trait combinations (Thompson *et al.*, 2021, 2022).

Here, we investigate drought response traits in an experimental hybrid population generated from crosses between inbred lines of *M. guttatus* and *M. nasutus* derived from Catherine Creek. We perform a common garden experiment using inductive light conditions that are typical of mid- to late-season day lengths at Catherine Creek when there is substantial phenological overlap between species and admixed individuals are abundant (Kenney & Sweigart, 2016). In a previous common garden experiment involving Catherine Creek inbred lines, we observed dramatic differences between *M. guttatus* and *M. nasutus* in survival and seed set under drought conditions (Mantel & Sweigart, 2019). This work implicated both plastic and genetic contributors to drought response, but additional inferences were somewhat limited because no recombinant genotypes were evaluated. Here, we extend these findings to address the following questions: 1) To what extent are *Mimulus* species differences in drought response determined by plasticity, genetic changes, or genetic-by-environment interactions (GxE)? 2) Are phenotypic traits genetically correlated and, if so, what are the consequences for ecological adaptation and isolation? 3) Which traits are most important for fitness in well-watered versus drought conditions and is there evidence for genetic tradeoffs? By dissecting the genetic and evolutionary mechanisms of *Mimulus*

divergence in drought response, we aim to evaluate the potential for ecological isolation to maintain species boundaries in nature.

Materials and Methods

Plant Material and Crossing Design

To investigate differences in drought response between sister taxa *Mimulus guttatus* and *M. nasutus*, we generated a population of recombinant inbred intercrossed individuals (RIXs). We constructed this RIX population from three independent *M. nasutus* x *M. guttatus* crosses between inbred lines derived from the sympatric site, Catherine Creek (CAC). In all three crosses, one *M. nasutus* inbred line (CAC9) served as the maternal parent, which we crossed to three distinct *M guttatus* inbred lines (CAC110, CAC162, and CAC415, selected to capture some of the genetic variation present in CAC *M. guttatus*) to produce three F1 hybrids. For each of these F1 hybrids, we self-fertilized a single plant to generate three corresponding F2 populations (N = 500 each). Beginning with these F2 hybrids (N = 1500), we performed four additional rounds of self-fertilization using single-seed-descent to produce three distinct populations of F6 recombinant inbred lines (denoted RIL Group 1, 2, and 3 respectively). We generated a total of 368 RILs (151 in Group 1, 128 in Group 2, and 89 in Group 3), much lower than the initial 1500 due to line extinction during the inbreeding process. To remove any potential effects of inbreeding depression, which is generally severe in outcrossing taxa such as *M. guttatus* (Husband & Schemske, 1996), we then intercrossed random pairs of F6 RILs to produce 301 unique sets of outbred RIX hybrids. Each RIX set consists of full-sib individuals that are expected to be genetically identical to each other and heterozygous at loci that differ between their RIL parents. We therefore refer to full-sib RIXs within a set as "replicates." The 301 RIX sets form six genetically distinct groups defined by their RIL parents (Group 1x1 RIXs, Group 2x2 RIXs, Group 3x3 RIXs, Group 1x2 RIXs, Group 2x3 RIXs, and Group 3x1 RIXs) (Figure 1).

Experimental Setup and Plant Growth Conditions

For each of the 301 RIX sets, we planted up to 30 seeds (μ = 14.04, sd = 2.94) into 2.5" pots filled with moist Fafard 3-B potting mix (Sun Grow Horticulture). We also planted approximately 50

seeds for each of the four parental inbred lines, and approximately 25 seeds for each of the three F1s. We stratified these seeds in the dark for seven days at 4°C before moving them to a Conviron growth chamber with lights set to 12-hr days and temperatures set to 23°C days/16°C nights (Figure S3.1). After moving pots to growth chambers, we misted and bottom-watered them to saturation and allowed seeds to germinate. For all temporal measurements, we set this day - when pots were moved to the growth chamber - as Day 0. Then, for each of the next 17 days (Day 1 – 17), we took overhead images to track the number of germinated seedlings in each pot. Using these images of cumulative germination, we determined the number of new seedlings that emerged each day. For each RIX set, we scored germination time as the average time to germination for the subset of seeds that germinated. We grew these seedlings as part of a larger experiment investigating four experimental growing conditions, only two of which are analyzed in this study.

From Days 7 to 17, we transplanted seedlings into 54x28-cm flats (Kord, HC Companies, Canada), with holes for drainage, filled with moist Fafard 3-B potting mix (Figure S3.1). Plants were grown communally in flats to minimize individual differences in soil environment. Each of the 46 experimental flats contained a 4x7 grid with one vacant position (to monitor soil drying rate) and 27 randomly assigned RIX, parental, or F1 plants. For any given flat, all individuals were transplanted into their assigned positions on the same day, although if the assigned RIX replicate was too small or had not yet germinated, we filled its position later (when seedlings were sufficiently large or, in the case of insufficient germination, with a new random RIX assignment). Across all flats, we transplanted a total of 1215 seeds, leaving 27 randomly distributed positions remained vacant due to insufficient germination and early seedling death. As flats were filled with plants, we moved them to a UGA greenhouse with 12-hr supplemental light and 23°C days/16°C nights and bottom-watered them to saturation until Day 17. To maintain this day length, we used black plastic sheeting to eliminate light contamination from neighboring greenhouses. Transplantation occurred in early to mid-January (2019) when natural

daylengths were less than 12 hours. When all flats were filled, we recorded flat positions within the greenhouse in north-to-south and east-to-west directions.

On Day 18, when all experimental plants had been transplanted, we increased supplemental light time to 16-hrs, mimicking daylengths in middle to late summer at CAC. Then, on Day 19, we established two different watering treatments: well-watered and dry-down, each in half of the 46 experimental flats. In the well-watered treatment, flats were bottom-watered every other day to maintain soil saturation, mimicking the wet microsites occupied by *M. guttatus* at CAC. In the dry-down treatment, we simulated the onset of terminal drought in the microsites occupied by *M. nasutus* at CAC by withholding any additional water and allowing flats to gradually dry from saturation (Figure S3.1). We established the 23 dry-down and 23 well-watered flats positioned in the greenhouse in an alternating pattern. Of the 301 RIX sets planted, 267 germinated in sufficient numbers to be transplanted into both experimental conditions. Additionally, a handful of RIX sets were present in only one of the two treatments (four in dry-down, 14 in well-watered). In all, each treatment included an average of just over two replicates for each RIX set present in that treatment ($\mu = 2.10$, sd = 0.75). At the initiation of these treatments, all individuals remained vegetative, but RIXs varied in size; from seedlings, with only one set of true leaves, to small rosettes, with their second set of true leaves. Outbred RIX individuals frequently germinated and grew much faster than parental and F1 plants, outcompeting them for light. Because this situation resulted in underdeveloped (or even dead) F1 and parental plants, they were removed from our analyses. In total, our experiment consisted of 1159 RIX plants (582 under well-watered, and 577 under dry-down).

To monitor soil moisture change over time in each of the treatments, we calculated dry weight basis soil moisture (θ_d) at eight timepoints from Day 18 to Day 38. At each timepoint, we took small (~35g) soil samples from the vacant positions of five randomly selected flats in each treatment and recorded wet soil mass (WM). We then dried these soil samples at 60°C in a drying oven for 24-48 hrs, or until their weights stabilized, and recorded dry soil mass (DM). We discontinued measurements after Day 38 because θ_d was no longer decreasing in dry-down flats. We calculated θ_d of each measured flat as (WM – DM)/DM. For each watering treatment, we calculated the average change in θ_d per day between Days

26 and 35, the period when drying occurred most rapidly. Average θ_d of well-watered flats remained constant over the course of the experiment, with an average rate of -0.039 θ_d/Day. Alternatively, θ_d of dry-down flats decreased substantially, with an average rate of -0.336 θ_d/Day (Figure S3.2).

To determine if drying rate varied across space in the greenhouse, we tested for an effect of flat position with a repeated measures ANOVA using a linear mixed effect model with the lmer command in the "lme4" package in R (v. 3.2.3). We used a model including "Day" as a repeated measure factor, "X _position" (fixed effect), "Y_position" (fixed effect), the two-way interaction effect, and "Treatment" (random effect). This model identified a significant X_position x Y_Position interaction effect on soil moisture (Figure S3.2). Additionally, a previous dry-down experiment (Mantel & Sweigart, 2019) with much more densely sampled soil moisture measurements showed significant effects of greenhouse position on soil moisture level. We therefore included "Block" as a random effect in all models to correct for differences in soil drying across the greenhouse.

Trait Measurements

To investigate variation in RIX response to decreased water availability, we measured a large number of phenotypes under the two experimental treatments (Table 3.1). We tracked plant survival and maturation by daily inspection. We recorded the day when each plant reached three life stages: (a) budding, (b) flowering, and (c) fruiting. We scored budding as the day when a bud was first visible at the primary inflorescence of each plant. We then scored flowering as the day when a fully emerged flower, displaying an open, receptive stigma, was visible at the first flower pair (*Mimulus* plants produce pairs of flowers in a sequential progression up the inflorescence). On the day after the first flower opened, we marked and hand-pollinated one of the flowers from that pair, with pollen donated from the Catherine Creek *M. guttatus* inbred line CAC134. This line was used as common pollen donor because of its large flower number, and consistently viable pollen (90%+). The remaining flower of that pair was left unmanipulated and allowed to self-pollinate. Following initial pollinations, the few marked flowers that remained receptive were hand-pollinated a second time to minimize pollen limitation. In the rare instance that a first flower was missed during daily inspection, the affected plant was excluded from subsequent

flower and seedset measurements. Finally, we scored fruiting as the day when the marked, outcrossed flower produced a brown, dehiscent fruit. We ended the experiment on Day 89, the day when the last pollinated flower produced a mature fruit. For each surviving plant, we calculated the number of days between RIX set average germination and budding, between budding and flowering, and between flowering and fruiting. Because we found almost no variation in flower maturation (days from budding to flowering), this phenotype was not considered further.

On the day of budding, we measured the length and width of the plant's largest leaf. The relative growth rate of leaf length (Leaf Length/Day Germ to Bud) was calculated by dividing measured leaf length by the number of days between that RIX set's average germination date and that plant's budding date. The relative growth rate of leaf width (Leaf Length/Day Germ to Bud) was calculated in the same way. On the day after flowering, we again measured leaf length and width of the largest leaf, and similarly calculated relative growth rates (e.g. Leaf Length/Day Bud to Flower = (Leaf Length at Flower – Leaf Length at Bud)/Days between budding and flowering). Additionally, on the marked flower, we measured two floral traits: corolla length as the distance from the base of the calyx to the end of the bottom petals when hand straightened, and corolla width as the distance between the widest point of the bottom petal lobes. We also determined peduncle length by measuring the distance from the base of the calyx to the stem when hand straightened. Ten days after flowering, we counted the total number of flowers on each plant, including senesced flowers that had dropped their corollas, open receptive flowers, and unopened buds. We collected and counted seedset on a per fruit basis for the marked, hand-pollinated flower (outcrossed seedset) and for the self-pollinated flower (selfed seedset).

Data Analysis

To determine how watering treatment, genetic variation (*M. guttatus* ancestry), and their interaction (GxE) affected RIX phenotypes, we ran linear mixed effect models (LMMs, using the lmer command in the 'lme4' package) or generalized linear mixed models (GLMMs, using the glmer command in the 'lme4' package). Because the genetic contribution of each *M. guttatus* founder differed among the six RIX groups, we used the cbind command in R to create the variable "*M. guttatus* ancestry"

by combining three variables representing the proportion of a RIX set's ancestry derived from each of the *M. guttatus* founders respectively. We calculated p-values using the anova command in the "lmerTest" package for LMM effects, and with likelihood ratio tests using the anova command comparing our main model with models lacking each fixed and interaction effect in turn, for GLMM effects, and determined significance based on a Bonferroni correction. From each model we calculated least square means (LSMs) for the two treatments using the emmeans function in the "emmeans" package, and performed post hoc Tukey-Kramer HSD tests with α=0.05, generating letter reports with the cld command in the "multcomp" package. All models and analyses were run in R (v. 3.2.3).

To investigate phenotypic plasticity across water treatments, we used one-way ANOVAs in JMP (v. 14.0.1, SAS Institute), to compare average phenotypes for each RIX set between the two treatments (excluding RIXs that survived in only one treatment). Based on a cutoff of 1.5x the Interquartile Range (IQR) above and below the mean, many traits contained outliers, which is not ideal for ANOVA. Thus, we performed all ANOVAs with and without outliers removed, as the removal of outliers did not affect any of our conclusions, we present only analyses with outliers removed. We also tested all traits for normality using Shapiro-Wilk normality tests. Because some traits were significantly non-normal and showed evidence of different variances between treatment groups, we also used Kruskal-Wallis (K-W) Tests (a non-parametric ANOVA alternative) and Welch's Tests (robust to unequal variances) to investigate differences between treatment groups. Again, these alternate tests did not change our conclusions.

To estimate narrow-sense heritability and genetic correlations, we used a Bayesian approach with an 'animal model' using the MCMCglmm package in R (v. 3.2.3) (Wilson *et al.*, 2010). This approach uses a mixed model restricted maximum likelihood (REML) process to analyze individual-level data while considering genetic relationships between individuals. MCMCglmm links individual plant phenotype data to a pedigree file, which identifies the parents of each plant, enabling accurate estimations of heritability and genetic correlations in populations composed of individuals with different degrees of relatedness. For example, in this experiment, RIX replicates are full siblings, various RIXs are half

siblings (share one RIL parent), while other RIXs are less related. To estimate phenotypic correlations, we first calculated an average value for each measured trait within each treatment for each RIX set. Using these values, we calculated phenotypic correlations within each watering treatment as Kendall's Tau (Kendall Rank Correlation Coefficient), which is a non-parametric measure of correlation robust to outliers and non-normal data (Kendall & Gibbons, 1990). These analyses were performed in JMP (v. 14.1.0, SAS Institute). We also used Levene's Test to compare phenotypic variance between treatments; for these tests, we used average trait values for RIX sets within each watering treatment.

To investigate which plant traits were favored in each environment, we conducted phenotypic selection analyses. We used LMMs to estimate selection coefficients on one (s) or multiple (β) traits using survival, flower number, outcrossed seedset, or selfed seedset as fitness proxies, and determined significance with a Bonferroni correction (Lande & Arnold, 1983). We utilized a hurdle model approach, by first estimating coefficients using survival as the fitness proxy, and subsequently including only individuals that survived when calculating coefficients using the remaining proxies. To avoid collinearity in highly correlated traits, we retained one trait of any pair of traits with $|\tau| \geq 0.5$ in both watering treatments (Lande & Arnold, 1983; Presotto *et al.*, 2019). To enable direct comparison of the strength of selection among traits, we calculated relative fitness for each individual (for each fitness proxy) and standardized all other phenotypes to a mean of 0 and a standard deviation of 1. To estimate standardized selection differentials (s') for each trait separately we modeled relative survival, relative flower number, relative outcrossed seedset, or relative selfed seedset with LMMs with the trait of interest as a fixed effect, and with block, RIX, and day of transplant as random effects. When available we also included which lab member made each measurement as a random effect. To estimate standardized selection gradients (β') for each trait we modeled relative survival, relative flower number, relative outcrossed seedset, or relative selfed seedset with an LMM with the all traits as fixed effects, and with block, RIX, and day of transplant as random effects.

To investigate the relative contributions of genetic variation and plasticity to RIX trait differences across environments, we grouped RIXs by their performance in the dry-down treatment. For each trait, we

used a one-way ANOVA in JMP (v. 14.1.0, SAS Institute) to compare well-watered phenotypes between two groups: RIX sets with at least one replicate that survived to be measured under dry-down and RIX sets with no replicates that survived to be measured under dry-down. We reasoned that if trait values differed between these groups under non-stressful, well-watered conditions, it is an indication that genetic differences (*i.e.*, in loci controlling the trait) contributed to survival under dry-down. Additionally, we split dry-down RIXs into a reproductive group that included all RIX sets with at least one replicate producing seeds (outcrossed, selfed, or both) and a non-reproductive group in which no replicates produced any seeds (outcrossed or selfed). To investigate genetic and plastic contributions to differences in maturation rates between these two groups, we used JMP (v. 14.0.1, SAS Institute) to generate Kaplan–Meier Plots and calculate Cox Proportional Hazards.

To test whether plastic trait changes under drought were adaptive or maladaptive, we calculated a realized plasticity value for each trait, which is relative measure of plasticity: (RIX set average under dry-down – RIX set average under well-watered)/(RIX set average under well-watered). Plasticity was calculated in this way to preserve directionality of the measurement, producing a negative plasticity value when phenotypes decreased under dry-down conditions relative to well-watered conditions. Because seedset under dry-down conditions was highly zero-inflated, we adopted a hurdle model approach. We first used logistic regression in JMP (v. 14.0.1, SAS Institute) to test the effect of relative plasticity in each trait on the probability of setting seeds under dry-down conditions (RIX sets with no replicates producing any seeds under dry-down were coded as 0, RIX sets with one or more replicates producing seeds were coded as 1). Next, for RIX sets that survived under dry-down conditions, we used simple linear regression in JMP to test how relative plasticity in each trait affected the relative change in flower number (ln(x+1) transformed), outcrossed seedset, and selfed seedset in turn. These fitness measures were calculated as: (RIX set average under dry-down – RIX set average under well-watered)/(RIX set average under well-watered), allowing for the relationship between plasticity and the effect of drought on fitness to be investigated. We again ran all of these models both with outliers included and with outliers removed, and note any differences in our conclusions.

Results

Phenotypic differences within and between treatments

The *M. nasutus*-*M. guttatus* RIX population showed substantial phenotypic variation within and between water treatments (Figure 3.2A), with simulated drought causing significant reductions in the means of all vegetative and floral trait values (Tables S2 and S3). Mortality was also much higher under drought, leading to steep declines in survival at all three measured life stages (budding, flowering, and fruiting), particularly between budding and flowering (Figure 3.2B, Tables S2 and S3). We observed these same plastic shifts when comparing RIX replicates across treatments, with the average value of all traits decreasing – often substantially – under dry-down (Table S3.4). Overall, plants grown in the dry-down treatment progressed more quickly through reproduction, had much smaller leaves and flowers, and produced far fewer seeds.

In addition to these strong treatment effects, we found a significant contribution of genetic variation – due to segregating alleles from the one *M. nasutus* and three *M. guttatus* parents – to variation in RIX phenotypes within each environment. For many traits, *M. guttatus* ancestry is a significant predictor of the phenotypic variation (Table S3.3), suggesting there is substantial genetic variation within *M. guttatus* at Catherine Creek. Additionally, narrow-sense heritability values for RIX plants are moderate for most traits, ranging from 0.01 to 0.73 (Table 3.2). In general, narrow-sense heritability values were lower in the dry-down treatment (mean = 0.283) than in well-watered conditions (mean = 0.358). Although this trend could be due to increased environmental variability and a depletion of genetic variation in the dry-down treatment, lower heritability estimates might also be a statistical artifact of the much smaller sample sizes in this treatment due to higher mortality (Figure 3.2B, Table S2).

We also found evidence for GxE interactions across the two treatments. Despite a general shift toward smaller trait values under drought (Figure 3.2A, Table S3.4), RIX sets varied considerably in the magnitude and even the direction of their plastic response (Figure 3.3), with a handful RIX sets showing negligible plasticity or an increase in trait values under drought. For many traits, we also found significant interaction effects between *M. guttatus* ancestry and treatment (Table S3.2).

Genetic and phenotypic correlations

We detected strong genetic correlations between many traits, indicating pleiotropy or genetic linkage of the underlying loci (Figure 3.4). We note that most genetic correlations were estimated exclusively from plants in the well-watered treatment because heritability values in the dry-down treatment were often prohibitively low (< 0.2). As expected from previous work (Fishman *et al.*, 2002), we found strong positive genetic correlations between flower size traits (corolla length and width) and between leaf size traits (leaf length and width). We also found strong positive correlations between budding time and leaf size early (measured at budding), with plants that delayed reproduction growing larger. Later budding also led to larger flowers, but this positive correlation was fairly weak (and significant only for corolla length, not width). Somewhat less expectedly, we found strong negative correlations between vegetative traits measured early and later: plants with leaves that grew larger and faster early (germination to budding), had leaves that grew more slowly later (between budding and flowering). This result suggests there is a limit on leaf size – individuals with leaves that grew larger before initiating reproduction, grew less after budding. We also found a significant, reasonably strong negative genetic correlation between budding time (days germ to bud) and the rate of fruit ripening (days bud to fruit), suggesting a constraint on rapid reproductive development across the life cycle.

Phenotypic correlations measured under well-watered conditions tracked closely with genetic correlations (compare above and below the diagonal in Figure 3.4) but differed somewhat from phenotypic correlations under dry-down (Figure S3.3). This result is not unexpected given treatment differences in heritability (Table 3.2) and phenotypic variance (as measured by Levene's test, Table S3.5).

Which traits are advantageous under drought?

Selection analysis suggests several traits contribute to *Mimulus* fitness under drought (Table 3.3). Survival under the dry-down treatment was much higher in RIXs that initiated budding more quickly (s' = -0.88). Likewise, RIX survival was higher for plants with larger leaves (at flowering), larger flowers, and longer peduncles. For any of these traits, a significant selection differential (Table 3.3) could be caused by a genetic predisposition of some RIXs to survive better under drought – for instance, if they carry alleles

that cause them to initiate reproduction more quickly. To investigate this possibility, we compared the phenotypes in well-watered conditions of RIX sets that did and did not survive to each trait's measurement in dry-down conditions (Table 3.4). Indeed, survivors under dry-down initiated budding more quickly, had smaller leaves early, faster growing leaves late, and had longer peduncles *even in the well-watered treatment*, suggesting a genetic contribution to RIX survival under drought (we note that because several of these traits are correlated, selection on some traits is likely indirect). On the other hand, flower size in the well-watered treatment was no different between RIXs that did and did not survive under dry-down. Thus, not only is there no evidence for an intrinsic cost of larger flowers under drought (dry-down survivors do not have smaller flowers under well-watered conditions), large flowers actually confer a survival advantage (Table 3.3), presumably because they are an attribute of healthier plants.

Our selection analysis also shows that several traits were associated with fitness differences and even tradeoffs in the two environments. We detected significant selection gradients for larger plants (leaves and flowers) in the well-watered treatment, but not in the dry-down treatment (Table 3.3). Fitness effects of peduncle length actually switched between the two environments, with shorter peduncles slightly favored in the well-watered treatment and long peduncles strongly favored under dry-down. For fruit ripening (days bud to fruit), effects on fitness were also markedly different between the two treatments – faster fruit ripening was favored in *both* environments, but selection (s and β) was much stronger under dry-down conditions. Taken together, these results suggest environmental variation in water availability can select for dramatically different phenotypes related to reproductive timing and plant size.

Next, we wanted to examine the degree to which RIX fitness under drought was affected by genetic causes and plastic changes in reproductive timing. We split dry-down RIXs into two groups: a reproductive group that included all RIX sets with at least one replicate producing seeds, and a non-reproductive group in which no replicates produced any seeds. Early in the life cycle (germination to budding), success of the reproductive group was almost entirely due to a genetic predisposition for earlier budding: even under well-watered conditions, the reproductive group was much more likely to produce

buds on any given day than the non-reproductive group (hazards ratio = 3.18, $p < 0.0001$, compare blue lines in Figure 3.5A). Plasticity during this period of the life cycle was modest with the reproductive group only slightly more likely to bud under dry-down than well-watered conditions (hazards ratio = 1.73, $p < 0.0001$, compare light red vs. light blue lines in Figure 3.5A). On the other hand, plastic changes were much more important to seed production later in the life cycle (budding to fruiting). During this time period, the reproductive and non-reproductive groups showed no significant difference in their propensity for producing a mature fruit under well-watered conditions (hazards ratio =0.95, $p = 0.6975$, compare blue lines in Figure 3.5B). However, reproductive RIXs showed a large plastic response with this group more than twice as likely to produce a mature fruit on any given day under dry-down than well-watered conditions (hazards ratio = 2.38, $p < 0.0001$, compare light red vs. light blue lines in Figure 3.5B). Taken together, these analyses suggest RIX success under dry-down was twofold: reproductive individuals carried alleles for faster budding and responded plastically to increase the rate of fruit ripening.

Given that plastic changes toward smaller trait values were ubiquitous under the drought treatment (Figure 3.2, Table S3.4), a key question is whether any of the changes were adaptive. Does a plastic shift toward smaller leaves or flowers confer a fitness advantage under drought (perhaps because it facilitates diverting resources toward seed production) or is it simply an indication of unhealthy plants? For nearly all traits, we observed a positive correlation between realized plasticity values (i.e., relative difference in trait value in well-watered vs. dry-down) and seed production (Figure 3.6), consistent with a maladaptive plastic response (i.e., sick plants). That is, the more positive a plant's plastic shift under dry-down (either because of negligible plasticity or even an increase in trait value), the more seeds it produced. Plants with strongly negative plastic shifts in dry-down (towards smaller trait values) produced fewer seeds. A notable exception to this pattern was for fruit ripening: a larger decrease in the number of days from bud to fruit was associated with higher seed production. In other words, a plastic shift toward faster fruit ripening under dry-down conditions appears to have been adaptive for this RIX population.

Discussion

Divergent adaptation to the abiotic environment is thought to be a major contributor to reproductive isolation between species, but it is unclear how often such ecologically-based barriers erode upon secondary contact. Here, we discovered evidence of genetic tradeoffs that might maintain boundaries between closely related species of *Mimulus* adapted to distinct hydrological microhabitats at Catherine Creek. In our experimental hybrid (RIX) population of *M. guttatus* and *M. nasutus*, different suites of traits were favored in the two water environments, driven in part by positive genetic correlations between flowering time and plant size. Similar to previous studies of variation within *M. guttatus* (Mojica & Kelly, 2010; Mojica *et al.*, 2012; Troth *et al.*, 2018), being "fast and small" was associated with much higher survival under water-stressed conditions, but being "slow and big" led to higher fecundity in the more benign, well-watered treatment. In terms of drought adaptation, however, the story is likely more complicated – the "fast and small" strategy is somewhat opposed by a negative genetic correlation between the speed of bud initiation and fruit ripening. With this genetic constraint impeding drought adaptation, it is notable that fruit ripening was the one trait with evidence of drought-induced adaptive plasticity. The general picture emerging from this and our previous work (Mantel and Sweigart 2019) is that divergent habitat adaptation is an important component of reproductive isolation and species maintenance between *M. guttatus* and *M. nasutus* in secondary sympatry.

Mimulus drought response involves genetic differences and plastic changes

Across our admixed population drought stress was, unsurprisingly, very costly, leading to substantial reductions in plant survival, growth and fitness. However, the fitness effects of drought stress did vary dramatically among RIXs, with some responding much more effectively than others. In investigating the possible phenotypic basis of these differences, we identified multiple phenotypes across the plants' life cycle contributing to success under drought conditions. It is important to note that our RIX population both includes allelic differences that vary between species (*M. guttatus* vs *M. nasutus* alleles contributing to phenotypic variance) as well as allelic differences that differ between the three *M. guttatus* parental lines, seen as a significant signal of *M. guttatus* ancestry and interaction effects for many traits

(Table S3.2). Additionally, the timing of the induction of our dry-down treatment likely influenced patterns of survival and reproductive success. Because dry-down flats were allowed to dry from saturation, plants in both conditions experienced relatively similar conditions early in life, and the dry-down treatment didn't see a 50% decrease in soil moisture until day 29, very near the peak of budding (Figure 3.2A). Immediately following budding, when the flower is maturing, may be an important developmental time that coincided with extremely decreased water availability for many plants, leading to increased mortality at this stage (Figure 3.2B). Once the flower has developed, fruit maturation may be a less water intensive process, leading to less mortality between flowering and fruiting. Still, given this experimental setup, plants which developed at different speeds likely experienced drought differently, influencing their phenotypes, as well as whether enough time existed to mount a plastic response at any given stage of growth. Our experimental dry-down treatment aimed to simulate conditions that plants experience in microhabitats occupied by *M. nasutus* at CAC, but due to these limitations, further experiments of plants growing in the field will be necessary to establish the degree to which relationships observed here are relevant to natural conditions.

Faster flowering time is a key drought escape strategy, frequently implicated in adaptation across systems. As expected, we find that a genetic predisposition for faster budding (measured as days from germination to budding here) enhances survival, allows plants to produce more flowers, and increases the probability of successfully setting seed under the dry-down treatment. As our experiment was conducted under an inductive photoperiod (16hr days), known genetic divergence in critical photoperiod (Friedman & Willis, 2013; Fishman *et al.*, 2014a), which promotes early-season flowering in *M. nasutus,* when natural daylengths are shorter, does not contribute to this pattern. Under natural conditions, critical photoperiod divergence likely facilitates drought escape to an even greater degree. In addition to flowering time, reproductive timing later in the life cycle, i.e., the speed of fruit ripening (measured here as days from budding to fruiting) is also important for success under drought. While more rapid fruit ripening is favored in both water treatments, this relationship is enhanced under drought, showing upwards of 6x stronger selection (Table 3.3). While a genetic component to fruit ripening does exist,

heritability is lower than that of phenotypes earlier in the life cycle, such as flowering time (Table 3.2), however a much clearer plastic shift in this fruit ripening exists (Figure 3.5) accompanied by a signal of adaptive plasticity (Figure 3.6, Table S3.6). Previous work would suggest that these adaptive alleles are presumably mostly derived from *M. nasutus* (Mantel & Sweigart, 2019).

A more speculative interpretation of our results is necessary to explain selection on peduncle length under the dry-down treatment. Long peduncles are strongly favored under drought stress (Table 3.3). Research in crop systems like wheat indicate that this could be due to sugar accumulation in peduncles under water limiting conditions, allowing for more rapid and more effective seed maturation under drought conditions in plants with longer peduncles (Bazargani *et al.*, 2012; Zhang *et al.*, 2015, 2020; Srivastava *et al.*, 2017).

Genetic correlations facilitate and impede drought adaptation

We also observed numerous and complex genetic correlations between the traits we measured, many of which are likely to either impede or facilitate drought adaptation in these species (Figure 3.4). As discussed above, rapid flowering is strongly correlated with reduced leaf size at budding, which is in turn correlated with more rapid vegetative growth later in life (i.e., between budding and flowering). However, in a seeming contradiction, leaf size at budding is also positively correlated with leaf size at flowering (individuals with large leaves at bud still have large leaves at flower, despite growing more slowly). This is likely explained both by the relatively short amount of time between budding and flowering, as well as large individuals which flowered slowly reaching an upper bound of leaf size before flowering. Additionally, flower size shows a negative genetic correlation with peduncle length (larger flowers are associated with shorter peduncles), and short peduncles are, in turn, genetically correlated with more rapid fruit ripening. To summarize, in general, we observed positive correlations between flowering time and size traits, meaning that individuals which budded more quickly were smaller. However, a notable exception being peduncle length, possibly implying peduncles are unique in that they grow continuously following bud initiation. Moreover, a strong negative correlation between reproductive timing across the life cycle was observed. The faster a plant initiates budding, for example, the slower it ripens its fruit

following fertilization. Could this be due to a developmental/genetic tradeoff between these life stages? Alternatively, this pattern may emerge if limited resources (i.e., photosynthetic tissue) in small fast budding individuals are less able to support rapid fruit ripening.

Another potentially important implication of these correlations stems from the negative genetic correlation observed between budding time and peduncle length. This relationship would seem to facilitate adaptation to drought conditions, as both more rapid budding and longer peduncles were strongly selected for under our dry-down treatment (Table 3.3). On the other hand, the fact that each of these traits is negatively correlated with fruit ripening is likely to constrain drought adaptation, as the optimal phenotypic strategy, fast budding, long peduncles, and fast fruit ripening is opposed by these correlations. However, we also observed a signature of adaptive plasticity in speed of fruit ripening. Could plasticity circumvent this constraint, and allow a more optimal phenotypic combination?

Do genetic tradeoffs contribute to ecological isolation?

Overall, previous studies have established a negative correlation between genetic divergence and recombination at CAC (Kenney & Sweigart, 2016) suggesting that there might be a substantial genome-wide cost to harboring *M. nasutus* alleles in an *M. gutattus* background. But, at Catherine Creek, as well as other sites where these species co-occur, widespread introgression raises the possibility that species boundaries could begin to degrade despite this. Indeed, in highly heterogeneous environments like CAC, some patches dry out much earlier than others, making it easy to envision introgression from *M. nasutus* being maintained to allow *M. guttatus* to more effectively survive in water stressed environments. This possibility seems to be supported by studies which have identified variation within allopatric populations of *M. guttatus*, that is maintained by environmental fluctuations across years (Mojica & Kelly, 2010; Mojica *et al.*, 2012; Troth *et al.*, 2018), or by large-scale spatial heterogeneity in water availability (Hall & Willis, 2006; Hall *et al.*, 2010). As outlined above, we see evidence of genetic tradeoffs in flowering time and plant size, with differential selection on these traits in our two water regimes. This tradeoff may mean then that introgression of *M. nasutus* alleles conferring drought adapted phenotypes may be selected against in more consistently wet habitat, or in wetter years, helping to resist hybridization. Moreover, as

under inductive photoperiod *M. nasutus* and *M. guttatus* bud at about the same time, this tradeoff is likely both due to species' differences, as well as allelic variation within our *M. guttatus* parental lines, making predicting the effect of introgression of these alleles even more difficult.

Additionally, we found no evidence of a cost to large leaves or flowers under dry-down conditions (Table 3.3), rather more rapid budding lead to overall smaller leaves and flowers due to the genetic correlations as described above. This result is unexpected, as it contradicts work in many other systems, showing that vegetative and floral investment is water and recourse intensive and decreases survival under drought (Carroll *et al.*, 2001; Opedal *et al.*, 2016; Gallagher & Campbell, 2017; Descamps *et al.*, 2018; Phillips *et al.*, 2018).

To conclude, our results suggest that drought adaptation is multifaceted, involving many phenotypes across the life cycle including, but likely not limited to flowering time differences (Friedman & Willis, 2013; Fishman *et al.*, 2014a), differences in plant size, growth, and developmental strategies. As many of these traits are expected to be highly polygenic (Fishman *et al.*, 2002), we might expect phenotypic mismatches (Thompson *et al.*, 2022), or maladapted intermediate phenotypes upon the formation of hybrids. With this framework it will be necessary to map the causal loci responsible for these drought response traits and investigate their phenotypic and fitness effects under benign and water limiting environments to fully understand how local adaptation shapes species boundaries in this system.

References

Arnegard ME, McGee MD, Matthews B, Marchinko KB, Conte GL, Kabir S, Bedford N, Bergek S, Chan YF, Jones FC, *et al.* 2014. Genetics of ecological divergence during speciation. *Nature* **511**: 307–311.

Bazargani MM, Hajirezaei M-R, Salekdeh GH, Bushehri A-AS, Falahati-Anbaran M, Moradi F, Naghavi M-R, Ehdaie B. 2012. A view on the role of metabolites in enhanced stem reserves remobilization in wheat under drought during grain filling. *Australian Journal of Crop Science*.

Berger JD, Ludwig C. 2014. Contrasting adaptive strategies to terminal drought-stress gradients in Mediterranean legumes: phenology, productivity, and water relations in wild and domesticated Lupinus luteus L. *Journal of Experimental Botany* **65**: 6219–6229.

Brandvain Y, Kenney AM, Flagel L, Coop G, Sweigart AL. 2014. Speciation and Introgression between Mimulus nasutus and Mimulus guttatus. *PLOS Genetics* **10**: e1004410.

Carroll AB, Pallardy SG, Galen C. 2001. Drought stress, plant water status, and floral trait expression in fireweed, Epilobium angustifolium (Onagraceae). *American Journal of Botany* **88**: 438–446.

Coughlan JM, Brown MW, Willis JH. 2021. The genetic architecture and evolution of life-history divergence among perennials in the Mimulus guttatus species complex. *Proceedings of the Royal Society B: Biological Sciences* **288**: 20210077.

Coyne JA, Orr HA. 2004. *Speciation*. Oxford, New York: Oxford University Press.

Descamps C, Quinet M, Baijot A, Jacquemart A-L. 2018. Temperature and water stress affect plant–pollinator interactions in Borago officinalis (Boraginaceae). *Ecology and Evolution* **8**: 3443–3456.

Dillon LS. 1970. Speciation and Changing Environment. *American Zoologist* **10**: 27–39.

Fishman L, Kelly AJ, Willis JH. 2002. Minor Quantitative Trait Loci Underlie Floral Traits Associated with Mating System Divergence in Mimulus. *Evolution* **56**: 2138–2155.

Fishman L, Sweigart AL, Kenney AM, Campbell S. 2014. Major quantitative trait loci control divergence in critical photoperiod for flowering between selfing and outcrossing species of monkeyflower (Mimulus). *The New Phytologist* **201**: 1498–1507.

Franks SJ. 2011. Plasticity and evolution in drought avoidance and escape in the annual plant Brassica rapa. *New Phytologist* **190**: 249–257.

Franks SJ, Weis AE. 2009. Climate change alters reproductive isolation and potential gene flow in an annual plant. *Evolutionary Applications* **2**: 481–488.

Friedman J, Willis JH. 2013. Major QTLs for critical photoperiod and vernalization underlie extensive variation in flowering in the Mimulus guttatus species complex. *The New Phytologist* **199**: 571–583.

Gallagher MK, Campbell DR. 2017. Shifts in water availability mediate plant–pollinator interactions. *New Phytologist* **215**: 792–802.

Hall MC, Lowry DB, Willis JH. **2010**. Is local adaptation in Mimulus guttatus caused by trade-offs at individual loci? *Molecular Ecology* **19**: 2739–2753.

Hall MC, Willis JH. **2006**. Divergent Selection on Flowering Time Contributes to Local Adaptation in Mimulus Guttatus Populations. *Evolution* **60**: 2466–2477.

Husband BC, Schemske DW. **1996**. Evolution of the Magnitude and Timing of Inbreeding Depression in Plants. *Evolution* **50**: 54–70.

Jiggins CD, Mallet J. **2000**. Bimodal hybrid zones and speciation. *Trends in Ecology & Evolution* **15**: 250–255.

Kendall MG, Gibbons JD. **1990**. *Rank correlation methods*. London; New York, NY: E. Arnold ; Oxford University Press.

Kenney AM, Sweigart AL. **2016**. Reproductive isolation and introgression between sympatric Mimulus species. *Molecular Ecology* **25**: 2499–2517.

Kiang YT, Hamrick JL. **1978**. Reproductive Isolation in the Mimulus guttatus M. nasutus Complex. *The American Midland Naturalist* **100**: 269–276.

Kleindorfer S, O'Connor JA, Dudaniec RY, Myers SA, Robertson J, Sulloway FJ. **2014**. Species Collapse via Hybridization in Darwin's Tree Finches. *The American Naturalist* **183**: 325–341.

Lande R, Arnold SJ. **1983**. The Measurement of Selection on Correlated Characters. *Evolution* **37**: 1210–1226.

Lee C-R, Mitchell-Olds T. **2011**. Quantifying effects of environmental and geographical factors on patterns of genetic differentiation. *Molecular Ecology* **20**: 4631–4642.

Lin Y-P, Lu C-Y, Lee C-R. **2020**. The climatic association of population divergence and future extinction risk of Solanum pimpinellifolium. *AoB PLANTS* **12**: plaa012.

Mantel SJ, Sweigart AL. **2019**. Divergence in drought-response traits between sympatric species of Mimulus. *Ecology and Evolution* **9**: 10291–10304.

Martin NH, Willis JH. **2007**. Ecological Divergence Associated with Mating System Causes Nearly Complete Reproductive Isolation between Sympatric Mimulus Species. *Evolution* **61**: 68–82.

Mojica JP, Kelly JK. **2010**. Viability selection prior to trait expression is an essential component of natural selection. *Proceedings of the Royal Society B: Biological Sciences* **277**: 2945–2950.

Mojica JP, Lee YW, Willis JH, Kelly JK. **2012**. Spatially and temporally varying selection on intrapopulation quantitative trait loci for a life history trade-off in Mimulus guttatus. *Molecular Ecology* **21**: 3718–3728.

Nosil P. **2012**. *Ecological Speciation*. Oxford: Oxford University Press.

Nosil P, Vines TH, Funk DJ. **2005**. Reproductive Isolation Caused by Natural Selection Against Immigrants from Divergent Habitats. *Evolution* **59**: 705–719.

Opedal ØH, Listemann J, Albertsen E, Armbruster WS, Pélabon C. **2016**. Multiple Effects of Drought on Pollination and Mating-System Traits in Dalechampia scandens. *International Journal of Plant Sciences* **177**: 682–693.

Phillips BB, Shaw RF, Holland MJ, Fry EL, Bardgett RD, Bullock JM, Osborne JL. **2018**. Drought reduces floral resources for pollinators. *Global Change Biology* **24**: 3226–3235.

Presotto A, Hernández F, Mercer KL. **2019**. Phenotypic selection under two contrasting environments in wild sunflower and its crop–wild hybrid. *Evolutionary Applications* **12**: 1703–1717.

Rieseberg LH, Raymond O, Rosenthal DM, Lai Z, Livingstone K, Nakazato T, Durphy JL, Schwarzbach AE, Donovan LA, Lexer C. **2003**. Major Ecological Transitions in Wild Sunflowers Facilitated by Hybridization. *Science* **301**: 1211–1216.

Rundle HD, Nosil P. **2005**. Ecological speciation. *Ecology Letters* **8**: 336–352.

Safran RJ, Scordato ESC, Symes LB, Rodríguez RL, Mendelson TC. **2013**. Contributions of natural and sexual selection to the evolution of premating reproductive isolation: a research agenda. *Trends in Ecology & Evolution* **28**: 643–650.

Sambatti JBM, Strasburg JL, Ortiz-Barrientos D, Baack EJ, Rieseberg LH. **2012**. Reconciling Extremely Strong Barriers with High Levels of Gene Exchange in Annual Sunflowers. *Evolution* **66**: 1459–1473.

Schluter D. **2000**. *The Ecology of Adaptive Radiation*. OUP Oxford.

Schluter D. **2009**. Evidence for Ecological Speciation and Its Alternative. *Science* **323**: 737–741.

Sobel JM, Chen GF, Watt LR, Schemske DW. **2010**. The Biology of Speciation. *Evolution* **64**: 295–315.

Srivastava A, Srivastava P, Sharma A, Sarlach RS, Bains NS. **2017**. Effect of stem reserve mobilization on grain filling under drought stress conditions in recombinant inbred population of wheat. *Journal of Applied and Natural Science* **9**: 1–5.

Sweigart AL, Willis JH. **2003**. Patterns of Nucleotide Diversity in Two Species of Mimulus Are Affected by Mating System and Asymmetric Introgression. *Evolution* **57**: 2490–2506.

Taylor EB, Boughman JW, Groenenboom M, Sniatynski M, Schluter D, Gow JL. **2006**. Speciation in reverse: morphological and genetic evidence of the collapse of a three-spined stickleback (Gasterosteus aculeatus) species pair. *Molecular Ecology* **15**: 343–355.

Thompson KA, Peichel CL, Rennison DJ, McGee MD, Albert AYK, Vines TH, Greenwood AK, Wark AR, Brandvain Y, Schumer M, *et al.* 2022. Analysis of ancestry heterozygosity suggests that hybrid incompatibilities in threespine stickleback are environment dependent. *PLOS Biology* **20**: e3001469.

Thompson KA, Urquhart-Cronish M, Whitney KD, Rieseberg LH, Schluter D. **2021**. Patterns, Predictors, and Consequences of Dominance in Hybrids. *The American Naturalist* **197**: E72–E88.

Troth A, Puzey JR, Kim RS, Willis JH, Kelly JK. **2018**. Selective trade-offs maintain alleles underpinning complex trait variation in plants. *Science* **361**: 475–478.

Vonlanthen P, Bittner D, Hudson AG, Young KA, Müller R, Lundsgaard-Hansen B, Roy D, Di Piazza S, Largiader CR, Seehausen O. **2012**. Eutrophication causes speciation reversal in whitefish adaptive radiations. *Nature* **482**: 357–362.

Wilson AJ, Réale D, Clements MN, Morrissey MM, Postma E, Walling CA, Kruuk LEB, Nussey DH. **2010**. An ecologist's guide to the animal model. *Journal of Animal Ecology* **79**: 13–26.

Zhang J, Chen W, Dell B, Vergauwen R, Zhang X, Mayer J, Van den Ende W. **2015**. Wheat genotypic variation in dynamic fluxes of WSC components in different stem segments under drought during grain filling. *Frontiers in Plant Science* **6**.

Zhang P, Liu Y, Li M, Ma J, Wang C, Su J, Yang D. **2020**. Abscisic acid associated with key enzymes and genes involving in dynamic flux of water soluble carbohydrates in wheat peduncle under terminal drought stress. *Plant Physiology and Biochemistry* **151**: 719–728.

Table 3.1: Measured traits included in this paper their units.

Trait	Units
Days to Germination	days
Survival to Bud	%
Days Germ to Bud	days
Leaf Length at Bud	mm
Leaf Width at Bud	mm
Leaf Length/Day Germ to Bud	mm/day
Leaf Width/Day Germ to Bud	mm/day
Survival to Flower	%
Leaf Length at Flower	mm
Leaf Width at Flower	mm
Leaf Length/Day Bud to Flower	mm/day
Leaf Width/Day Bud to Flower	mm/day
Corolla Length	mm
Corolla Width	mm
Peduncle Length	mm
Number of Flowers	flowers/plant
Survival to Fruit	%
Days Bud to Fruit	days
Outcrossed Seedset	seeds/fruit
Selfed Seedset	seeds/fruit

Table 3.2: Narrow-sense heritability (H^2) estimates for survival as well as each measured trait in each watering regime, well-watered (WW) and dry-down (DD). Lower and upper 95% credible intervals are represented as L95 and U95 respectively. H^2 estimates with credible intervals that don't overlap zero are shown in bold. Asterisks indicate traits with non-overlapping credible intervals in the two watering regimes.

	WW			DD		
	H^2	L95	U95	H^2	L95	U95
Survival to Bud	**0.63**	**0.36**	**0.84**	**0.73**	**0.61**	**0.83**
Days Germ to Bud	**0.52**	**0.43**	**0.60**	**0.56**	**0.46**	**0.65**
Leaf Length at Bud	**0.50**	**0.41**	**0.59**	**0.33**	**0.21**	**0.45**
Leaf Width at Bud	**0.50**	**0.41**	**0.59**	**0.28**	**0.16**	**0.41**
Leaf Length/Day Germ to Bud	**0.23**	**0.12**	**0.34**	0.13	0.00	0.24
Leaf Width/Day Germ to Bud	**0.22**	**0.11**	**0.34**	0.09	0.00	0.20
Survival to Flower	**0.59**	**0.34**	**0.81**	**0.72**	**0.62**	**0.82**
Leaf Length at Flower*	**0.18**	**0.08**	**0.28**	0.01	0.00	0.07
Leaf Width at Flower	0.08	0.00	0.19	0.11	0.00	0.30
Leaf Length/Day Bud to Flower	**0.26**	**0.17**	**0.36**	0.10	0.00	0.27
Leaf Width/Day Bud to Flower	**0.39**	**0.29**	**0.48**	0.20	0.00	0.37
Corolla Length	**0.36**	**0.25**	**0.46**	0.10	0.00	0.29
Corolla Width*	**0.41**	**0.31**	**0.51**	0.08	0.00	0.27
Peduncle Length	**0.30**	**0.20**	**0.40**	**0.34**	**0.19**	**0.50**
Number of Flowers	**0.28**	**0.14**	**0.40**	**0.67**	**0.20**	**1.00**
Survival to Fruit	**0.55**	**0.29**	**0.80**	**0.63**	**0.51**	**0.75**
Days Bud to Fruit	**0.24**	**0.13**	**0.34**	0.07	0.00	0.30
Outcrossed Seed set	**0.21**	**0.09**	**0.33**	0.13	0.00	0.36
Selfed Seed set	**0.36**	**0.25**	**0.48**	0.08	0.00	0.29

Table 3.3: Selection coefficients for eight measured traits calculated as a function of relative survival, relative flower number 10 days after first flower, relative outcrossed seedset per fruit, and relative selfed seedset per fruit. A) Standardized selection differentials (s') and their standard errors (SE) are shown. Significant p-values are shown in bold, significance was determined using a Bonferroni correction of $\alpha = 0.0063$. B) Standardized selection gradients (β') and their standard errors (SE) are shown.

A)

	Survival			Survivor Number of Flowers						Survivor Outcrossed Seedset						Survivor Selfed Seedset					
	DD			WW			DD			WW			DD			WW			DD		
Phenotype	s'	SE	p	s'	SE	p	s'	SE	p	s'	SE	p	s'	SE	p	s'	SE	p	s'	SE	p
Days Germ to Bud	**-0.88**	**0.06**	**<0.0001**	**-0.10**	**0.03**	**0.0024**	**-1.08**	**0.14**	**<0.0001**	**0.09**	**0.03**	**0.0046**	-0.69	0.37	0.0667	0.04	0.03	0.1860	**-2.40**	**0.50**	**<0.0001**
Leaf Length at Bud	-0.21	0.08	0.0130	**0.09**	**0.03**	**0.0027**	-0.13	0.12	0.2930	**0.18**	**0.03**	**<0.0001**	-0.40	0.28	0.1593	**0.18**	**0.03**	**<0.0001**	**-1.72**	**0.43**	**0.0002**
Leaf Length at Flower	0.32	0.12	0.0080	**0.24**	**0.03**	**<0.0001**	**0.47**	**0.09**	**<0.0001**	**0.16**	**0.03**	**<0.0001**	-0.20	0.30	0.4924	**0.17**	**0.03**	**<0.0001**	0.42	0.58	0.4743
Leaf Width at Flower	**0.34**	**0.12**	**0.0047**	**0.25**	**0.03**	**<0.0001**	**0.51**	**0.09**	**<0.0001**	**0.16**	**0.03**	**<0.0001**	0.40	0.30	0.1906	**0.17**	**0.03**	**<0.0001**	1.45	0.57	0.0135
Leaf Length/Day Bud to Flower	**0.41**	**0.11**	**0.0004**	**0.14**	**0.03**	**<0.0001**	**0.40**	**0.10**	**0.0001**	-0.04	0.03	0.2280	0.11	0.29	0.7207	0.00	0.03	0.9220	1.04	0.46	0.0266
Corolla Length	**0.43**	**0.10**	**0.0001**	**0.17**	**0.03**	**<0.0001**	**0.45**	**0.09**	**<0.0001**	**0.11**	**0.03**	**0.0002**	0.60	0.30	0.0502	0.08	0.03	0.0112	0.05	0.52	0.9216
Peduncle Length	**0.36**	**0.11**	**0.0012**	0.04	0.03	0.2260	**0.60**	**0.09**	**<0.0001**	**-0.10**	**0.03**	**0.0010**	**0.92**	**0.26**	**0.0007**	-0.05	0.03	0.1290	**1.41**	**0.40**	**0.0010**
Days Bud to Fruit	-	-	-	-0.03	0.03	0.2140	-0.21	0.12	0.0744	**-0.23**	**0.03**	**<0.0001**	**-1.21**	**0.24**	**<0.0001**	**-0.14**	**0.03**	**<0.0001**	**-1.32**	**0.46**	**0.0052**

B)

	Survivor Number of Flowers						Survivor Outcrossed Seedset						Survivor Selfed Seedset					
	WW			DD			WW			DD			WW			DD		
Phenotype	β'	SE	p	β'	SE	p	β'	SE	p	β'	SE	p	β'	SE	p	β'	SE	p
Days Germ to Bud	**-0.20**	**0.04**	**<0.0001**	**-0.97**	**0.23**	**<0.0001**	0.01	0.04	0.7775	0.18	0.51	0.7199	-0.08	0.04	0.0885	-0.70	0.78	0.3714
Leaf Length at Bud	**0.50**	**0.18**	**0.0066**	0.57	1.04	0.5873	-0.28	0.17	0.0933	-0.12	2.53	0.9616	-0.03	0.19	0.8928	0.86	3.84	0.8241
Leaf Length at Flower	-0.31	0.17	0.0686	0.04	0.96	0.9646	0.29	0.16	0.0700	-0.54	2.33	0.8189	0.14	0.18	0.4363	-1.64	3.52	0.6427
Leaf Width at Flower	**0.15**	**0.04**	**0.0005**	0.16	0.18	0.3832	**0.10**	**0.04**	**0.0187**	0.62	0.41	0.1359	0.08	0.05	0.0884	0.39	0.67	0.5651
Leaf Length/Day Bud to Flower	**0.34**	**0.14**	**0.0171**	0.08	0.95	0.9349	-0.25	0.13	0.0555	-0.88	2.30	0.7041	-0.14	0.14	0.3254	1.08	3.55	0.7620
Corolla Length	**0.06**	**0.03**	**0.0360**	0.13	0.11	0.2425	**0.07**	**0.03**	**0.0204**	0.43	0.26	0.1035	0.03	0.03	0.3930	-0.51	0.40	0.2110
Peduncle Length	-0.02	0.03	0.5580	**0.34**	**0.16**	**0.0303**	**-0.07**	**0.03**	**0.0149**	**0.95**	**0.34**	**0.0064**	-0.05	0.03	0.0941	**1.35**	**0.53**	**0.0121**
Days Bud to Fruit	-0.01	0.03	0.8458	**-0.27**	**0.10**	**0.0064**	**-0.21**	**0.03**	**<0.0001**	**-1.12**	**0.23**	**<0.0001**	**-0.10**	**0.03**	**0.0007**	**-1.05**	**0.43**	**0.0168**

Note: Model information given in Appendix 1.
A) s' as a function of relative survival was only calculated under dry-down conditions due to near complete survival under well watered conditions.
B) β' as a function of relative survival was not calculated under either watering regime due to insufficient variance in survival to accurately fit a model containing the necessary fixed effects.

Table 3.4: Results of ANOVAs testing for differences in average phenotype under well-watered conditions between groups of RIXs which survived to be measured under dry-down conditions (Measured, M), and those which did not (Unmeasured, U). Significant p-values are shown in bold, significance was determined using a Bonferroni correction of $\alpha = 0.0038$.

		N	μ	SE	*F*	*p*
Days Germ to Bud	M	221	29.65	0.33	**68.94**	**<0.0001**
	U	51	35.98	0.69		
Leaf Length at Bud	M	223	45.36	1.01	**33.86**	**<0.0001**
	U	52	58.87	2.09		
Leaf Width at Bud	M	223	37.43	0.80	**19.36**	**<0.0001**
	U	52	45.53	1.65		
Leaf Length/Day Germ to Bud	M	224	1.51	0.02	3.90	0.0494
	U	52	1.62	0.05		
Leaf Width/Day Germ to Bud	M	224	1.25	0.02	0.10	0.7501
	U	52	1.26	0.04		
Leaf Length at Flower	M	127	65.40	1.09	7.52	0.0065
	U	142	69.52	1.03		
Lead Width at Flower	M	128	54.73	0.77	0.11	0.7453
	U	142	54.38	0.73		
Leaf Length/Day Bud to Flower	M	130	2.40	0.07	**131.18**	**<0.0001**
	U	140	1.28	0.07		
Leaf Width/Day Bud to Flower	M	131	1.99	0.07	**126.12**	**<0.0001**
	U	140	0.87	0.07		
Corolla Length	M	130	23.01	0.32	0.06	0.8148
	U	143	23.11	0.30		
Corolla Width	M	131	17.31	0.31	1.29	0.2578
	U	143	16.83	0.29		
Peduncle Length	M	129	27.51	0.50	**22.72**	**<0.0001**
	U	142	24.23	0.48		
Days Bud to Fruit	M	102	36.77	0.33	0.69	0.4078
	U	159	36.42	0.27		

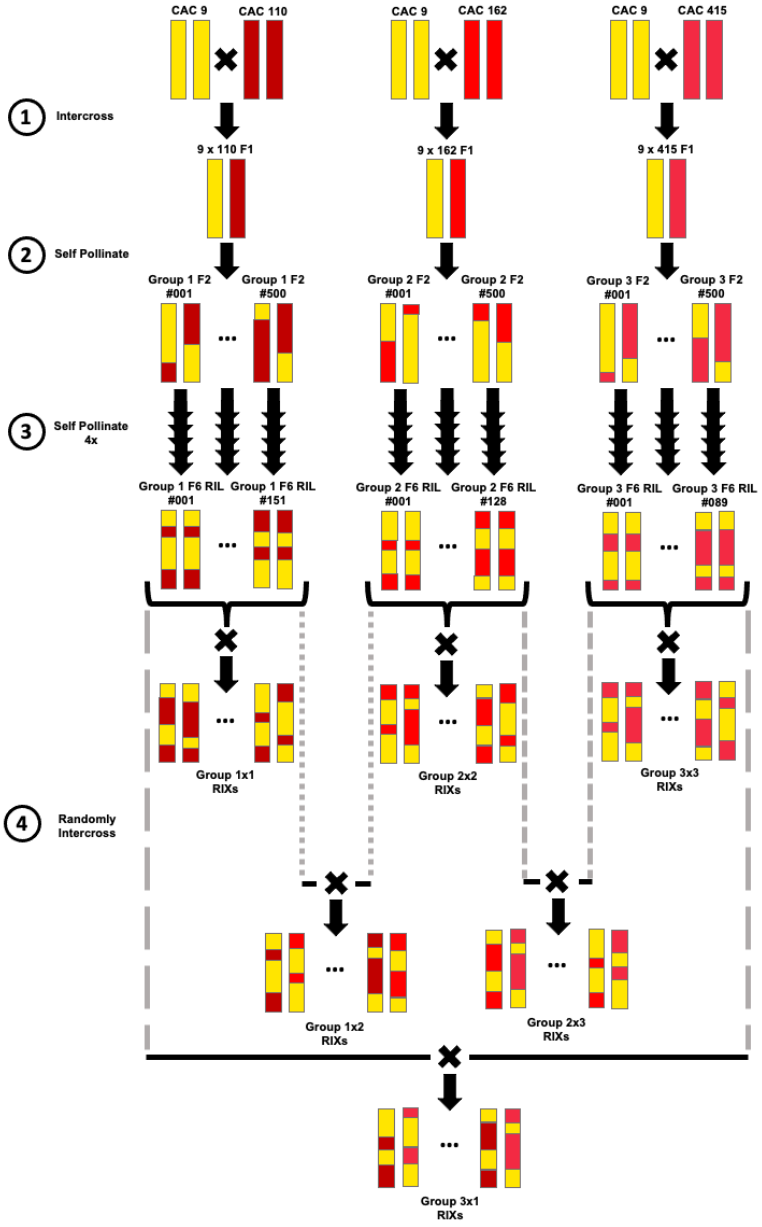
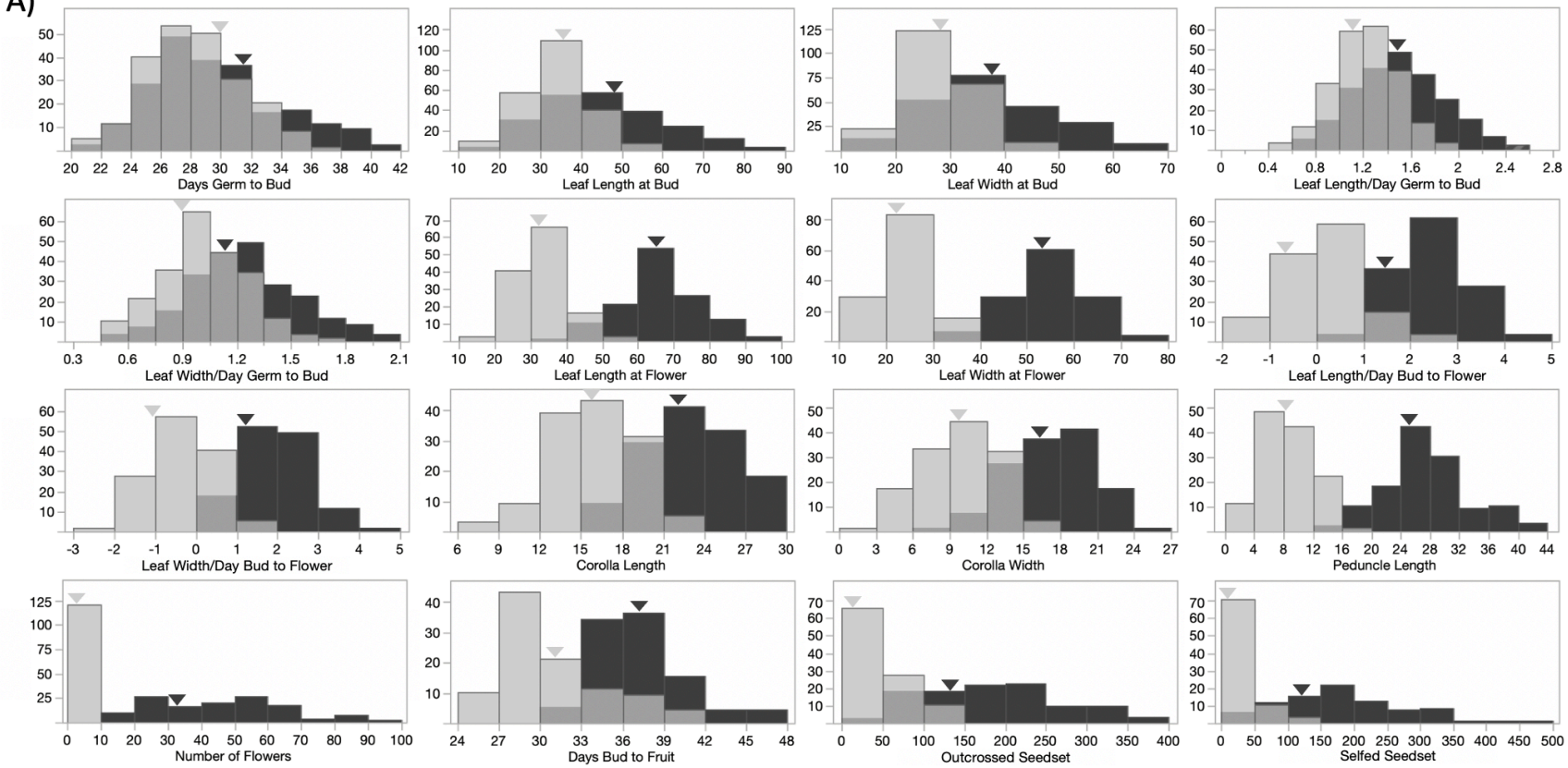


Figure 3.1: Overview of the crossing scheme used to produce the mapping population used in the experiment. (1) Three individuals of the Catherine Creek (CAC) derived *M. nasutus* inbred line CAC 9 acted as maternal parents in crosses to three distinct CAC derived *M. guttatus* inbred lines, CAC 110, CAC 162, and CAC 415. (2) We self-pollinated one F1 individual from each cross to produce 500 F2 individuals in each family (Fam 1, Fam 2, and Fam 3 respectively). (3) We self-pollinated these F2 individuals 4 additional generations, with single seeds descent, producing three F6 recombinant inbred line populations (RILs) of the same names. Due to line extinction during inbreeding, 151, 128, and 89 RILs respectively (368 total) remained at the F6 generation. (4) We then randomly intercrossed individuals from these RIL populations to produce F1 recombinant inbred intercrossed lines (RIXs) in 6 distinct groups such that each Family served as the maternal parent for two groups (Fam 1 x Fam 1, Fam 2 x Fam 2, Fam 3 x Fam 3, Fam 1 x Fam 2, Fam 2 x Fam 3, and Fam 3 x Fam 1). We then grew these RIX individuals under our various experimental conditions.

A)


Days Germ to Bud
Leaf Length at Bud
Leaf Width at Bud
Leaf Length/Day Germ to Bud
Leaf Width/Day Germ to Bud
Leaf Length at Flower
Leaf Width at Flower
Leaf Length/Day Bud to Flower
Leaf Width/Day Bud to Flower
Corolla Length
Corolla Width
Peduncle Length
Number of Flowers
Days Bud to Fruit
Outcrossed Seedset
Selfed Seedset

B)

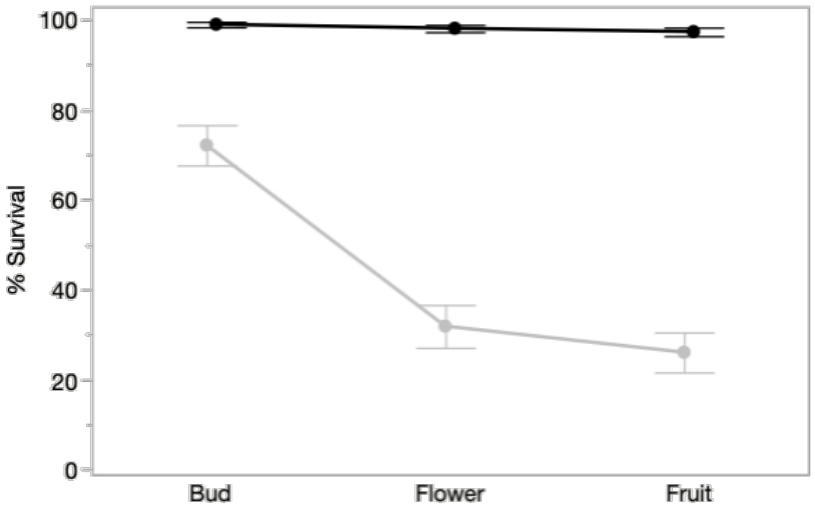


Figure 3.2: A) Phenotypic distributions of RIX set mean values grown under well-watered conditions (black) and dry-down conditions (gray) for each measured trait. Triangles indicate the treatment mean value, estimated from GLMMs shown in Table S2. B) Survival percentages to each life stage (bud, flower, and fruit) under well-watered conditions (black) and dry-down conditions (gray).

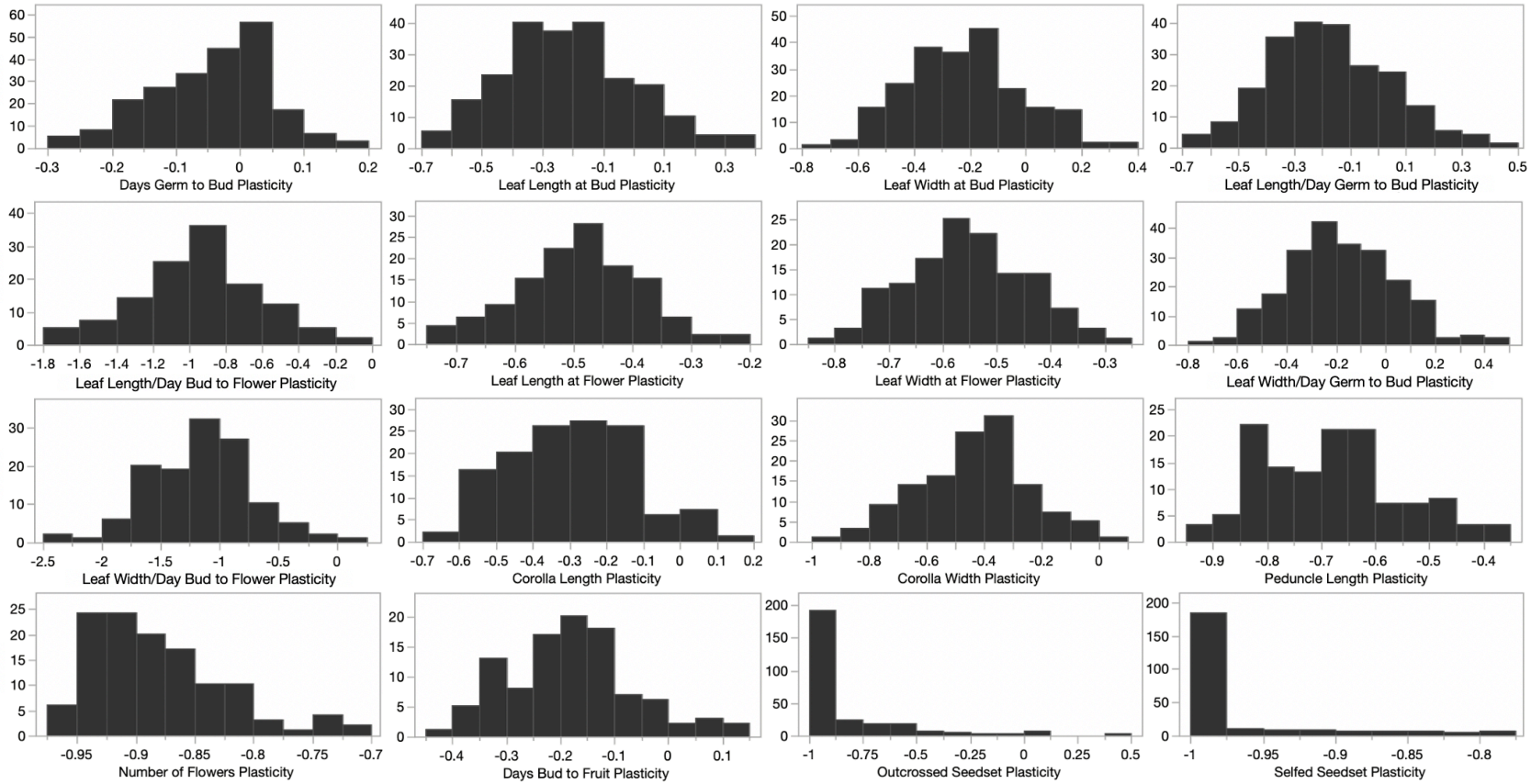
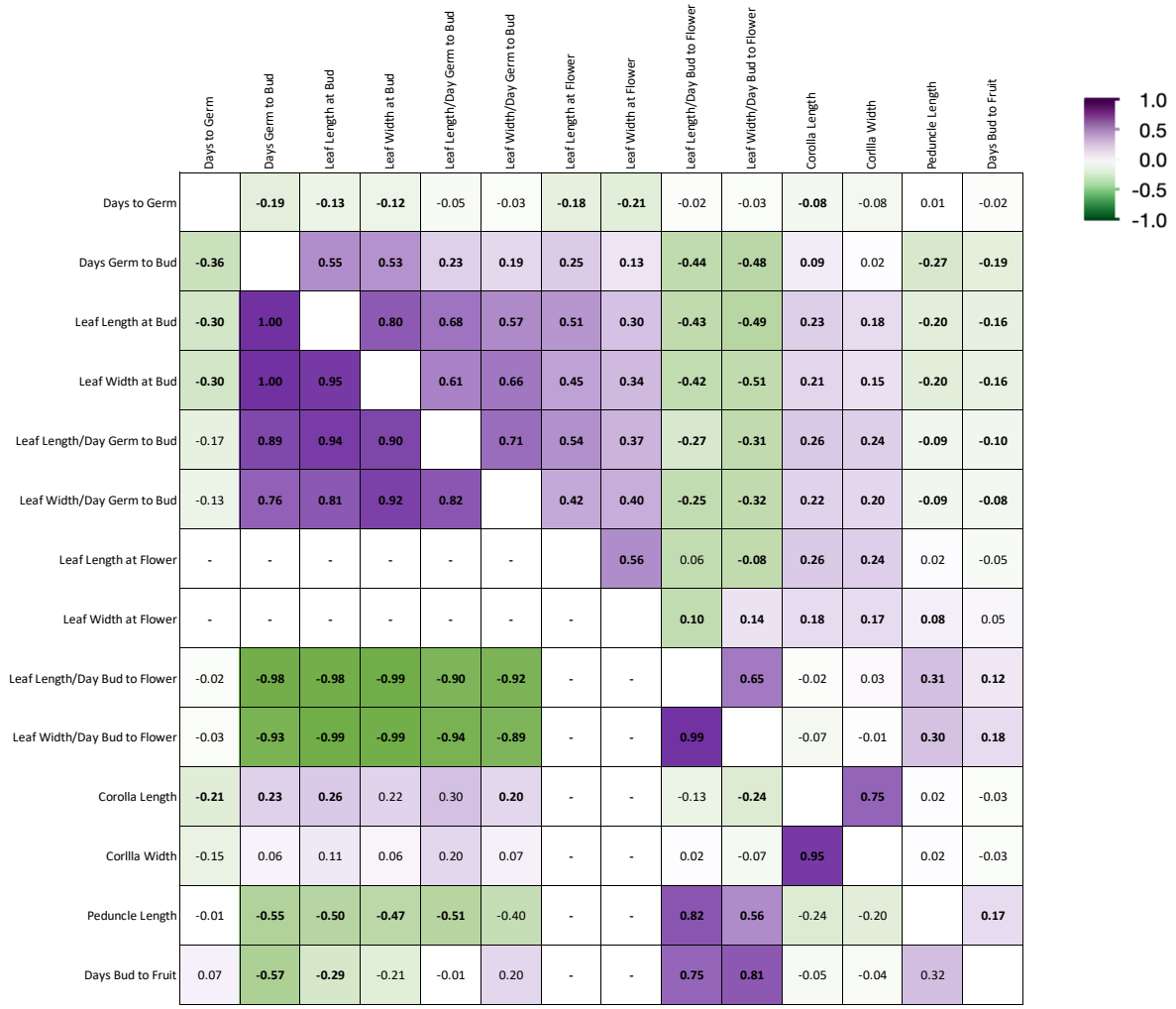


Figure 3.3: Distributions of RIX set average plasticity between well-watered and dry-down conditions for each measured trait. Plasticity was calculated for each RIX set as (RIX average phenotype under dry-down conditions – RIX average phenotype under well-watered conditions)/(RIX average phenotype under well-watered conditions).



	Days to Germ	Days Germ to Bud	Leaf Length at Bud	Leaf Width at Bud	Leaf Length/Day Germ to Bud	Leaf Width/Day Germ to Bud	Leaf Length at Flower	Leaf Width at Flower	Leaf Length/Day Bud to Flower	Leaf Width/Day Bud to Flower	Corolla Length	Corlla Width	Peduncle Length	Days Bud to Fruit
Days to Germ		**-0.19**	**-0.13**	**-0.12**	-0.05	-0.03	**-0.18**	**-0.21**	-0.02	-0.03	**-0.08**	-0.08	0.01	-0.02
Days Germ to Bud	**-0.36**		**0.55**	**0.53**	**0.23**	**0.19**	**0.25**	**0.13**	**-0.44**	**-0.48**	**0.09**	0.02	**-0.27**	**-0.19**
Leaf Length at Bud	**-0.30**	**1.00**		**0.80**	**0.68**	**0.57**	**0.51**	**0.30**	**-0.43**	**-0.49**	**0.23**	**0.18**	**-0.20**	**-0.16**
Leaf Width at Bud	**-0.30**	**1.00**	**0.95**		**0.61**	**0.66**	**0.45**	**0.34**	**-0.42**	**-0.51**	**0.21**	**0.15**	**-0.20**	**-0.16**
Leaf Length/Day Germ to Bud	-0.17	**0.89**	**0.94**	**0.90**		**0.71**	**0.54**	**0.37**	**-0.27**	**-0.31**	**0.26**	**0.24**	**-0.09**	**-0.10**
Leaf Width/Day Germ to Bud	-0.13	**0.76**	**0.81**	**0.92**	**0.82**		**0.42**	**0.40**	**-0.25**	**-0.32**	**0.22**	**0.20**	**-0.09**	**-0.08**
Leaf Length at Flower	-	-	-	-	-	-		**0.56**	0.06	**-0.08**	**0.26**	**0.24**	0.02	-0.05
Leaf Width at Flower	-	-	-	-	-	-	-		**0.10**	**0.14**	**0.18**	**0.17**	**0.08**	0.05
Leaf Length/Day Bud to Flower	-0.02	**-0.98**	**-0.98**	**-0.99**	**-0.90**	**-0.92**	-	-		**0.65**	-0.02	0.03	**0.31**	**0.12**
Leaf Width/Day Bud to Flower	-0.03	**-0.93**	**-0.99**	**-0.99**	**-0.94**	**-0.89**	-	-	**0.99**		-0.07	-0.01	**0.30**	**0.18**
Corolla Length	**-0.21**	**0.23**	**0.26**	0.22	0.30	**0.20**	-	-	-0.13	**-0.24**		**0.75**	0.02	-0.03
Corlla Width	-0.15	0.06	0.11	0.06	0.20	0.07	-	-	0.02	-0.07	**0.95**		0.02	-0.03
Peduncle Length	-0.01	**-0.55**	**-0.50**	**-0.47**	**-0.51**	-0.40	-	-	**0.82**	**0.56**	-0.24	-0.20		**0.17**
Days Bud to Fruit	0.07	**-0.57**	**-0.29**	-0.21	-0.01	0.20	-	-	**0.75**	**0.81**	-0.05	-0.04	0.32	

Figure 3.4: Phenotypic correlations calculated as Kendall's Tau (**τ**, Kendall Rank Correlation Coefficient) above diagonal, genetic correlations below diagonal, under well-watered conditions. Significant correlations at the $\alpha = 0.05$ level are shown in bold. Negative correlations are shown in green, positive correlation are shown in purple. Genetic correlations are only estimated between pairs of traits which both have estimated $H^2 \geq 0.20$.

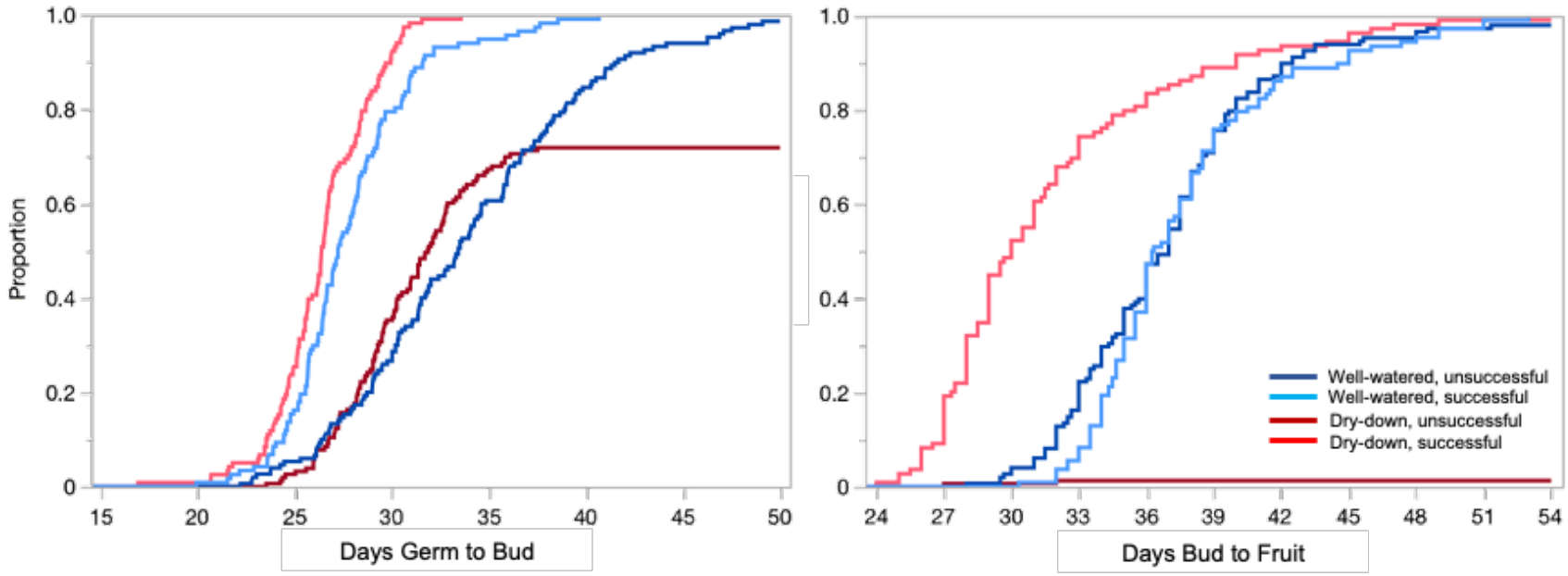
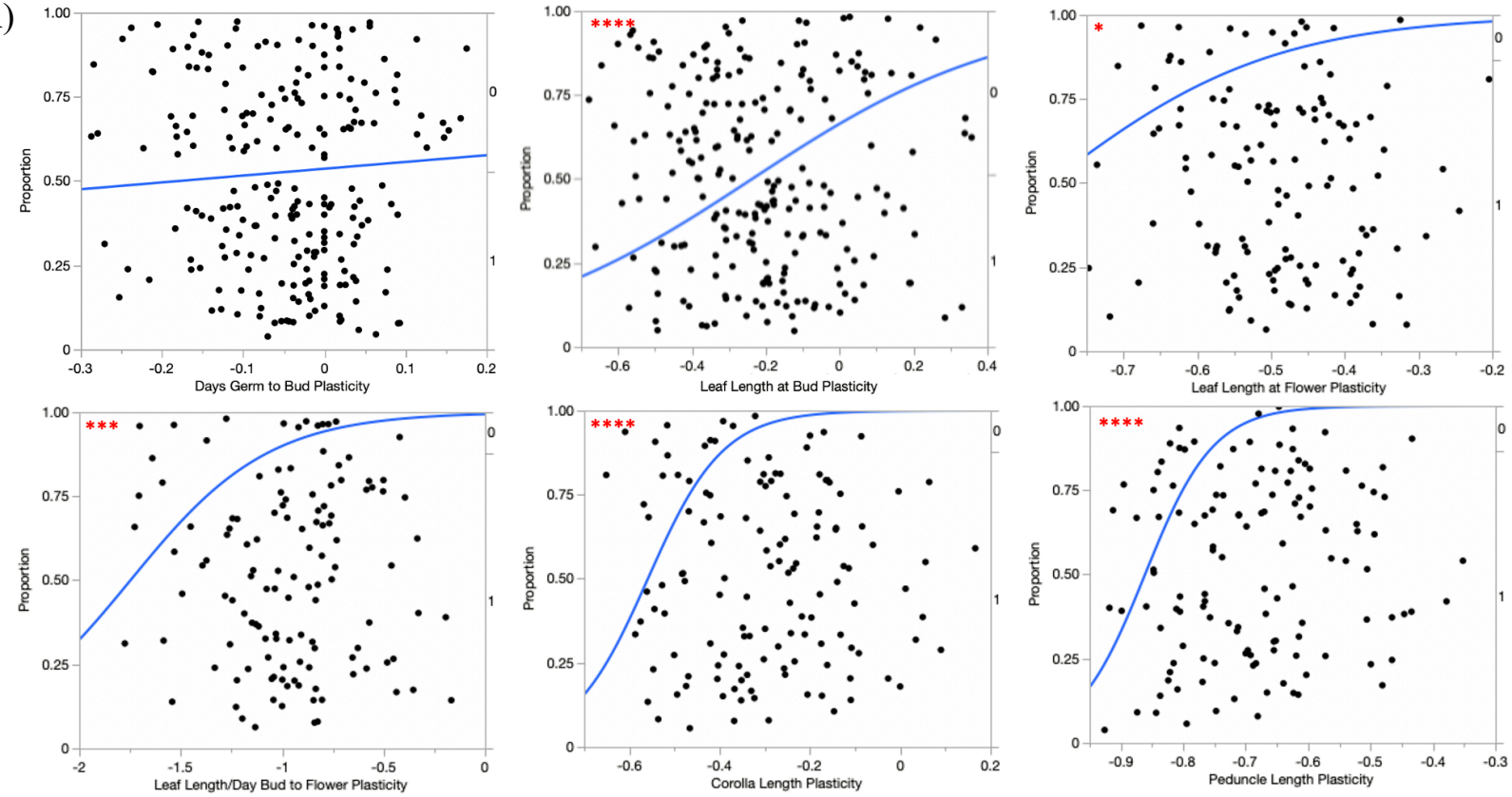
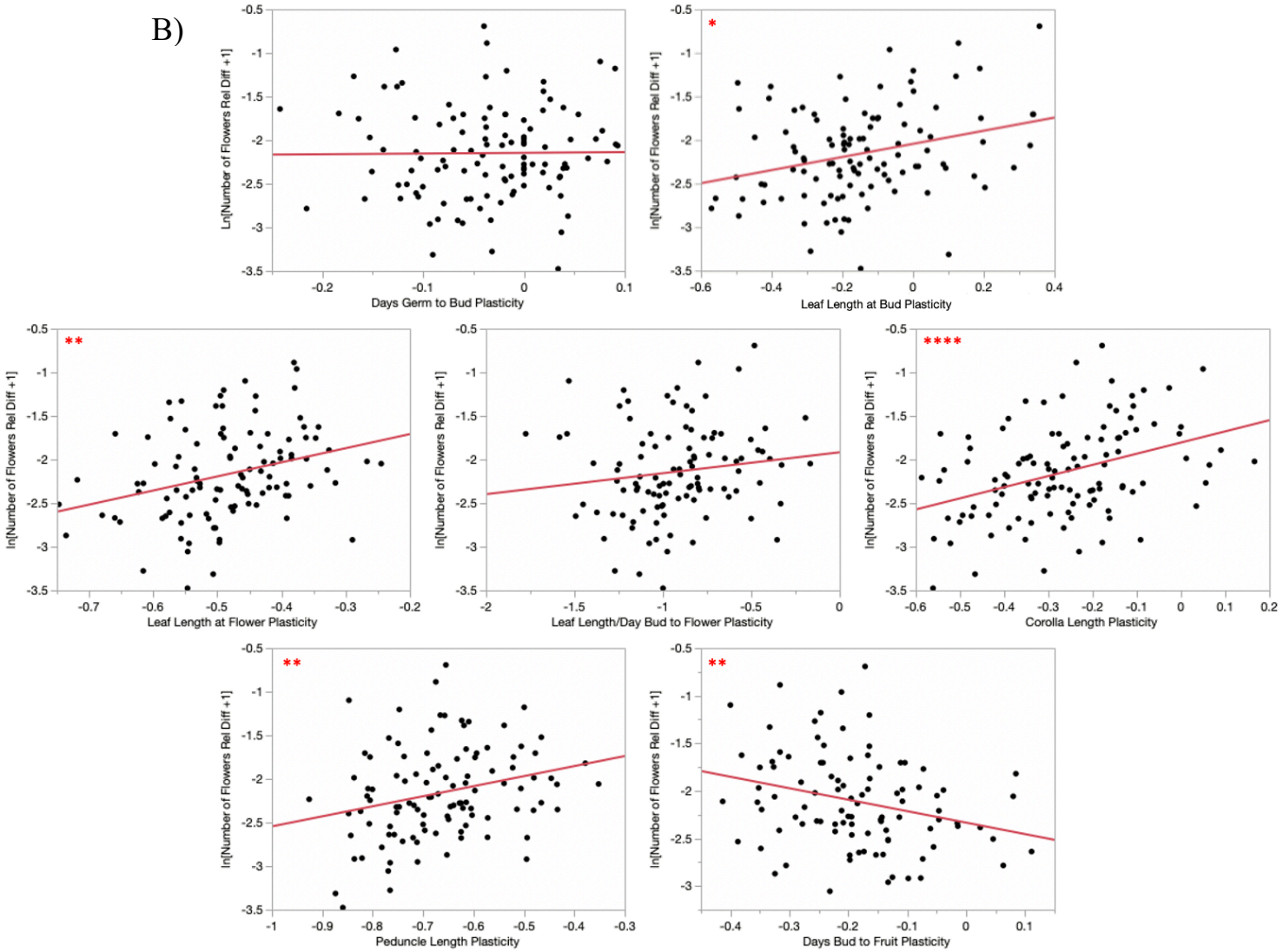


Figure 3.5: Divergence in developmental response to experimental drought between groups of RIXs separated by reproductive success under both experimental conditions. Kaplan-Meier Plots show the progression from germination to budding (left) and from budding to fruiting (right), of reproductively successful RIXs (darker shades), and reproductively unsuccessful RIXs (lighter shades), grown under well-watered conditions (shades of blue), and dry-down conditions (shades of red).

A)


Proportion
Days Germ to Bud Plasticity
Leaf Length at Bud Plasticity
Leaf Length at Flower Plasticity
Leaf Length/Day Bud to Flower Plasticity
Corolla Length Plasticity
Peduncle Length Plasticity
****
*
***
0
1

B)


Ln[Number of Flowers Rel Diff +1]
Days Germ to Bud Plasticity
*
Leaf Length at Bud Plasticity
**
Leaf Length at Flower Plasticity
Leaf Length/Day Bud to Flower Plasticity
****
Corolla Length Plasticity
**
Peduncle Length Plasticity
**
Days Bud to Fruit Plasticity

C)

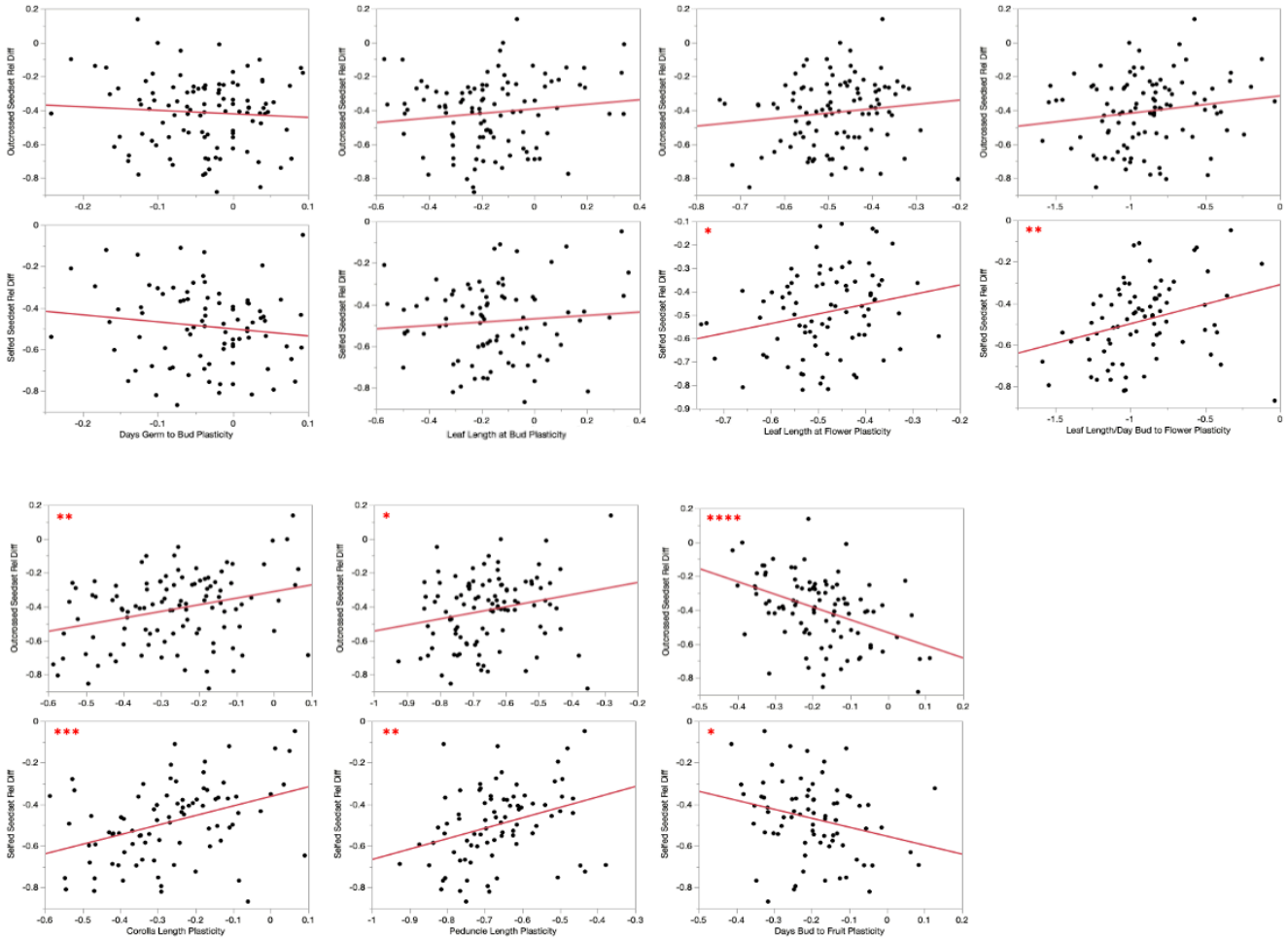


Figure 3.6: Selection on plasticity in seven measured traits as a function of A) survival proportion under dry-down conditions (modeled with logistic regression), B) Relative difference in number of flowers (WW->DD in survivors, ln(x+1) transformed, modeled with simple linear regression), C) Relative difference in outcrossed seedset and selfed seedset (WW->DD in survivors, modeled with simple linear regression). Only one trait was retained for all pairs of traits with $\boldsymbol{\tau} \geq 0.5$ under both watering treatments. Due to a lack of samples which produced a fruit, but no seeds, days bud to fruit is excluded from panel A. Negative plasticity values indicate smaller phenotypes under dry-down conditions. *p>0.05, **p>0.01, ***p>0.001, ****p>0.0001
Note: Removal of outliers raised p-values above 0.05 for relative difference in flower number regressed against leaf length at bud plasticity, as well as relative difference in outcrossed seedset regressed against peduncle length plasticity.

CHAPTER IV

QTL MAPPING OF DIVERGENT DROUGHT RESPONSE TRAITS IN *MIMULUS GUTTATUS* AND *M. NASUTUS*[3]

[3]Mantel S.J. and A.L. Sweigart, To be submitted to *Evolution.*

Abstract

Previous chapters have shown strong evidence for a complex phenotypic and genetic basis to drought response divergence between *Mimulus guttatus* and *M. nasutus*. To fully understand the implications of this differential adaptation, the action of the alleles involved, and their impact on hybridization and introgression between the species, mapping of important traits is necessary. Here, we interrogate the history of introgression of the three *M. guttatus* lines which served as parents to our mapping population, as well as investigating transmission ratio distortion (TRD) present in the three recombinant inbred line (RIL) populations we produced, before mapping a subset of important drought related traits. We find that both introgression in our parental lines and TRD is common across the genome, and discuss the implications of these results for both our QTL mapping and introgression in nature. Additionally, mapping results confirm the highly polygenic nature of drought adaptation in this system, yet reveal potentially important QTL which act pleiotropically to influence correlated traits. These results serve as a solid foundation to continue to investigate ecological isolation between these sympatric species, and how it may act to maintain species boundaries in the face of geneflow.

Introduction

Postzygotic reproductive isolation, hybridization which leads to the formation of unfit offspring, frequently contributes to species boundaries in sympatry (Coyne & Orr, 2004). This isolation may be intrinsic, in the case of hybrid incompatibilities between species (Bateson, 1909; Dobzhansky, 1937; Muller, 1942), or extrinsic, in which locally adapted alleles produce maladaptive phenotypes in an alternative habitat, or in an alternative genetic background (Coyne & Orr, 2004; Arnegard *et al.*, 2014; Thompson *et al.*, 2021; Coughlan *et al.*, 2021). Local adaptation to divergent ecological conditions, causing extrinsic postzygotic isolation, is therefore a main driver of ecological isolation (Dillon, 1970; Schluter, 2000, 2009; Rundle & Nosil, 2005; Nosil, 2012; Safran *et al.*, 2013; Lin *et al.*, 2020), making adaptation to differing habitats one of the most important forces both driving speciation, and consequently, maintaining extant lineages (Sobel *et al.*, 2010).

A largely outstanding question, though, is how effective ecological isolation is expected to be at maintaining species boundaries in the face of hybridization during secondary contact. To investigate this question, we use quantitative trait locus (QTL) mapping to examine the genetic basis of drought response differences, between *Mimulus guttatus* and *M. nasutus*, closely related (~200 KYA diverged, Brandvain et al. 2014), sister species which, though broadly allopatric, frequently occur in secondary sympatry. These species, present across much of western North America, have diverged both in mating system: *M. guttatus,* while self-compatible, is primarily bee pollinated and outcrossing, while *M. nasutus* is frequently cleistogamous, and almost exclusively selfing. Furthermore, these species differ in seasonal flowering time (Kiang and Hamrick 1978, Martin and Willis 2007, Kenney and Sweigart 2016), caused at least in part, by divergence in critical photoperiod, causing *M. nasutus* to begin flowering early in spring, while *M. guttatus* waits for the longer days of early summer to initiate reproduction (Fishman *et al.*, 2014a; Kenney & Sweigart, 2016). Additionally, in areas of sympatry, the species are ecologically divergent, with *M. guttatus* growing within more permanently wet seeps and streams, and *M. nasutus* occupying more ephemerally wet habitats with shallow soils that dry out quickly (Kiang and Hamrick 1978). *M. nasutus'* ability (and *M. guttatus'* inability) to flower earlier in the season, before the onset of summer drought, reflects a drought escape strategy (Franks and Weis, 2009; Franks, 2011; Berger and Ludwig, 2014) and likely contributes to the fine scale distribution of these species. As such, critical photoperiod divergence is likely a major contributor to ecological isolation between these species (Fishman et al. 2014, Kenney and Sweigart 2016). However, the species also diverge in other drought-response traits, including increased rate of fruit maturation under drought conditions in *M. nasutus* (Mantel and Sweigart 2019).

Despite these abundant reproductive barriers, ongoing hybridization at sympatric sites such as Catherine Creek (CAC), a system of ephemeral streams and seeps flowing into the Columbia River, is observed (Kenney and Sweigart, 2016) and the genomic signatures of introgression, largely unidirectionally from *M. nasutus* to *M. guttatus* (Sweigart and Willis, 2003; Brandvain et al., 2014) are present. How, then, do we expect this hybridization to affect the maintenance of these ecological barriers?

Will adaptive introgression easily allow *M. guttatus* to acquire drought adapted alleles, and dissolve ecological barriers between the species, or is this divergence robust to hybridization?

The strength of ecological barriers, and their resistance to geneflow between lineages is largely dependent upon the genetic basis and architecture of the loci involved. For example, the number of alleles controlling important locally adapted traits, the effect sizes of these loci, as well as dominance and epistatic relationships between them (and potential for transgressive phenotypes) are all expected to influence the propensity of introgression to homogenize lineages (Orr, 2005; Turner *et al.*, 2005; Martin *et al.*, 2005; Hall & Willis, 2006; Nosil *et al.*, 2009; Michel *et al.*, 2010). Additionally, the extent of pleiotropy or close linkage between loci is expected to influence the ease of adaptive introgression, with pleiotropic loci underlying suites of correlated adaptive traits expected to allow for more homogenizing introgression (Abbott *et al.*, 2013; Friedman *et al.*, 2015). Alternatively, many additional loci with uncorrelated effects may be more difficult to introgress effectively (Barton & Bengtsson, 1986). Finally, we may expect extrinsic postzygotic isolation to be stronger if antagonistically pleotropic alleles with genetic tradeoffs at important loci underly ecological isolation (Nosil *et al.*, 2005). On the other hand, alleles which are conditionally neutral (advantageous in one environment and selectively neutral in another) are expected to introgress more freely (Sambatti *et al.*, 2012).

Ultimately, to understand the effects of ongoing hybridization between divergent lineages at a broad scale, it is also likely necessary to understand the effect of genetic variation present within the populations of interest, on hybridization outcomes. Segregating variation, both in genetic sequence and genome architecture, may mean that the relative fitness of hybrids is highly variable based on the genotype of their parents (Rieseberg, 2001; Noor *et al.*, 2001; Liti *et al.*, 2006; Zuellig & Sweigart, 2018a). As such, it is important to assess the strength of ecological isolation among a sample of the genomic variation present at a hybridizing site. As *M. guttatus* is highly genetically diverse(Sweigart & Willis, 2003; Wu *et al.*, 2008; Brandvain *et al.*, 2014), we here analyze data from intercrosses between three independent recombinant inbred line (RIL) populations. This genetic variation is also likely to cause variation in transmission ratio distortion (TRD), or the nonmendelian inheritance of one allele over

another, which could be caused by a myriad of mechanisms including drivers during meiosis, pollen competition, gamete killers, or by genetic incompatibilities either during haploid gamete phase or during zygote development, all of which could influence the ability of important locally adapted loci to introgress freely (Harushima *et al.*, 2001; Hall & Willis, 2005; Fishman & McIntosh, 2019; Coughlan *et al.*, 2021). TRD is also relevant to the mapping of important loci as, if extreme, it may influence our ability to accurately map traits, as TRD can both bias estimated distances between markers (Lorieux *et al.*, 1995) and reduce the sample size of informative genotypes (Xu, 2003), biasing QTL estimation.

In previous studies (Mantel & Sweigart, 2019, Chapter III) we identified key phenotypes that contribute to differential adaptation to low water conditions in *M. guttatus* and *M. nasutus*, and demonstrated that pleiotropic or linked alleles may contribute to important tradeoffs between environments. We also showed that drought adaptation is likely both phenotypically and genetically complex, involving many small effect alleles across the genome working together to produce highly quantitative traits which mediate the ability to successfully survive and reproduce under water limiting conditions. Here we expand this work, mapping some of these important traits and investigating genetic and genomic variation present within these hybridizing populations, giving a better understanding of the likely outcomes of ongoing hybridization at CAC.

Materials and Methods

Library Preparation, and Sequencing

To map the genetic loci underlying drought-response divergence and subsequent ecological isolation between *M. guttatus* and *M. nasutus* we produced three parallel RIL populations, as outlined in the previous chapter. At the F6 generation we grew a single individual of each of the 368 RILs for tissue collection, DNA extraction, and crossing in the greenhouse at the University of Georgia. We planted ~5 to 10 seeds into 2.5” pots filled with moist Fafard 3-B potting mix (Sun Gro Horticulture). We stratified seeds for seven days at 4°C to promote germination, and then moved them to a Conviron growth chamber with lights set to 16-hr days and temperatures set to 23°C days/16°C nights. Following germination, we transplanted a single individual of each RIL into a new 2.5” pot in the UGA greenhouses under the same

light and temperature conditions, and bottom watered daily. We collected bud and meristem tissue from each individual into 96 well plates on dry ice, stored them at -80°C, and extracted DNA using a modified CTAB method (Doyle & Doyle, 1987). We quantified DNA using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) and diluted it to between 5 and 10 ng/ul. We then constructed a ddRADseq library for our RIL population using a modified version of RAD Capture (Ali *et al.*, 2016), "BestRAD" (dx.doi.org/10.17504/protocols.io.6awhafe). Briefly, we digested 50-100 ng of genomic DNA with BfaI and PstI, ligated 48 unique barcoded i5 adaptors to each sample, isolated tagged fragments, and pooled samples into 8 pools of 48 or fewer samples each. We attached a unique NEBNext Adaptor for Illumina i7 adaptor to each pool of samples, and enriched the samples with PCR, finally pooling the samples in equimolar concentrations. This ddRADseq library was sequenced, along with WGS libraries, for each of the four parental lines (CAC9, CAC110, CAC162, and CAC415) on two lanes of Illumina Hi-seq 4000 for paired end 150 bp reads at the Genome Sequencing Facility at Duke University.

Genotyping

After Illumina adaptors were removed using Trimmomatic (Bolger *et al.*, 2014) we aligned WGS reads from each of the parental lines to the *Mimulus guttatus* v5.0 refence genome (phytozome-next.jgi.doe.gov) using BWA-MEM (Li & Durbin, 2009; Li, 2013) removed reads with alignment qualities below Q29 with SAMtools view (Li *et al.*, 2009), and processed reads with Picard AddOrReplaceReadGroups, FixMateInformation, and MarkDuplicates (broadinstitute.github.io/picard/). Using GATK HaplotypeCaller in GVCF mode we called SNPs, removing heterozygous calls (likely to be errors or miss-mapped reads in our highly inbred parental lines) and filtered SNPs to a minimum depth of 3x. We then joint genotyped each pair of parental lines (CAC9 & CAC110, CAC9 & CAC162, and CAC9 & CAC415) using GATK GenotypeGVCFs. We filtered our called SNPs based on quality (QD < 2.0, FS > 60.0, MQ < 40.0, MQRankSum < -12.5, ReadPosRankSum < -8.0) and retained only biallelic SNPs using GATK VariantFiltration and GATK SelectVariants. From these filtered VCFs, we extracted a full list of diagnostic SNPs (those homozygous for different alleles in each of the parental pairs).

Introgression at CAC occurs nearly exclusively unidirectionally, from *M. nasutus* into *M. guttatus* (Kenney & Sweigart, 2016). To identify putative genomic regions of introgression between *M. nasutus* (CAC9) and our three *M. guttatus* parental lines (CAC110, CAC162, and CAC415), we used a custom perl script to calculate nucleotide diversity (p) between each of the pairs of parental lines in non-overlapping 100kb windows across the genome. We identified a region of the genome as introgressed by visually inspecting plots of nucleotide diversity across the *Mimulus guttatus* v5.0 refence genome in each pair of parental lines, identifying regions of the genome in which nucleotide diversity decreased abruptly when compared to other windows on the same linkage group and in which a large number of consecutive 100kb windows had $p < 0.005$. As some regions of the genome, which are likely transposon and repeat rich, show overall reduced nucleotide diversity, we identified putatively introgressed regions conservatively.

Our ddRADseq data was demultiplexed by i7 index following sequencing at the Genome Sequencing Facility at Duke University. We removed PCR duplicates from each sub-library, using the unique molecular barcode of the i5 adaptor with a custom Python script, and then demultiplexed each pool to the individual sample level using Flip2BeRAD (github.com/tylerhether/Flip2BeRAD) and Stacks process_radtags (Catchen *et al.*, 2011, 2013). We removed Illumina adaptors using Trimmomatic, aligned reads to the *Mimulus guttatus* v5.0 refence genome using BWA-MEM and processed reads with Picard AddOrReplaceReadGroups and FixMateInformation, (broadinstitute.github.io/picard/). Using GATK HaplotypeCaller in GVCF mode we called SNPs, and then joint genotyped each group of RILs with the parental lines from which it was derived using GATK GenotypeGVCFs. We filtered called SNPs based on quality, as above, retaining only biallelic SNPs, and removed sites that were only genotyped in parental samples, using GATK VariantFiltration and GATK SelectVariants. Finally, using the list of diagnostic SNPs between each pair of parental lines, we removed non-ancestry informative positions.

We genotyped each RIL across the genome by calculating genotype probabilities in 25 kb windows using modified Python scripts from the program GOOGA (Flagel *et al.*, 2019), along with custom R scripts. Briefly, we assigned parental ancestry, allowing each SNP to be identified as having *M.*

guttatus ancestry, or *M. nasutus* ancestry. We then averaged individual SNP genotypes across each window to produce a consensus call, using a 95% homozygosity cutoff. From these windowed genotypes, we calculated genotype error rates and recombination rates for each RIL sample and estimated genotype probabilities across the genome, allowing for genotyping of windows that contained few or no RADseq reads. From the genotype probabilities produced by this pipeline, we created genotype matrices containing genotypes at confidently called windows, by including genotype calls with an estimated genotype probability of >0.75. Of the original 368 RILs, we generated genotype data for 317 of them (140 of the original 151 from Group 1, 96/128 from Group 2, and 81/89 from Group 3).

Linkage Mapping

Using the resulting genotype matrices, we constructed a high density linkage map for each of the three RIL groups using LepMAP3 (Rastas, 2017). We used the affx2post.awk script to calculate posterior probabilities and then ParentCall2 (default parameters), SeparateChromosomes2 (lodLimit=1, theta=0.15), and OrderMarkers2 (grandparentPhase=1, useKosambi=1, sexAveraged=1) to produce a linkage map for each chromosome of the v5.0 reference genome separately. Linkage groups were numbered and oriented in accordance with the *Mimulus guttatus* v5.0 refence genome. We then used the map2genotypes.awk script to export a final genotype matrix with associated map positions, for each linkage group of each RIL group. This procedure produced three linkage maps containing between ~5,300 and 6,500 markers per RIL group (Group 1: 5,343, Group 2: 6,493, Group 3: 6,503).

Comparison of Linkage Maps

We used JoinMap 4.0 (Van Ooijen, 2006, 2011) to create comparison plots visualizing differences in marker order and mapping distance between shared markers in the three linkage maps we produced. We interpreted blocks of markers that spanned large physical distances in the *Mimulus guttatus* v5.0 refence genome, but mapped to the same, or nearly the same, genetic position, as putative inversions between the *M. guttaus* and *M. nasutus* parents used to produce that RIL group. The *Mimulus guttatus* v5.0 refence genome was produced using a line of the perennial ecotype of *M. guttatus*. Thus, we interpreted blocks of colinear markers that displayed a reversal in linkage order relative to physical

marker order in the reference genome as putative inversions between the perennial *M. guttatus* orientation and the orientation shared by annual *M. guttatus* and *M. nasutus*. We used this information to identify genomic rearrangements that appear to be segregating within annual *M. guttatus* at CAC (i.e., rearrangements that appear in some but not all of the three RIL groups).

Transmission Ratio Distortion

Using the final genotype matrix outputs from LepMAP3 we used chi-square tests to test for significant transmission ratio distortion (TRD) across the genome in each of the three RIL groups produced. Any genomic region in which three or more consecutive recombination-informative markers showed evidence of distortion from the Mendelian 1:1 expectation at the $\alpha = 0.05$ level was considered a putative region of TRD. As the three maps produced varied in both marker order and overall size, to identify if regions of significant TRD colocalized in the three maps, we overlayed regions of TRD on the linkage maps and identified a marker within each distorted region that was present in the map of all three RIL groups, plotting the location of said marker in all three maps.

Creating a consensus linkage map

Because linkage maps differed greatly between the three RIL groups, we used the program LPmerge (Endelman & Plomion, 2014) in R (v. 3.2.3) to create a consensus map including the linkage information from all three RIL group maps, running each linkage group separately. We calculated consensus maps using a max interval size ranging from 1 to 30 markers and selected the consensus map with the lowest overall root mean-squared error (RSME). We then pruned the final consensus map to 1,132 recombination informative sites.

QTL Mapping

RILs were crossed to form outbred recombinant inbred intercrossed lines (RIXs), which were grown under four experimental treatments (12hr days well-watered, 12hr days dry-down, 16hr days well-watered, and 16hr days dry-down) and phenotyped as described in Chapter III. As in Chapter III, here we present results only from the portion of the plants grown under 16hr days. Using the known parentage of each of the RIXs created, we combined the RIL group genotype matrices in excel to infer the genotypes

of each RIX at each marker. A genotype was coded as missing in the RIX dataset if either of the parental RILs was genotyped as heterozygous at that marker. Although this crossing design produces outbred individuals harboring alleles from up to two of the three *M. guttatus* parental lines, in addition to alleles from the *M. nasutus* parent, CAC9, for this first pass at QTL mapping we coded all *M. guttatus* alleles identically, to investigate fixed differences between *M. guttatus* and *M. nasutus* in our population. We performed quantitative trait locus (QTL) mapping using R/qtl (Broman & Sen, 2009) in R (v. 4.0.0) separately in each of the three largest groups of RIX individuals, those created via crosses between RIL individuals from group 1 and group 2 (1x2), group 2 and group 3 (2x3), and group 3 and group 1 (3x1).

We determined the best multiple QTL model using the stepwiseqtl command using Haley–Knott regression from genotype probabilities of markers (and pseudomarkers in gaps >1cM). We ran 5,000 permutations to determine LOD thresholds ($\alpha = 0.05$) and employed automated forward-backward stepwise model selection, which scans for additive and epistatic QTL at each step, refining QTL positions and fitting the model with ANOVA to calculate effect size and percentage variance explained for each QTL. To minimize false positives we took a conservative approach, only reporting QTL which were both identified via this stepwise model selection procedure, and were also outliers in simple interval mapping (SIM), and/or composite interval mapping (CIM) scans ran on the same data. We further, confirmed QTL locations by examining genotypic averages at the marker or pseudomarker identified with the largest LOD score of each QTL.

For this first-pass, although we measured many additional traits in our mapping population, for preliminary analysis, we chose six phenotypes to investigate (four measured under well-watered conditions: days from germination to budding, leaf length at budding, peduncle length, and days from budding to fruiting, and two under dry-down conditions: survival to fruiting and outcrossed seedset, all grown under 16hr days). Days from germination to budding and leaf length at budding were chosen to investigate their strong positive genetic correlation and the association between these genetically constrained (big and slow/small and fast strategies) phenotypes with survival under dry-down conditions as identified in Chapter III. Similarly, peduncle length and days bud to fruit were analyzed to investigate

their relationship with seedset under dry-down conditions, and positive genetic correlation, which may prevent efficient selection to drought conditions, as discussed in Chapter III. Phenotypes other than fitness proxies were mapped under well-watered conditions to investigate the innate genetic differences responsible for variation in these traits under benign conditions, rather than a more complex combination of those effects and plastic responses to the dry-down treatment. Larger samples sizes under well-watered conditions also allow for the detection of QTL with smaller effects.

For each QTL found we estimated additive (a) and dominance (d) effects and used those values to calculate the additive dominance ratio (d/|a|). We used the 'summary' command in the qtl package to calculate a and d for the marker (or pseudomarker) with the largest LOD score in each identified QTL, accounting for all other QTLs included in the model. This effectively calculates the mean phenotype for each genotype class and sets the *M. nasutus* allele homozygote mean to 0, the *M. guttatus* allele homozygotes to 2a, and heterozygotes to a+d, however because this calculation includes information about linked QTL included in the full model, these calculated values may differ from a and d values calculated directly from genotypic means.

On this scale, positive values of a indicate that the *M. guttatus* allele is associated with the larger phenotype, while negative values indicates that the *M. nasutus* allele is associated with the larger phenotype. Positive values of d indicate that the allele associated with the smaller phenotype is dominant over the allele associated with the larger phenotype, and negative values of d indicate the reverse. These relationships are such that additive dominance ratio values below -1 indicate underdominance, a value near -1 indicates complete dominance of the "small" allele, a value between -1 and 0 indicates partial dominance of the "small" allele, a value around 0 indicates additivity, a values between 0 and 1 indicates partial recessivity of the "small" allele, a value near 1 indicates complete recessivity of the "small" allele, and a value above 1 indicated overdominance.

Results

Variation in introgression among parental lines

In comparing putative areas of introgression between *M. nasutus* and the three *M. guttatus* parents used as paternal lines in our mapping population, we observed substantial variation in both the proportion of the genome introgressed, and the loci introgressed (Table 4.1, Figure S4.1). Two of the parental lines, seemed to have only a small proportion of *M. nasutus* ancestry (CAC162: ~2.5%, and CAC415: ~1.2% *M. nasutus* ancestry genome wide), while one appears to have a substantially hybrid genome (CAC110: ~20.5% *M. nasutus* ancestry genome wide). These putative blocks of introgression are also largely non-overlapping, with very few areas of introgression shared between the *M. guttatus* lines we studied, implying they are likely more recent, independent introgression events. Still, a few consistent areas of introgression are present. A small area of *M. nasutus* introgression, present on chromosome 8 (~0.9Mb) is shared among all three *M. guttatus* lines, and another area on Chr 2 (~0.7Mb) is shared between CAC110 and CAC415. Both of these overlapping regions, are a part of larger blocks of non-overlapping introgression in multiple lines, implying that they may be derived from independent hybridization events, however adaptive introgression could be implicated in regions of consistent introgression in these regions. Further, most chromosomes showed regions of introgression in at least one of the *M. guttatus* lines tested, with chromosomes 3 and 7 the only to show no obvious sign of introgression in any of the three *M. guttatus* lines.

Structural variation within and between species

Overall, linkage map size also varied between RIL groups (Figure 4.1, Table 4.2). While groups 1 and 3, had similar global map sizes (~1279cM and ~1277cM respectively) and recombination rates (4.94 and 4.93 cM/Mb respectively), group 2 had a substantially shorter map (~983 cM, 3.80 cM/Mb). Additionally, variation in map size from chromosome to chromosome was also present, which at its most extreme, for example, involved chromosome 11 in group 1 having a nearly 2.5x greater recombination rate (8.12 cM/Mb) than Chromosome 3 of group 2 (3.26 cM/Mb).

Much of the difference in recombination rates appears to be driven by substantial structural variation between the four parental lines (Table 4.3, Figure 4.1). From maps made from crosses using our three *M. guttatus* parental lines, we identified 6 potential genomic regions with structural variation segregating within *M. guttatus* at CAC, as well as one potentially *M. nasutus* specific structural variant, which has likely been introduced to *M. guttatus* via introgression. Lastly, we were able to recapitulate the presence of two previously identified structural variants, one specific to the perennial ecotype of *M. guttatus* (Lowry & Willis, 2010), and another specific to the annual ecotype of M. guttatus in Oregon (Holeski *et al.*, 2014; Flagel *et al.*, 2019).

Extensive transmission ratio distortion across the genome

Large areas of significant transmission ratio distortion were also found across the genome in all three RIL groups (Figure 4.2). Regions of TRD vary substantially in the location, direction, and severity of distortion among RIL groups. The most distorted regions, (Those with more than 80% transmission of one allele over the other) for example, are unique to each RIL group. In group 1: a large portion of chromosome 5 distorted toward *M. guttatus* ancestry, in group 2: a portion of chromosome 13 is distorted toward *M. nasutus* ancestry, and in group 3: a large portion of chromosome 10 is distorted toward *M. guttatus* ancestry. The only chromosome showing no sign of significant distortion in any of the three groups is chromosome 9. Despite the high levels of variation, there are a few consistently distorted regions across our three RIL groups (Figure 4.2). The beginning of chromosome 5, for example, is consistently distorted towards *M. guttatus* ancestry, and parts of chromosome 8 are consistently distorted towards *M. naustus* ancestry. This consistent region of TRD on chromosome 8 may be of particular interest as it overlaps with the consistent region of introgression found on that chromosome. This seemingly species specific TRD may help to explain why introgression on this portion of chromosome 8 seems to be more common in CAC *M. guttatus*.

QTL mapping of important traits

We identified a total of 48 QTL across the six phenotypes investigated here, in our three distinct RIX groups (Figure 4.3, Table 4.4). These QTL vary substantially among crosses, with very few loci that

colocalize between groups. These traits also appear largely polygenic, as the variance explained by each QTL is generally low, with the largest effect QTL explaining ~25% of the total phenotypic variance. Within crosses, although some clustering/colocalization of QTL was detected, identified QTL appear largely randomly distributed across the genome, with at least two QTL identified on each chromosome. Additionally, as expected, 7 of the 8 identified QTL associated with our two fitness proxies (survival, and outcrossed seedset) showed additive effects indicating that *M. nasutus* alleles conferred increased fitness under drought conditions.

As expected from previous results showing a strong genetic correlation between vegetative size and budding time, we identified multiple, well supported QTL associated with leaf length at bud and days from germination to budding within RIX groups; however QTL unique to each of these traits were also common (Figure 4.3, Table 4.4). Additive effect estimates of the QTL identified for these two traits are both positive and negative, indicating that the directionality of QTL effects is not species-specific. For example, *M. guttatus* alleles are associated with increased budding time at some loci, and decreased budding time at other loci (the same is true of *M. nasutus*). Additionally, QTL for budding time and survival under dry-down conditions colocalize on chromosome 8. This region is of particular interest because it is both very near (within 1cM) the shared region of introgression and overlaps with the shared region of TRD identified on this chromosome (Table 4.1, Figure 4.2, Figure S4.1). This QTL was estimated to explain ~6% of variance in budding time and ~8.5% of the variance in survival, with values indicating *M. nasutus* alleles at this locus are associated with increased survival, and more rapid budding (Figure 4.3, Table 4.4). Further, inspection of genotype class averages at these peak loci reveals larger variance in phenotypes associated with *M. guttatus* alleles, implying that the *M. guttatus* parental lines used may harbor distinct alleles with disparate effects on the phenotype.

Given their potential importance for drought adaptation, we also identified well supported QTL associated with peduncle length and fruit ripening time. As expected, these traits showed less QTL colocalization than vegetative size and budding time. Most identified QTL appear unique to a trait,

however one QTL, on chromosome 3 appears associated with both traits in one of the three groups of RIXs we produced.

Discussion

The three parallel RIL populations we produced to create our mapping population were used as opposed to a single RIL population in an effort to capture some of the genetic and genomic variation present within *M. guttatus* at CAC. We were successful in doing so, as we found substantial differences in putative areas of introgression between the *M. guttatus* parents, in chromosomal structure (i.e., inversions and translocations) and genetic map length, and in transmission ratio distortion across RIL groups. The totality of this variation present both within *M. guttatus* and between *M. nasutus* and *M. guttatas* at CAC is likely to have profound effects on the fate of hybrid individuals, with the identity of the *M. guttatus* parent contributing to probability of the hybrid fitness.

Introgression among parental lines

Both the amount and location of introgression varied greatly among the *M. guttatus* lines used in this study. It appears that even lines phenotypically similar to *M. guttatus* can have a cryptic history of hybridization, as CAC110 showed evidence of substantial *M. nasutus* ancestry (~20.5%). These blocks of introgression in CAC110 were frequently large, covering most of chromosomes 11 and 13, for example (Table 4.1, Figure S4.1), suggesting recent hybridization. On the other hand, CAC162 and CAC415 appear to carry much less *M. nasutus* ancestry – in smaller blocks – aligning with our expectation of genome wide selection against *M. nasutus* ancestry in an *M. guttatus* background (Brandvain et al. 2014; Kenney & Sweigart, 2016). Still, introgression was common, we identified only two chromosomes (3 and 7) with no obvious sign of introgression in any of the three *M. guttatus* lines. Although rare, we identified two locations of consistent introgression across the genome in our sample of three *M. guttatus* lines, largely consistent with previous work at CAC (Kenney & Sweigart, 2016) which identified frequent introgression in a large sample of wild individuals, with some loci more commonly introgressed than expected by chance. Additionally, this result appears largely consistent with previous work that found blocks of putative introgression between CAC *M. nasutus* (CAC9) and a different CAC *M. guttatus*

inbred line (CAC6), including one that overlaps with the putative introgressions we found on chromosome 2 (Brandvain *et al.*, 2014). These overlapping areas of introgression warrant further investigation, as they may be evidence of universally adaptive loci, favored in both species and/or favored in both microsites. While our data indicate adaptive introgression is likely not ubiquitous, there may be small regions of *M. nasutus* variation (e.g., on chromosomes 2 and 8) that are being preferentially retained in *M. guttatus* genomes.

Recombination rates across and among RIL populations

In our three RIL mapping populations we also observed variable recombination rates. In particular, Group 2 RILs (CAC9 x CAC162) exhibited substantially less recombination, resulting in an overall shorter linkage map when compared to the maps made from the other two RIL groups (Table 4.2, Figure 4.1). This suppressed recombination in group 2 RILs is likely to lead to decreased precision in QTL identification. Across the three RIL populations we observed recombination rates comparable to those found in previous linkage maps of *M. guttatus* and *M. nasutus* (Fishman *et al.*, 2014b; Holeski *et al.*, 2014). Much of this variation in recombination rate between maps, and among chromosomes within maps, is likely due to segregating structural variation. The structural variants we identified are spread across the genome, and common in the small sample of three *M. guttatus* lines used here. This segregating variation in a hybridizing population likely means that the effect of hybridization varies based on the *M. guttatus* genotype involved.

Segregating structural variation

Lending confidence to our linkage mapping procedure, we recapitulated two known structural variants in *Mimulus* (Table 4.3). One is an inversion on chromosome 8 that is specific to the perennial ecotype of *M. guttatus* (Lowry & Willis, 2010). The other is an *M. guttatus*-specific inversion on chromosome 10 that has so far been found only in annual Oregon populations (Holeski *et al.*, 2014; Flagel *et al.*, 2019). On the other hand, there are two other known inversions that we did not detect in these CAC RIL mapping populations. One of these is another inversion specific to the perennial ecotype of *M. guttatus*. This inversion is on chromosome 5, in a region of low confidence in our linkage maps;

additional analyses may show evidence of this inversion, which is likely carried by the reference genome. We also found no evidence of a previously identified inversion on chromosome 6 (Lee *et al.*, 2016) that is segregating – and perhaps restricted to – the Iron Mountain population of *M. guttatus*, located approximately 200 kilometers south of Catherine Creek. Additionally, we identified multiple previously unidentified structural variants segregating within *M. guttatus* at CAC (Table 4.3), likely to have implications for the success of natural hybrids.

Pervasive transmission ratio distortion

Transmission ratio distortion in our mapping population was also pervasive and frequently extreme (Figure 4.2). This distortion can be interpreted in a number of ways, each of which have potentially important implications for species maintenance. These distorted loci may reflect selfish evolution, when an allele manipulates its own transmission, or alternatively, postzygotic isolation selecting against incompatible allele combinations. Further analysis will be needed to determine likely agents causing the pervasive TRD observed in this experiment. These distorted loci likely also have implications for their propensity to introgress. In a backcrossing scenario, in which hybrid individuals largely backcross into an *M. guttatus* genetic background (Kenney & Sweigart, 2016), *M. naustus* alleles that are preferentially inherited over *M. guttatus* alleles, may be much more likely to introgress irrespective of their fitness consequences. On the other hand, *M. guttatus* alleles that are inherited over *M. naustus* alleles, may be much less likely to introgress, even if introgression could be selectively advantageous in an *M. guttatus* background. Additionally, regions of TRD are frequently variable in there RIL groups, with the identity of the *M. guttatus* allele involved influencing patterns of distortion. At a single locus, for example, some alleles derived from *M. guttatus* are favored over *M. nasutus* alleles while others are disfavored, or inherited in a Mendelian fashion. Similar patterns, in which distinct crosses show vastly different patterns of TRD, is consistent with previous work in *Mimulus* (Hall & Willis, 2005), however we also found a small number of consistently distorted regions across our three RIL groups. As such, this shared TRD on chromosomes 5 and 8 may warrant further investigation for loci contributing to distortion in admixed populations. Intriguingly, the distorted region on chromosome 8, which shows an

excess of *M. nasutus* homozygotes, overlaps with a region of shared introgression in our parental *M. guttatus* lines. Thus, it is possible that distortion favoring the inheritance of *M. nasutus* alleles contributes to this block of *M. nasutus* ancestry being retained in *M. guttatus* in our lines, however further investigation of this region of TRD and introgression will be necessary.

QTL mapping of important traits

To remove the potential effects of inbreeding depression, and to allow for future mapping under natural conditions in the field, we performed QTL mapping in outcrossed RIX individuals. This crossing design means that outbred RIX individuals derived from an intercross between different RIL groups potentially harbor two distinct *M. guttatus* alleles (originating from distinct *M. guttatus* progenitor lines). To account for these individual allelic effects in *M. guttatus*, we performed QTL mapping separately in the three largest groups of RIX individuals (Groups 1x2, 2x3, and 3x1); however, this method decreases the sample size of each mapping population considerably, compromising our power to detect QTL. As such, a more thorough analysis using a model capable of accounting for the presence of four different alleles at a single locus in the full RIX population (Yu *et al.*, 2008; Hu *et al.*, 2018; Scott *et al.*, 2020) will be necessary to more fully identify line-specific alleles associated with important drought response related phenotypes.

For the phenotypes analyzed thus far, we see that the traits we have identified as important for drought response differences between *M. guttatus* and *M. nasutus* are genetically complex and highly polygenic, with each allele contributing to a relatively small proportion of the variance in the phenotype population wide, although some larger effect loci, explaining upwards of 20% of the phenotypic variance were identified (Table 4.4). This pattern is not unexpected, with previous investigations of floral and vegetative traits also finding evidence of polygenic adaptation (Fishman *et al.*, 2002; Troth *et al.*, 2018). The complex genetic basis of these traits, would imply that introgression of a small number of locally adapted loci would not be sufficient to introduce the more effective drought response strategy of *M. nasutus* into *M. guttatus*. Thus, it is possible that polygenic adaptation to the abiotic environment contributes to strong ecological isolation in secondary sympatry.

We have shown in Chapter III that a strong genetic correlation between vegetative size and budding time may create a genetic constraint producing plants that employ one of two broad strategies: reproducing late and growing large, or reproducing early and remaining small. As expected from this result, multiple colocalized QTL associated with these two phenotypes were identified. As such these phenotypes are likely to introgress together, maintaining this genetic constraint in admixed populations (Figure 4.3, Table 4.4). Given our previous results, it seems likely that these alleles act pleiotropically to impact both reproductive timing and vegetative size, rather than being separate, but tightly linked loci. Due to strong selection seen for faster budding and smaller plants under drought conditions mediated through plant survival, we expected to also observe colocalization with QTL associated with survival under drought stress. Little evidence of this pattern was found however, with a single colocalized QTL between budding time and survival identified in one of the three RIX groups (Figure 4.3, Table 4.4). This lack of colocalization is likely due to a lack of power to detect line-specific QTL with the current analysis method, but it may also be due to distinct loci controlling plasticity or trait expression specifically under dry-down conditions. However, the identified case of QTL colocalization between survival and budding time is of particular interest given the patterns of introgression and TRD identified. These QTL, with *M. nasutus* alleles associated with increased survival under drought and more rapid budding, colocalize with the identified regions of *M. nasutus* introgression, and TRD favoring *M. nasutus* alleles over *M. guttatus* alleles. Together, these results suggest that selection for rapid budding under drought-stressed conditions and/or distortion favoring *M. nasutus* alleles may result in elevated introgression in this region.

Additionally, we identified multiple QTL associated with peduncle length and speed of fruit ripening, two other phenotypes previously identified as important factors influencing reproductive success under dry-down conditions in our mapping population. Just as before, more thorough analysis of the individual allelic effects on these important traits will be necessary to get a full picture of the level of pleiotropy between these traits and reproductive success, as little colocalization was identified with the current analysis.

While we have identified multiple QTL likely to be partially responsible for the genetic correlations, and selection under drought conditions we observed in Chapter III, further analysis is still sorely needed. QTL mapping of these traits in plants grown under dry-down conditions will allow us to identify if QTL identified so far show signs of antagonistic pleiotropy, conditional neutrality, or are likely to introgress adaptively. Additionally, investigating other potentially important traits that we measured in this mapping population will allow for a more complete picture of the genetic basis of divergence in drought adaptation between *M. guttatus* and *M. nasutus*, as well as its implications for ecological isolation between them.

Overall, our results indicate the need for sampling of individuals over space and time in hybridizing populations such as CAC to get more complete, population wide, understanding of hybridization and introgression. This more comprehensive look at population features likely to influence the outcome of hybridization and introgression will allow us to better understand whether species boundaries are likely to remain intact, or dissolve with further secondary contact. Ultimately, as genetic, genomic, and environmental factors also vary at other sympatric sites, similar studies of hybridization, introgression, and the maintenance of species boundaries across sites will be necessary to broadly understand the ecological conditions which may facilitate both continued divergence between the species, for their homogenization.

References

Abbott R, Albach D, Ansell S, Arntzen JW, Baird SJE, Bierne N, Boughman J, Brelsford A, Buerkle CA, Buggs R, *et al.* 2013. Hybridization and speciation. *Journal of Evolutionary Biology* **26**: 229–246.

Ali OA, O'Rourke SM, Amish SJ, Meek MH, Luikart G, Jeffres C, Miller MR. **2016**. RAD Capture (Rapture): Flexible and Efficient Sequence-Based Genotyping. *Genetics* **202**: 389–400.

Arnegard ME, McGee MD, Matthews B, Marchinko KB, Conte GL, Kabir S, Bedford N, Bergek S, Chan YF, Jones FC, *et al.* 2014. Genetics of ecological divergence during speciation. *Nature* **511**: 307–311.

Barton N, Bengtsson BO. **1986**. The barrier to genetic exchange between hybridising populations. *Heredity* **57**: 357–376.

Bateson W. 1909. Heredity and variation in modern lights. *Darwin and modern science* **85**:101.

Bolger AM, Lohse M, Usadel B. **2014**. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**: 2114–2120.

Brandvain Y, Kenney AM, Flagel L, Coop G, Sweigart AL. **2014**. Speciation and Introgression between Mimulus nasutus and Mimulus guttatus. *PLOS Genetics* **10**: e1004410.

Broman KW, Sen S. **2009**. *A Guide to QTL Mapping with R/qtl*. New York, NY: Springer New York.

Catchen JM, Amores A, Hohenlohe P, Cresko W, Postlethwait JH. **2011**. Stacks: Building and Genotyping Loci De Novo From Short-Read Sequences. *G3 Genes|Genomes|Genetics* **1**: 171–182.

Catchen J, Hohenlohe PA, Bassham S, Amores A, Cresko WA. **2013**. Stacks: an analysis tool set for population genomics. *Molecular Ecology* **22**: 3124–3140.

Coughlan JM, Brown MW, Willis JH. **2021**. The genetic architecture and evolution of life-history divergence among perennials in the Mimulus guttatus species complex. *Proceedings of the Royal Society B: Biological Sciences* **288**: 20210077.

Coyne JA, Orr HA. **2004**. *Speciation*. Oxford, New York: Oxford University Press.

Dillon LS. **1970**. Speciation and Changing Environment. *American Zoologist* **10**: 27–39.

Dobzhansky T. 1937. Genetics and the Origin of Species. *Columbia University Press.*

Doyle J, Doyle J. 1987. Genomic plant DNA preparation from fresh tissue-CTAB method. *Phytochem Bull* **19**:11-15

Endelman JB, Plomion C. **2014**. LPmerge: an R package for merging genetic maps by linear programming. *Bioinformatics* **30**: 1623–1624.

Fishman L, Kelly AJ, Willis JH. **2002**. Minor Quantitative Trait Loci Underlie Floral Traits Associated with Mating System Divergence in Mimulus. *Evolution* **56**: 2138–2155.

Fishman L, McIntosh M. **2019**. Standard Deviations: The Biological Bases of Transmission Ratio Distortion. *Annual Review of Genetics* **53**: 347–372.

Fishman L, Sweigart AL, Kenney AM, Campbell S. **2014a**. Major quantitative trait loci control divergence in critical photoperiod for flowering between selfing and outcrossing species of monkeyflower (Mimulus). *The New Phytologist* **201**: 1498–1507.

Fishman L, Willis JH, Wu CA, Lee Y-W. **2014b**. Comparative linkage maps suggest that fission, not polyploidy, underlies near-doubling of chromosome number within monkeyflowers (Mimulus; Phrymaceae). *Heredity* **112**: 562–568.

Flagel LE, Blackman BK, Fishman L, Monnahan PJ, Sweigart A, Kelly JK. **2019**. GOOGA: A platform to synthesize mapping experiments and identify genomic structural diversity. *PLOS Computational Biology* **15**: e1006949.

Friedman J, Twyford AD, Willis JH, Blackman BK. **2015**. The extent and genetic basis of phenotypic divergence in life history traits in Mimulus guttatus. *Molecular Ecology* **24**: 111–122.

Hall MC, Willis JH. **2005**. Transmission Ratio Distortion in Intraspecific Hybrids of *Mimulus guttatus*: Implications for Genomic Divergence. *Genetics* **170**: 375–386.

Hall MC, Willis JH. **2006**. Divergent Selection on Flowering Time Contributes to Local Adaptation in Mimulus Guttatus Populations. *Evolution* **60**: 2466–2477.

Harushima Y, Nakagahra M, Yano M, Sasaki T, Kurata N. **2001**. A Genome-Wide Survey of Reproductive Barriers in an Intraspecific Hybrid. *Genetics* **159**: 883–892.

Holeski LM, Monnahan P, Koseva B, McCool N, Lindroth RL, Kelly JK. **2014**. A High-Resolution Genetic Map of Yellow Monkeyflower Identifies Chemical Defense QTLs and Recombination Rate Variation. *G3: Genes|Genomes|Genetics* **4**: 813–821.

Hu J, Guo C, Wang B, Ye J, Liu M, Wu Z, Xiao Y, Zhang Q, Li H, King GJ, *et al.* 2018. Genetic Properties of a Nested Association Mapping Population Constructed With Semi-Winter and Spring Oilseed Rapes. *Frontiers in Plant Science* **9**.

Kenney AM, Sweigart AL. **2016**. Reproductive isolation and introgression between sympatric Mimulus species. *Molecular Ecology* **25**: 2499–2517.

Lee YW, Fishman L, Kelly JK, Willis JH. **2016**. A Segregating Inversion Generates Fitness Variation in Yellow Monkeyflower (Mimulus guttatus). *Genetics* **202**: 1473–1484.

Lee C-R, Mitchell-Olds T. **2011**. Quantifying effects of environmental and geographical factors on patterns of genetic differentiation. *Molecular Ecology* **20**: 4631–4642.

Li H. **2013**. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv:1303.3997 [q-bio]*.

Li H, Durbin R. **2009**. Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics* **25**: 1754–1760.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome Project Data Processing Subgroup. **2009**. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**: 2078–2079.

Lin Y-P, Lu C-Y, Lee C-R. **2020**. The climatic association of population divergence and future extinction risk of Solanum pimpinellifolium. *AoB PLANTS* **12**: plaa012.

Liti G, Barton DBH, Louis EJ. **2006**. Sequence Diversity, Reproductive Isolation and Species Concepts in Saccharomyces. *Genetics* **174**: 839–850.

Lorieux M, Goffinet B, Perrier X, de León DG, Lanaud C. **1995**. Maximum-likelihood models for mapping genetic markers showing segregation distortion. 1. Backcross populations. *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik* **90**: 73–80.

Lowry DB, Willis JH. **2010**. A Widespread Chromosomal Inversion Polymorphism Contributes to a Major Life-History Transition, Local Adaptation, and Reproductive Isolation. *PLOS Biology* **8**: e1000500.

Mantel SJ, Sweigart AL. **2019**. Divergence in drought-response traits between sympatric species of Mimulus. *Ecology and Evolution* **9**: 10291–10304.

Martin NH, Bouck AC, Arnold ML. **2005**. Loci affecting long-term hybrid survivorship in Louisiana irises: implications for reproductive isolation and introgression. *Evolution; International Journal of Organic Evolution* **59**: 2116–2124.

Michel AP, Sim S, Powell THQ, Taylor MS, Nosil P, Feder JL. **2010**. Widespread genomic divergence during sympatric speciation. *Proceedings of the National Academy of Sciences* **107**: 9724–9729.

Noor MAF, Grams KL, Bertucci LA, Reiland J. **2001**. Chromosomal inversions and the reproductive isolation of species. *Proceedings of the National Academy of Sciences* **98**: 12084–12088.

Nosil P. **2012**. *Ecological Speciation*. Oxford: Oxford University Press.

Nosil P, Harmon LJ, Seehausen O. **2009**. Ecological explanations for (incomplete) speciation. *Trends in Ecology & Evolution* **24**: 145–156.

Nosil P, Vines TH, Funk DJ. **2005**. Reproductive Isolation Caused by Natural Selection Against Immigrants from Divergent Habitats. *Evolution* **59**: 705–719.

Orr HA. **2005**. The genetic theory of adaptation: a brief history. *Nature Reviews Genetics* **6**: 119–127.

Rastas P. **2017**. Lep-MAP3: robust linkage mapping even for low-coverage whole genome sequencing data. *Bioinformatics* **33**: 3726–3732.

Rieseberg LH. **2001**. Chromosomal rearrangements and speciation. *Trends in Ecology & Evolution* **16**: 351–358.

Rundle HD, Nosil P. **2005**. Ecological speciation. *Ecology Letters* **8**: 336–352.

Safran RJ, Scordato ESC, Symes LB, Rodríguez RL, Mendelson TC. **2013**. Contributions of natural and sexual selection to the evolution of premating reproductive isolation: a research agenda. *Trends in Ecology & Evolution* **28**: 643–650.

Sambatti JBM, Strasburg JL, Ortiz-Barrientos D, Baack EJ, Rieseberg LH. **2012**. Reconciling Extremely Strong Barriers with High Levels of Gene Exchange in Annual Sunflowers. *Evolution* **66**: 1459–1473.

Schluter D. **2000**. *The Ecology of Adaptive Radiation*. OUP Oxford.

Schluter D. **2009**. Evidence for Ecological Speciation and Its Alternative. *Science* **323**: 737–741.

Scott MF, Ladejobi O, Amer S, Bentley AR, Biernaskie J, Boden SA, Clark M, Dell'Acqua M, Dixon LE, Filippi CV, *et al.* 2020. Multi-parent populations in crops: a toolbox integrating genomics and genetic mapping with breeding. *Heredity* **125**: 396–416.

Sobel JM, Chen GF, Watt LR, Schemske DW. **2010**. The Biology of Speciation. *Evolution* **64**: 295–315.

Sweigart AL, Willis JH. **2003**. Patterns of Nucleotide Diversity in Two Species of Mimulus Are Affected by Mating System and Asymmetric Introgression. *Evolution* **57**: 2490–2506.

Thompson KA, Urquhart-Cronish M, Whitney KD, Rieseberg LH, Schluter D. **2021**. Patterns, Predictors, and Consequences of Dominance in Hybrids. *The American Naturalist* **197**: E72–E88.

Troth A, Puzey JR, Kim RS, Willis JH, Kelly JK. **2018**. Selective trade-offs maintain alleles underpinning complex trait variation in plants. *Science* **361**: 475–478.

Turner TL, Hahn MW, Nuzhdin SV. **2005**. Genomic Islands of Speciation in Anopheles gambiae. *PLOS Biology* **3**: e285.

Van Ooijen JW. **2011**. Multipoint maximum likelihood mapping in a full-sib family of an outbreeding species. *Genetics Research* **93**: 343–349.

Wu CA, Lowry DB, Cooley AM, Wright KM, Lee YW, Willis JH. **2008**. Mimulus is an emerging model system for the integration of ecological and genomic studies. *Heredity* **100**: 220–230.

Xu S. **2003**. Theoretical Basis of the Beavis Effect. *Genetics* **165**: 2259–2268.

Yu J, Holland JB, McMullen MD, Buckler ES. **2008**. Genetic Design and Statistical Power of Nested Association Mapping in Maize. *Genetics* **178**: 539–551.

Zuellig MP, Sweigart AL. **2018**. A two-locus hybrid incompatibility is widespread, polymorphic, and active in natural populations of Mimulus*. *Evolution* **72**: 2394–2405.

Table 4.1 Summary putative introgression, areas which show abruptly decreased nucleotide diversity and multiple windows of $\pi > 0.05$, in the three *M. guttatus* parental lines used.

	Chromosome	Position (Mb)
CAC110	1	6.5 - 9.7
	2	1.2 - 3.4
	4	0.0 - 4.2
	5	14.0 - 16.7
	8	5.3 - 6.4
	8	7.8 - 13.7
	8	17.3 - 17.8
	9	0.0 - 1.2
	10	3.3 - 4.3
	10	6.1 - 7.6
	11	2.1 - 15.5
	13	0.0 - 14.7
	14	1.4 - 2.8
CAC162	6	3.9 - 4.6
	8	1.4 - 2.3
	8	16.8 - 21.6
CAC415	2	0.5 - 1.9
	8	17.3 - 18.2
	12	15.9 - 16.6

Table 4.2 Chromosome by chromosome summary of intrascaffold recombination rate estimates for each of the three linkage maps. Group 1: CAC9xCAC110, group 2: CAC9xCAC162, group 3: CAC9xCAC415.

		Group 1		Group 2		Group 3	
Chromosome	Reference Length (Mb)	Map Length (cM)	cM/Mb	Map Length (cM)	cM/Mb	Map Length (cM)	cM/Mb
1	11.8	78.344	6.64	53.183	4.51	72.013	6.10
2	17.8	91.734	5.15	62.537	3.51	87.839	4.93
3	16.8	86.103	5.13	54.714	3.26	68.621	4.08
4	20.1	80.862	4.02	73.546	3.66	91.236	4.54
5	18.9	83.193	4.40	67.672	3.58	86.621	4.58
6	17.9	113.134	6.32	64.164	3.58	102.446	5.72
7	16.7	71.614	4.29	61.769	3.70	84.391	5.05
8	24.4	102.230	4.19	83.893	3.44	109.343	4.48
9	14.4	50.823	3.53	67.320	4.68	77.591	5.39
10	19.5	80.811	4.14	85.945	4.41	100.356	5.15
11	16.1	130.659	8.12	64.922	4.03	82.136	5.10
12	18.9	95.910	5.07	67.261	3.56	72.031	3.81
13	18.8	79.826	4.25	88.942	4.73	125.554	6.68
14	26.6	133.820	5.03	87.280	3.28	116.390	4.38
Global	258.7	1279.063	4.94	983.148	3.80	1276.568	4.93

Table 4.3 Summary of identified rearrangements among the four parental lines used. Citations indicate previous identification of the same rearrangement. Bolded entries indicate overlap between a putative segregating rearrangement and putative regions of introgression. “Forward” and “reverse” orientation designations are based on the physical orientation in the *Mimulus guttatus* v5.0 refence genome. Species distribution codes: S = segregating within Catherine Creek *M. guttatus*, N = *M. nasutus*-specific, P = perennial ecotype *M. guttatus*-specific, G-CAC = CAC *M. guttatus*-specific, G-OR = Oregon annual ecotype *M. guttatus*-specific.

Chr	Approximate Position (Mb)	Notes	Species Distribution	Previous Identification
1	0 - 3.5	Segregating inversion, "forward" orientation present in CAC9 and CAC162, "reverse" orientation present in CAC110 and CAC415	S	-
1	**6.5 - 9.8**	**Segregating inversion, "forward" orientation present in CAC162 and CAC415, "reverse" orientation present in CAC9 and CAC110**	**N?**	**-**
4	4.2 - 14.5	Segreagating inversion, "forward" orientation present in CAC9 and CAC415, "reverse" orientation present in CAC110 and CAC162, could also be explained by variability is supression of recombination surrounding the centromere	S	-
6	0 - 4.5	Segregating translocation, present in CAC162	S	-
7	6.3 - 16.6	Segregating inversion, "forward" orientation present in CAC9, CAC162, and CAC415, "reverse" orientation present in CAC110	S	-
8	1.3 - 5.2	Previously identified inversion, "reverse" orientation present in CAC9, CAC110, CAC162, and CAC415	P	Lowry & Willis, 2010
8	**7.8 - 12.7**	**Segregating inversion, "forward" orientation present in CAC9 and CAC110, "reverse" orientation present in CAC162 and CAC415**	**S or G-CAC**	**-**
10	5.7 - 16.8	Segregating inversion, "forward" orentation present in CAC9, "reverse" orientation present in CAC110, CAC162, and CAC415	G-OR	Holeski *et al* ., 2014 Flagel *et al* ., 2019
13	**0 - 13**	**Segregating inversion, "forward" orientation present in CAC9 and CAC110, "reverse" orientation present in CAC162 and CAC415**	**S or G-CAC**	**-**

Table 4.4 Summary of identified QTL peak locations, lower bound (LB) and upper bound (UB) of the QTL location calculated as the most distant positions with significant LOD scores (alpha=0.05 from 5,000 permutations) surrounding the peak. Percent variance explained (%VE), additive effect estimates (a), dominance effect estimates (d), and the dominance to additive ratio for each peak is shown.

RIX Group	Phenotype	Chromosome	Position	LB	UB	%VE	a	d	d/|a|
1x2	Outcrossed Seedset DD	7	9.1	7.7	71.2	1.08	-1.94	6.39	3.30
		11	14.6	13.1	81.2	6.42	22.49	-45.38	-2.02
	Survival to Fruit DD	8	79.3	28.3	100.0	8.42	-0.29	0.12	0.42
	Peduncle Length WW	3	28.7	25.2	46.2	4.06	-0.70	-2.37	-3.40
		5	66.1	52.9	73.0	12.47	-3.76	1.92	0.51
		10	80.0	31.5	86.8	17.87	5.67	1.42	0.25
		13	16.9	26.42	18.1	17.04	-2.19	5.15	2.36
	Days Bud to Fruit WW	3	26.7	22.2	33.4	7.20	-0.19	-1.71	-9.01
	Leaf Length at Bud WW	2	52.2	46.5	57.4	1.47	4.11	6.84	1.67
		3	0.4	0.0	19.7	15.89	-12.76	-4.37	-0.34
		4	66.4	63.0	74.7	2.25	0.56	3.73	6.72
		7	55.2	44.0	71.2	9.14	5.82	-13.22	-2.27
		10	36.4	31.5	72.6	2.75	-9.96	-9.19	-0.92
		14	75.5	70.9	90.6	18.71	14.46	-0.17	-0.01
	Days Germ to Bud WW	3	0.0	0.0	3.1	1.96	-4.95	-3.54	-0.72
		5	52.9	47.0	73.0	6.31	5.69	-4.70	-0.83
		7	55.2	36.9	71.2	20.96	8.81	-7.32	-0.83
		8	75.8	75.0	100.1	6.00	1.57	5.48	3.49
		10	38.4	31.5	54.3	22.92	-3.35	-8.51	-2.54
		14	87.8	76.3	101.5	23.85	0.13	-9.78	-73.26
2x3	Outcrossed Seedset DD	7	10.7	0.0	13.4	9.95	-28.53	44.72	1.57
		11	13.1	0.0	13.5	5.16	-12.03	-34.55	-2.87
		13	84.6	83.2	84.6	20.73	-12.06	44.31	3.67
	Peduncle Length WW	5	0.0	0.0	28.1	3.57	-1.66	-2.89	-1.74
		10	70.8	54.4	82.9	6.73	4.98	-3.79	-0.76
		14	66.6	58.4	72.3	10.85	0.82	7.08	8.63
	Days Bud to Fruit WW	1	5.6	3.7	16.0	1.29	0.12	2.83	23.85
		2	8.1	3.0	22.6	4.11	-0.49	6.22	12.77
		14	63.1	58.4	63.4	2.52	-7.71	0.54	0.07
	Leaf Length at Bud WW	5	65.2	0.0	73.0	24.06	18.35	-14.60	-0.80
		6	95.1	71.6	96.0	9.72	8.12	13.77	1.69
	Days Germ to Bud WW	2	82.0	47.3	84.0	2.31	-0.96	-3.29	-3.44
		6	84.9	1.7	96.0	3.71	2.00	-3.28	-1.64
		12	34.0	32.5	35.5	4.26	3.85	-7.95	-2.06
		13	75.3	57.1	78.5	4.04	5.89	-1.44	-0.24
3x1	Outcrossed Seedset DD	4	0.5	0.5	7.9	9.68	-44.00	-21.92	-0.50
		11	31.5	21.4	35.4	14.96	-45.98	63.14	1.37
	Peduncle Length WW	1	35.6	2.7	68.6	8.76	-1.95	-8.02	-4.12
		9	19.1	3.6	67.3	4.26	-2.21	1.58	0.71
		12	77.2	68.8	78.1	8.18	-33.82	21.99	0.65
	Days Bud to Fruit WW	8	49.0	41.2	55.9	10.81	-2.57	-4.91	-1.91
		9	20.5	19.5	20.8	1.39	2.22	-2.60	-1.17
		14	58.4	57.0	59.4	8.16	-6.69	-4.99	-0.74
	Leaf Length at Bud WW	6	40.0	32.4	96.0	5.82	0.33	10.81	33.26
		10	83.9	78.1	90.6	3.75	-7.28	-8.02	-1.10
	Days Germ to Bud WW	6	40.0	33.3	43.3	10.81	-2.57	-4.91	-1.91
		10	82.9	71.6	100.4	1.39	2.22	-2.60	-1.17
		14	17.6	11.1	34.6	8.16	-6.69	-4.99	-0.74

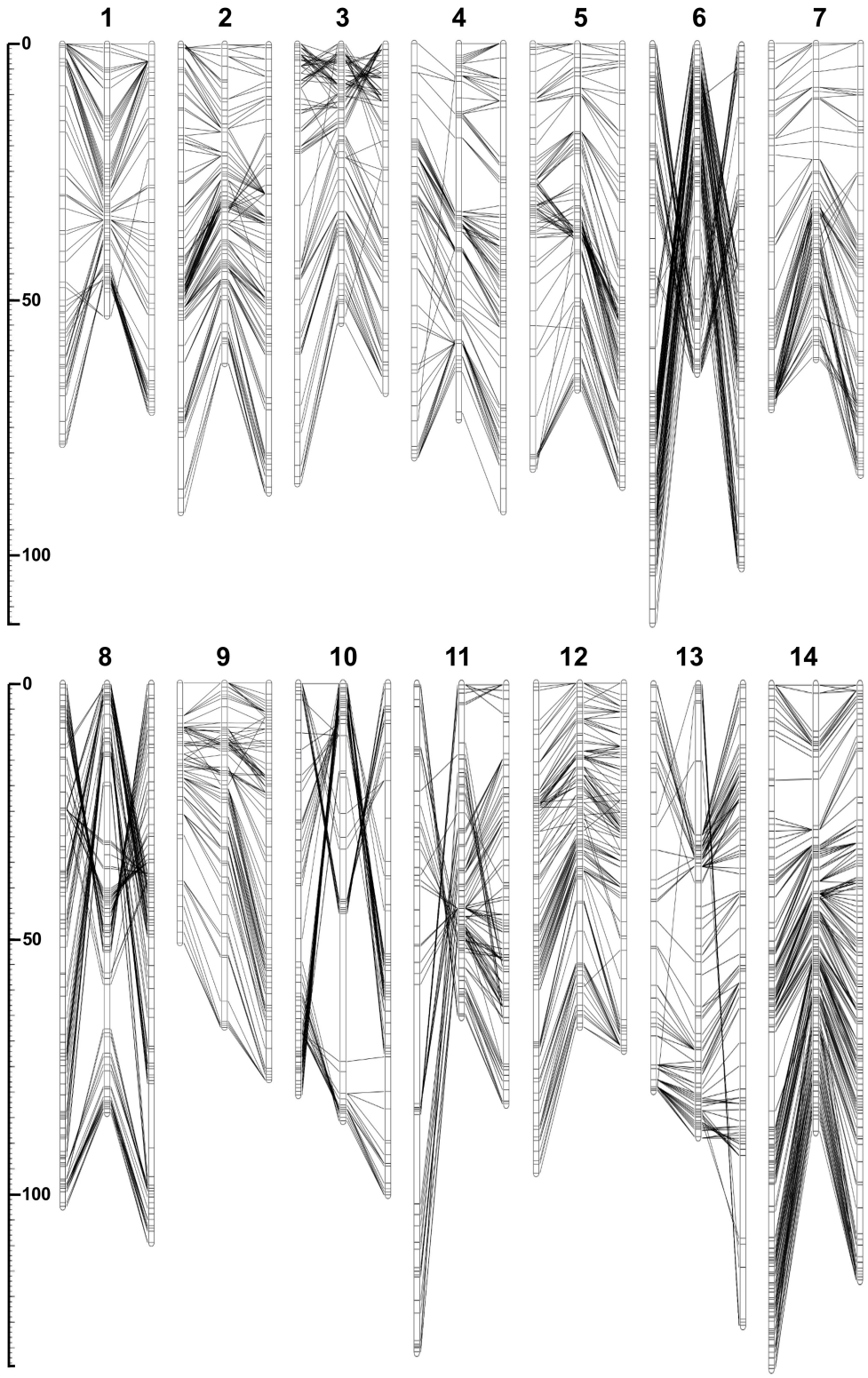


Figure 4.1 Comparison of overall size and marker position in the three independent linkage maps produced for the three RIL populations produced for this comparison. Scale bar units: cM.

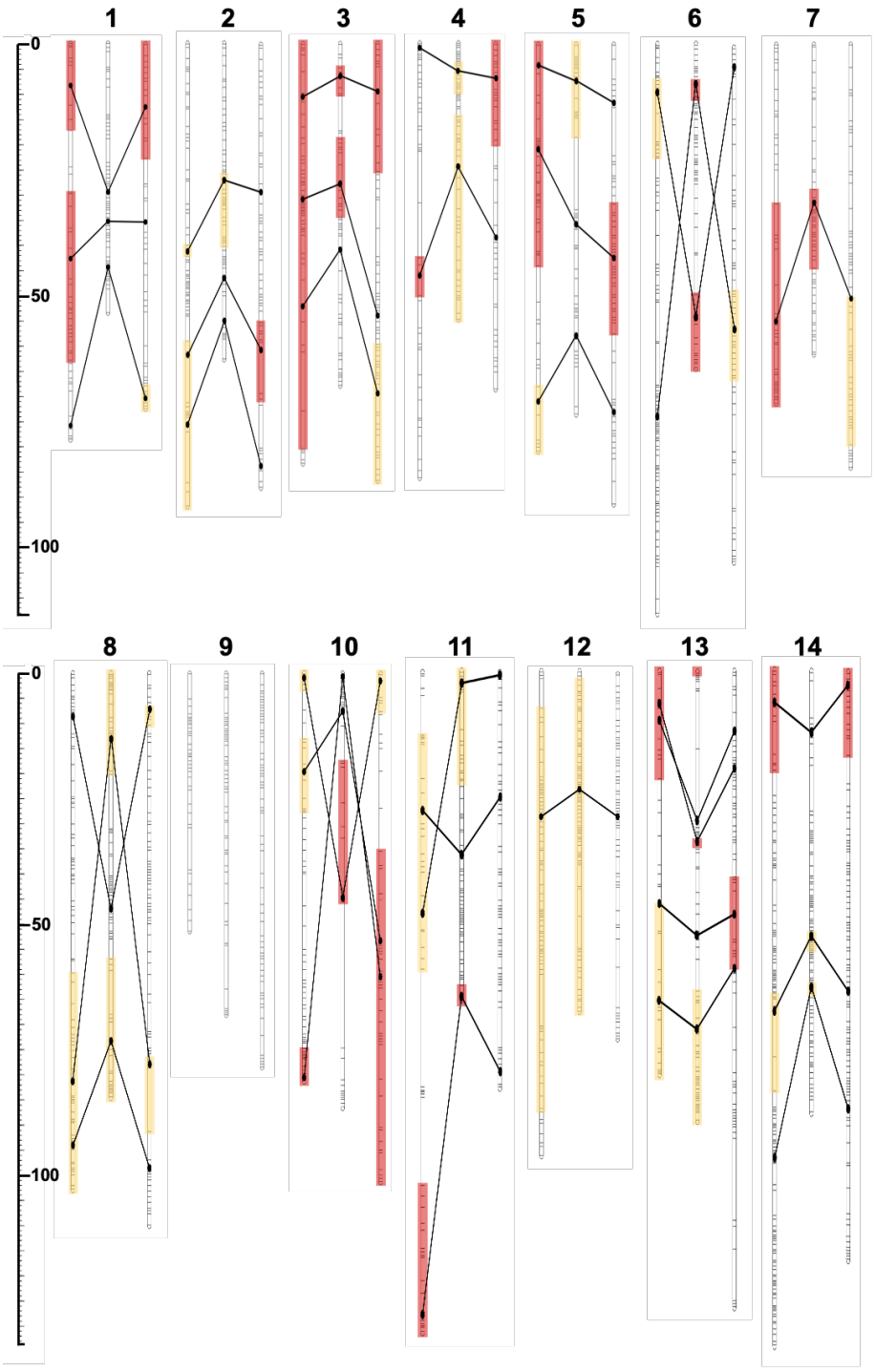


Figure 4.2 Transmission ratio distortion in the three RIL groups produced. Regions showing significant distortion at the α = 0.05 level are highlighted. Regions highlighted in red indicate distortion towards *M. guttatus* ancestry, and regions highlighted in yellow indicate distortion towards *M. nasutus* ancestry. The location of a single marker, shared between all three maps, from each of the significantly distorted regions is indicated with a dot to allow for locational comparisons where marker order differs between maps. Scale bar units: cM.

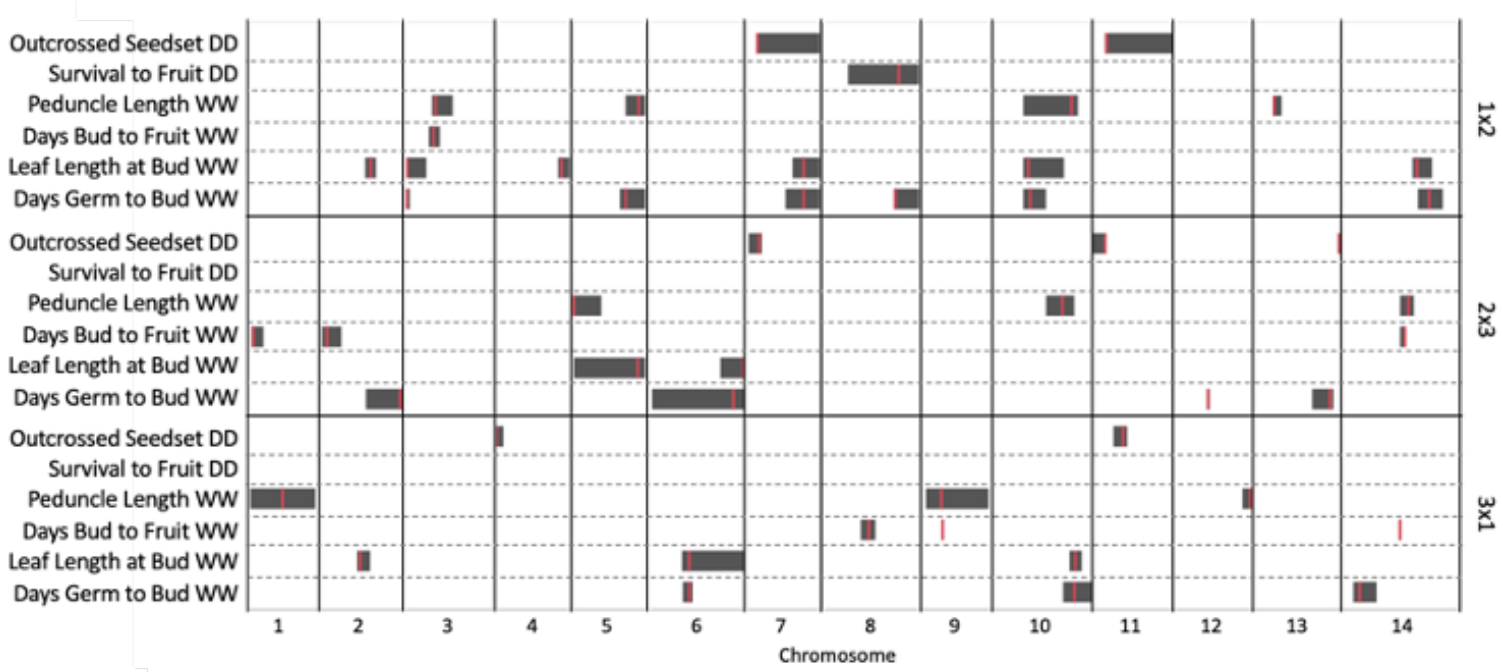


Figure 4.3 Locations of identified QTL plotted against the map position of the genome. The red vertical line corresponds to the estimated location of the QTL peak, with the width of the grey rectangles indicating the full range of positions with significant LOD scores (alpha=0.05 from 5,000 permutations) surrounding the peak.

CHAPTER V

CONCLUSION AND FUTURE DIRECTIONS

Taken as a whole, the work outlined in this dissertation adds to a growing body of knowledge on the forces responsible for the maintenance of biodiversity, particularly ecological isolation, caused by phenotypic variation in drought response between closely related, hybridizing species. This work provides a strong groundwork for future investigations that will continue to identify important ecological factors leading to such isolation, as well as the traits and genetic loci underlying reproductive barriers and the mechanisms driving their evolution.

First, in lines derived from the sympatric site Catherine Creek (CAC), we provided evidence of numerous drought response traits differing between sympatric *Mimulus guttatus* and *M. nasutus* (Mantel & Sweigart, 2019). It was previously understood that *M. nasutus* employed a drought escape strategy to avoid terminal drought through early-season reproductive initiation, mediated by a shorter critical photoperiod than that of *M. gutattus* (Fishman *et al.*, 2014). We showed that, even under an inductive photoperiod, when the two species flower at around the same time, *M. nasutus* is considerably more capable of successfully setting seeds under extreme drought stress than is *M. guttatus*. Therefore, additional adaptations, beyond reduced critical photoperiod, are present in CAC *M. nasutsus* that allows it to more successfully survive and set seeds under drought than *M. guttatus*. Most notably more rapid fruit development specifically, plasticity in fruit ripening, induced by low water availability was identified as an important axis of divergence between species.

We then further investigated this divergence in drought response between species, focusing on understanding its implications for ecological isolation between the species. In an admixed, custom-made mapping population we thoroughly investigated drought response traits related to plant growth and reproduction. We demonstrated that species differences are due to a combination of plasticity, genetic differences, and genetic-by-environment interactions in multiple genetically correlated traits, which in different instances are expected to both facilitate and constrain adaptation to drought conditions.

Additionally, these correlated traits also show evidence of genetic tradeoffs between environments, likely to contribute to ecological isolation between *M. guttatus* and *M. nasutus* at CAC.

In this same mapping population, we then began the process of mapping the genetic loci associated with the important phenotypic differences we observed. In doing so we observed the signatures of variable introgression present in the three *M. guttatus* lines used as parents of our mapping population. We also discovered extensive and variable transmission ratio distortion in the three RIL populations we created, finding potentially important regions of shared distortion across RIL populations which may indicate important incompatibility loci or genetic drivers. Finally, we identified numerous QTL associated with the subset of six phenotypes we analyzed here, further demonstrating the polygenic nature of the important traits involved in drought response in these *Mimulus* species. QTL generally had small estimated effects, and were spread throughout the genome. However, as expected given high genetic correlations between some of the traits investigated, we did discover polygenic loci which seem to be associated with multiple traits and therefore likely influence the effectiveness of selection at maximizing drought adaptation. Of notable interest was a QTL implicated in both survival under drought conditions and budding time found near a region of shared introgression between all three *M. guttatus* lines, and within a region of significant shared TRD among all three RIL populations.

While the work described here has expanded our knowledge of the action of ecological isolation, there is still much that needs to be addressed. Most obviously, we have not yet mapped all measured traits. Traits such as leaf length at flower and leaf growth rate were shown to be under selection under drought conditions, and are therefore likely to be important for drought adaptation in this system. Additionally, it will undoubtedly be informative to map the genetic basis of plasticity, both because in cases where plasticity was shown to be maladaptive, alleles conferring a lack of plasticity may be integral to plant success, and because adaptive plasticity in fruit ripening appears to be an important *M. nasutus* specific innovation allowing for additional reproductive success under drought conditions.

To fully establish expectations about the likelihood of an identified allele to resist introgression, and contribute to ecological isolation between species, or to introgress freely, it will also be necessary to

map these same traits under dry-down conditions. This will allow us to look directly at if the alleles we have identified are conditionally neutral, antagonistically pleiotropic, or even likely to introgress adaptively. Given previous findings showing genome wide selection against *M. nasutus* alleles in an *M. guttatus* background (Kenney & Sweigart, 2016) combined with the expectations that variable environmental conditions over space and time can maintain variation in these drought related traits in *Mimulus* (Mojica & Kelly, 2010; Mojica *et al.*, 2012; Troth *et al.*, 2018) both patterns are likely common.

Lastly, and very importantly, the strategy employed here for identifying QTL is far from ideal. We therefore need to develop a more appropriate mapping technique that will allow for QTL mapping in our full, multi-parental population (e.g., Yu *et al.*, 2008; Hu *et al.*, 2018; Scott *et al.*, 2020). In doing so we will obtain a finer scale look at the genetic variation present in our mapping population underlying the drought response traits measured. Identifying individual alleles and their effects, rather than species specific effects as we have analyzed so far, is sure to open the door for future research into the action and evolution of numerous loci integral to the maintenance of species boundaries between sympatric *M. nasutus* and *M. guttatus* at CAC.

References

Fishman L, Sweigart AL, Kenney AM, Campbell S. **2014**. Major quantitative trait loci control divergence in critical photoperiod for flowering between selfing and outcrossing species of monkeyflower (Mimulus). *The New Phytologist* **201**: 1498–1507.

Friedman J, Willis JH. **2013**. Major QTLs for critical photoperiod and vernalization underlie extensive variation in flowering in the Mimulus guttatus species complex. *The New Phytologist* **199**: 571–583.

Hu J, Guo C, Wang B, Ye J, Liu M, Wu Z, Xiao Y, Zhang Q, Li H, King GJ, *et al.* 2018. Genetic Properties of a Nested Association Mapping Population Constructed With Semi-Winter and Spring Oilseed Rapes. *Frontiers in Plant Science* **9**.

Kenney AM, Sweigart AL. **2016**. Reproductive isolation and introgression between sympatric Mimulus species. *Molecular Ecology* **25**: 2499–2517.

Mantel SJ, Sweigart AL. **2019**. Divergence in drought-response traits between sympatric species of Mimulus. *Ecology and Evolution* **9**: 10291–10304.

Mojica JP, Kelly JK. **2010**. Viability selection prior to trait expression is an essential component of natural selection. *Proceedings of the Royal Society B: Biological Sciences* **277**: 2945–2950.

Mojica JP, Lee YW, Willis JH, Kelly JK. **2012**. Spatially and temporally varying selection on intrapopulation quantitative trait loci for a life history trade-off in Mimulus guttatus. *Molecular Ecology* **21**: 3718–3728.

Scott MF, Ladejobi O, Amer S, Bentley AR, Biernaskie J, Boden SA, Clark M, Dell'Acqua M, Dixon LE, Filippi CV, *et al.* 2020. Multi-parent populations in crops: a toolbox integrating genomics and genetic mapping with breeding. *Heredity* **125**: 396–416.

Troth A, Puzey JR, Kim RS, Willis JH, Kelly JK. **2018**. Selective trade-offs maintain alleles underpinning complex trait variation in plants. *Science* **361**: 475–478.

Yu J, Holland JB, McMullen MD, Buckler ES. **2008**. Genetic Design and Statistical Power of Nested Association Mapping in Maize. *Genetics* **178**: 539–551.

APPENDIX A

SUPPLEMENTARY TABLES AND FIGURES FROM CHAPTER III

Table S3.1: Results of a Type III repeated measures ANOVA using Satterthwaite's method for dry weight basis soil moisture (θ_d) measured in dry-down flats. The model includes "Day" (repeated measure factor), "X_position" (fixed effect), "Y_position" (fixed effect), the two way interaction effect. "X_position" refers to the location of each flat, measured as distance from the cooling rack in an east/west direction, in each greenhouse room. "Y_position" refers to the location of each flat, measured as distance from the northmost window in a north/south direction, in each greenhouse room. Position values in both directions were standardized to a mean of zero and a standard deviation of one to avoid scale effects.

	SS	*df*	MS	*F*	*p*
Dry Weight Basis Soil Moisture (θd)					
Day	26.0344	7, 67.00	3.7192	11.4525	**<0.0001**
X_Position	0.0047	1, 67.03	0.0047	0.0145	0.9045
Y_Position	0.0246	1, 67.03	0.0246	0.0759	0.7839
X_Position*Y_Position	1.7590	1, 67.01	1.7590	5.4165	**0.0230**

Abbreviations: SS, Sum-of-Squares; *df,* degrees of freedom; MS, Mean-Squares.

Table S3.2: RIX population least squares means for growth and fitness related traits when grown under the two watering regimes, well-watered (WW), and dry-down (DD).

	Survival to Bud (%)	Days Germ to Bud	Leaf Length at Bud	Leaf Width at Bud	Leaf Length/Day Germ to Bud	Leaf Width/Day Germ to Bud	Survival to Flower (%)	Corolla Length	Corolla Width	Peduncle Length	Leaf Length at Flower
WW	98.94 (0.60, 582)	31.80 (0.51, 569)	48.45 (4.11, 565)	38.26 (1.86, 565)	1.44 (0.08, 565)	1.16 (0.05, 565)	98.07 (0.80, 582)	22.06 (0.69, 562)	16.41 (0.49, 562)	25.53 (0.72, 561)	64.57 (3.03, 559)
DD	72.11 (4.49, 577)	30.04 (0.52, 441)	35.81 (4.13, 440)	28.02 (1.89, 440)	1.12 (0.08, 440)	0.90 (0.05, 440)	31.85 (4.77, 577)	15.44 (0.77, 225)	9.13 (0.60, 225)	8.11 (0.92, 225)	31.90 (3.30, 226)

Leaf Width at Flower	Leaf Length/Day Bud to Flower	Leaf Width/Day Bud to Flower	Number of Flowers	Survival to Fruit (%)	Days Bud to Fruit	Outcrossed Seedset	Selfed Seedset
51.98 (1.96, 555)	1.37 (0.51, 552)	1.08 (0.31, 548)	30.90 (4.15, 505)	97.32 (0.95, 582)	37.19 (0.43, 547)	138.31 (18.27, 582)	116.61 (19.07, 582)
21.46 (2.18, 226)	-0.74 (0.52, 226)	-1.07 (0.32, 226)	3.06 (0.43, 570)	26.05 (4.45, 577)	31.15 (0.55, 174)	11.98 (1.51, 577)	6.45 (1.06, 577)

Note: Standard error and sample size given in parentheses. Model information given in Appendix 1, with model output statistics given in Table S3.

Table S3.3: GLMM or LMM output statistics for each measured phenotype using Satterthwaite's method in a type III ANOVA. For GLMMs $\chi 2$ and p-values were calculated using likelihood ratio tests comparing our main model with models lacking each fixed and interaction effect in turn. Significant p-values are shown in bold, significance was determined using a Bonferroni correction of $\alpha = 0.0026$.

		SS	*df*	MS	*F*	χ^2	*p*
Survival to Bud							
	Treatment	49.92	1	49.92	49.92	87.36	**<0.0001**
	Guttatus Ancestry	12.19	2	6.10	6.10	19.46	**0.0006**
	Treatment*Guttatus Ancestry	4.05	2	2.02	2.02	5.44	0.0659
Days Germ to Bud							
	Treatment	229.94	1	229.94	23.90	-	**<0.0001**
	Guttatus Ancestry	291.22	2	145.61	15.13	-	**<0.0001**
	Treatment*Guttatus Ancestry	69.17	2	34.58	3.59	-	0.0280
Leaf Length at Bud							
	Treatment	21194.60	1	21194.60	201.10	-	**<0.0001**
	Guttatus Ancestry	2374.70	2	1187.35	11.27	-	**<0.0001**
	Treatment*Guttatus Ancestry	2153.00	2	1076.50	10.21	-	**<0.0001**
Leaf Width at Bud							
	Treatment	16537.20	1	16537.20	232.95	-	**<0.0001**
	Guttatus Ancestry	1718.90	2	859.45	12.11	-	**<0.0001**
	Treatment*Guttatus Ancestry	1426.80	2	713.40	10.05	-	**<0.0001**
Leaf Length/Day Germ to Bud							
	Treatment	18.26	1	18.26	202.45	-	**<0.0001**
	Guttatus Ancestry	0.78	2	0.39	4.30	-	0.0144
	Treatment*Guttatus Ancestry	1.05	2	0.53	5.85	-	0.0030
Leaf Width/Day Germ to Bud							
	Treatment	9.92	1	9.92	159.50	-	**<0.0001**
	Guttatus Ancestry	0.69	2	0.34	5.51	-	0.0045
	Treatment*Guttatus Ancestry	0.63	2	0.31	5.03	-	0.0068
Survival to Flower							
	Treatment	171.06	1	171.06	171.06	128.14	**<0.0001**
	Guttatus Ancestry	16.62	2	8.31	8.31	28.12	**<0.0001**
	Treatment*Guttatus Ancestry	7.83	2	3.92	3.92	10.19	0.0061
Corolla Length							
	Treatment	1812.47	1	1812.47	156.12	-	**<0.0001**
	Guttatus Ancestry	209.93	2	104.97	9.04	-	**0.0001**
	Treatment*Guttatus Ancestry	12.35	2	6.18	0.53	-	0.5877
Corolla Width							
	Treatment	2180.52	1	2180.52	222.67	-	**<0.0001**
	Guttatus Ancestry	273.95	2	136.98	13.99	-	**<0.0001**
	Treatment*Guttatus Ancestry	5.30	2	2.65	0.27	-	0.7629
Peduncle Length							
	Treatment	18089.80	1	18089.80	499.10	-	**<0.0001**
	Guttatus Ancestry	142.90	2	71.45	1.97	-	0.1408
	Treatment*Guttatus Ancestry	199.80	2	99.90	2.76	-	0.0645
Leaf Length at Flower							
	Treatment	44218.00	1	44218.00	296.72	-	**<0.0001**
	Guttatus Ancestry	598.00	2	299.00	2.01	-	0.1360
	Treatment*Guttatus Ancestry	970.00	2	485.00	3.25	-	0.0394
Leaf Width at Flower							
	Treatment	44744.00	1	44744.00	479.18	-	**<0.0001**
	Guttatus Ancestry	238.00	2	119.00	1.28	-	0.2805
	Treatment*Guttatus Ancestry	80.00	2	40.00	0.43	-	0.6503
Leaf Length/Day Bud to Flower							
	Treatment	190.51	1	190.51	184.72	-	**<0.0001**
	Guttatus Ancestry	2.55	2	1.27	1.23	-	0.2927
	Treatment*Guttatus Ancestry	7.68	2	3.84	3.72	-	0.0248
Leaf Width/Day Bud to Flower							
	Treatment	149.94	1	149.94	198.52	-	**<0.0001**
	Guttatus Ancestry	6.40	2	3.20	4.23	-	0.0153
	Treatment*Guttatus Ancestry	0.76	2	0.38	0.50	-	0.6049
Number of Flowers							
	Treatment	1232.71	1	1232.71	1232.71	160.26	**<0.0001**
	Guttatus Ancestry	2.51	2	1.26	1.26	10.32	0.0354
	Treatment*Guttatus Ancestry	7.88	2	3.94	3.94	7.82	0.0200
Survival to Fruit							
	Treatment	211.67	1	211.67	211.67	123.09	**<0.0001**
	Guttatus Ancestry	8.09	2	4.05	4.05	19.38	**0.0007**
	Treatment*Guttatus Ancestry	8.32	2	4.16	4.16	10.12	0.0063
Days Bud to Fruit							
	Treatment	1634.32	1	1634.32	73.18	-	**<0.0001**
	Guttatus Ancestry	365.69	2	182.85	8.19	-	**0.0003**
	Treatment*Guttatus Ancestry	107.57	2	53.79	2.41	-	0.0907
Outcrossed Seed set							
	Treatment	298.21	1	298.21	298.21	434.38	**<0.0001**
	Guttatus Ancestry	5.65	2	2.83	2.83	348.96	**<0.0001**
	Treatment*Guttatus Ancestry	342.35	2	171.18	171.18	343.45	**<0.0001**
Selfed Seed set							
	Treatment	274.24	1	274.24	274.24	420.15	**<0.0001**
	Guttatus Ancestry	12.13	2	6.07	6.07	342.69	**<0.0001**
	Treatment*Guttatus Ancestry	330.64	2	165.32	165.32	330.94	**<0.0001**

Abbreviations: df, degrees of freedom; MS, Mean-Squares; SS, Sum-of-Squares.

Table S3.4: Population average percent decrease from well-watered conditions to dry-down conditions for each measured trait. Test statistics and p-values for 2-way ANOVA, Kruskal - Wallis test, and Welch's test are shown. Significance was determined using a Bonferroni correction of $\alpha = 0.0031$.

			ANOVA		Kruskal - Wallis Test		Welch's Test	
	N	% Decrease	*F*	*p*	χ^2	*p*	*F*	*p*
Days Germ to Bud	218	5.10	17.85	<0.0001	11.29	0.0008	17.85	<0.0001
Leaf Length at Bud	217	24.81	102.83	<0.0001	76.08	<0.0001	102.83	<0.0001
Leaf Width at Bud	219	24.86	104.45	<0.0001	76.04	<0.0001	104.45	<0.0001
Leaf Length/Day Germ to Bud	222	19.15	86.90	<0.0001	69.29	<0.0001	86.90	<0.0001
Leaf Width/Day Germ to Bud	223	19.10	87.16	<0.0001	70.87	<0.0001	87.16	<0.0001
Leaf Length at Flower	125	48.90	747.03	<0.0001	182.90	<0.0001	747.03	<0.0001
Leaf Width at Flower	127	56.07	1273.49	<0.0001	189.28	<0.0001	1273.49	<0.0001
Leaf Length/Day Bud to Flower	130	95.26	529.89	<0.0001	177.26	<0.0001	529.89	<0.0001
Leaf Width/Day Bud to Flower	130	116.74	574.16	<0.0001	183.04	<0.0001	574.16	<0.0001
Corolla Length	130	30.83	308.45	<0.0001	151.28	<0.0001	308.45	<0.0001
Coroall Width	131	43.61	359.56	<0.0001	160.29	<0.0001	359.56	<0.0001
Peduncle Length	124	68.99	1029.32	<0.0001	184.77	<0.0001	1029.32	<0.0001
Number of Flowers	119	89.79	523.45	<0.0001	178.25	<0.0001	523.45	<0.0001
Days Bud to Fruit	98	17.33	157.33	<0.0001	88.34	<0.0001	157.33	<0.0001
Outrossed Seedset	234	93.82	910.38	<0.0001	350.76	<0.0001	910.38	<0.0001
Selfed Seedset	218	98.70	605.95	<0.0001	341.64	<0.0001	605.95	<0.0001

Table S3.5: Results of Levene's test for each measured trait, identifying differences in the variance of RIX average phenotypes under well-watered (WW) and dry-down (DD) conditions. Significant p-values are shown in bold, significance was determined using a Bonferroni correction of $\alpha = 0.0038$.

	N	Treatment	SD	*p*
DGtB	218	WW	4.2412	**<0.0001**
		DD	3.1396	
LLaB	217	WW	14.3159	**<0.0001**
		DD	7.6950	
LWaB	219	WW	11.6954	**<0.0001**
		DD	6.4819	
LLRGR1	222	WW	0.3751	**<0.0001**
		DD	0.2697	
LWRGR1	223	WW	0.3034	**0.0004**
		DD	0.2305	
LLaFl	125	WW	11.1991	**<0.0001**
		DD	6.7574	
LWaFl	127	WW	8.1184	**<0.0001**
		DD	5.2423	
LLRGR2	130	WW	0.7757	0.5684
		DD	0.8274	
LWRGR2	130	WW	0.7903	0.7073
		DD	0.7643	
CL	130	WW	3.3743	0.4530
		DD	3.1322	
CW	131	WW	3.3143	0.5044
		DD	3.1261	
PL	124	WW	5.6721	**<0.0001**
		DD	3.2943	
DBtFr	98	WW	3.2249	0.0543
		DD	3.8804	

Table S3.6: Model statistics for tests of adaptive plasticity of measured traits. Logistic regression unit odds ratio (UOR) and 10% odds ratio with chi-square tests are listed for the regression of the plasticity in each trait against survival. Effect estimates (EE) of linear regression with 10% effect estimates and F-statistics for regression of relative plasticity in each trait against number of flowers (transformed ln(x+1)), outcrossed seedset, and selfed seedset. Tests which indicate significant signals of maladaptive plasticity are bolded and labeled M, Tests which indicate significant signals of adaptive plasticity are bolded and labeled A.

							Number of Flowers						Outcrossed Seedset						Selfed Seedset					
Phenotype	N	UOR	10%	$\chi 2$	*p*		N	EE	10%	*F*	*p*		N	EE	10%	*F*	*p*		N	EE	10%	*F*	*p*	
Days Germ to Bud	220	0.81	0.08	0.30	0.5809	-	110	0.36	0.04	0.16	0.6927	-	103	-0.21	-0.02	0.50	0.4796	-	85	-0.34	-0.03	1.48	0.2271	-
Leaf Length at Bud	**220**	**2.83**	**0.28**	**17.59**	**<0.0001**	**M**	105	0.83	0.08	4.83	0.0302	-	102	0.13	0.01	1.64	0.2037	-	84	0.08	0.01	0.68	0.4127	-
Leaf Length at Flower	126	6.47	0.65	6.06	0.0138	-	**108**	**4.05**	**0.40**	**11.00**	**0.0012**	**M**	101	0.26	0.03	1.69	0.1966	-	82	0.41	0.04	4.55	0.0360	-
Leaf Length/Day Bud to Flower	**121**	**3.22**	**0.32**	**12.60**	**0.0004**	**M**	103	0.32	0.03	2.19	0.1423	-	101	0.10	0.01	2.61	0.1094	-	**78**	**0.19**	**0.02**	**8.01**	**0.0059**	**M**
Corolla Length	**131**	**12.00**	**1.20**	**29.06**	**<0.0001**	**M**	**113**	**2.59**	**0.26**	**19.26**	**<0.0001**	**M**	**106**	**0.39**	**0.04**	**10.25**	**0.0018**	**M**	**85**	**0.46**	**0.05**	**14.78**	**0.0002**	**M**
Peduncle Length	**126**	**18.24**	**1.82**	**32.14**	**<0.0001**	**M**	**106**	**2.36**	**0.24**	**7.92**	**0.0058**	**M**	102	0.36	0.04	4.94	0.0285	-	**81**	**0.50**	**0.05**	**9.16**	**0.0033**	**M**
Days Bud to Fruit	-	-	-	-	-	-	**96**	**-0.69**	**-0.07**	**7.79**	**0.0064**	**A**	**96**	**-0.75**	**-0.08**	**20.98**	**<0.0001**	**A**	75	-0.43	-0.04	5.28	0.0245	-

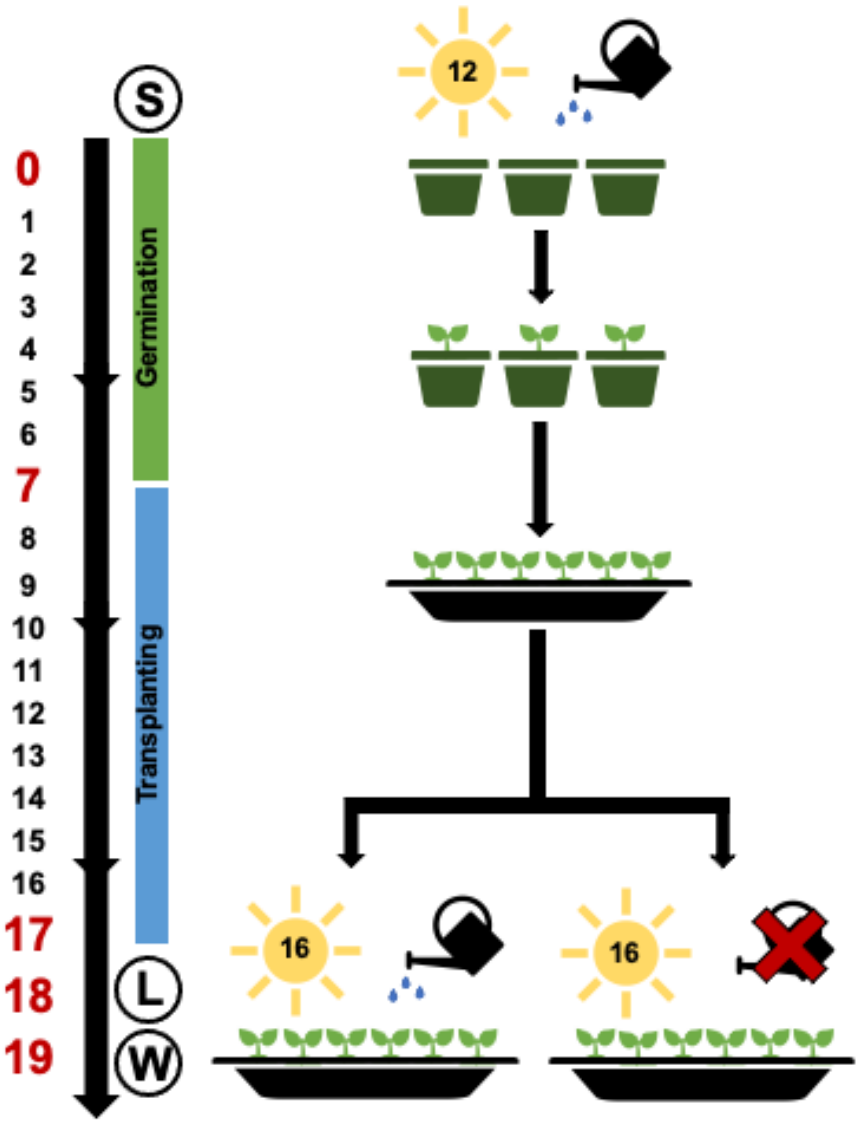


Figure S3.1: Overview of the experimental timeline used to test RIX individuals grown under our various experimental conditions. We planted a pre-counted number of seeds from each RIX genotype into individual pots. These seeds were stratified (S) in the dark at 4°C for 7 days before being moved to a growth chamber under 12-hr light conditions to germinate. We set this day as day 0. We allowed seeds to germinate, and transplanted them into communal flats in 4 x 7 grid on days 7 thought 17. When all experimental seedlings had been transplanted into their experimental flats, we moved half of the flats (containing two full sets of genotypes in replicate) to a new greenhouse room under 16-hr light conditions. We initiated these lighting treatments (L) on day 18. The next day, we suspended watering for half of the flats in each room (containing one full set of genotypes in replicate). We initiated these watering treatments (W) on day 19.

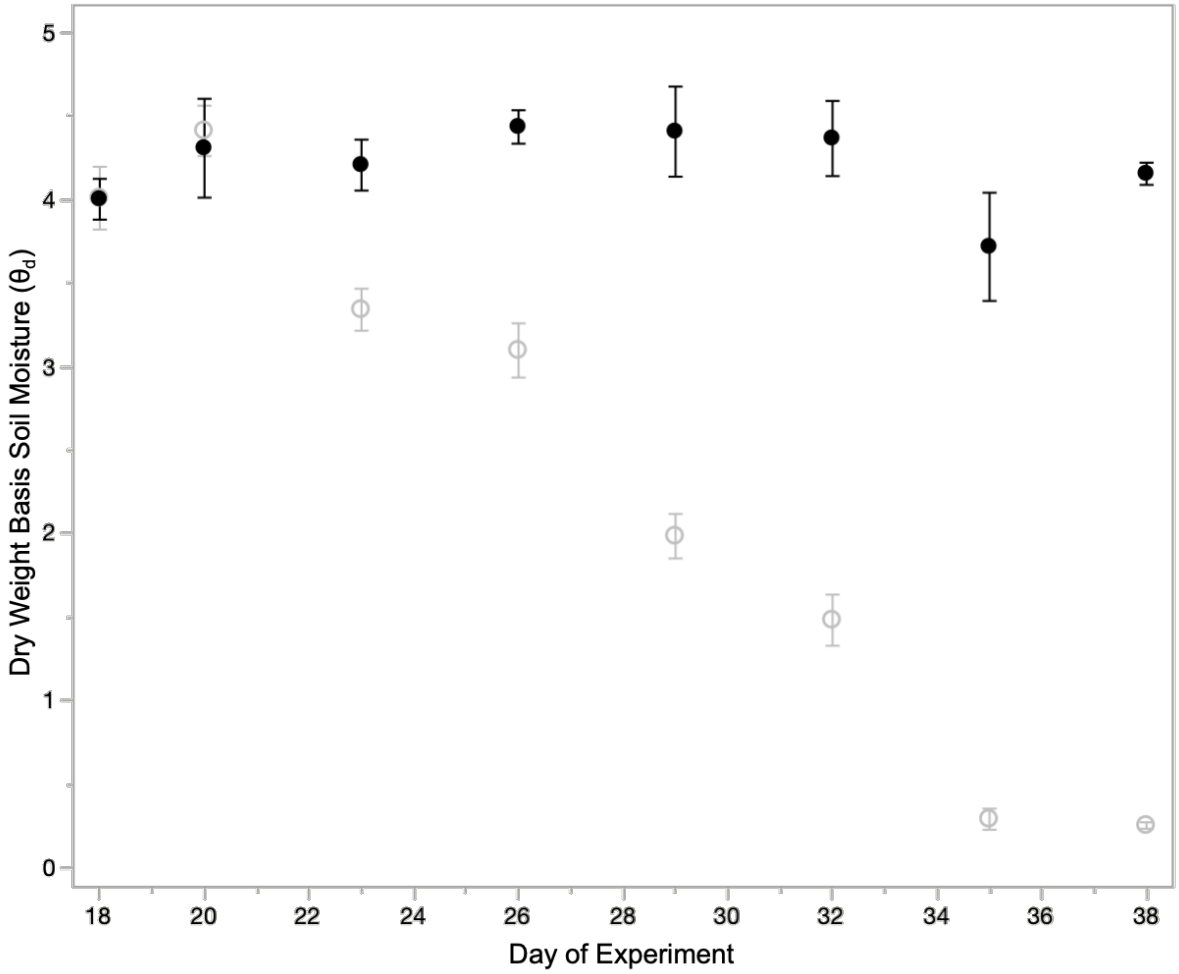
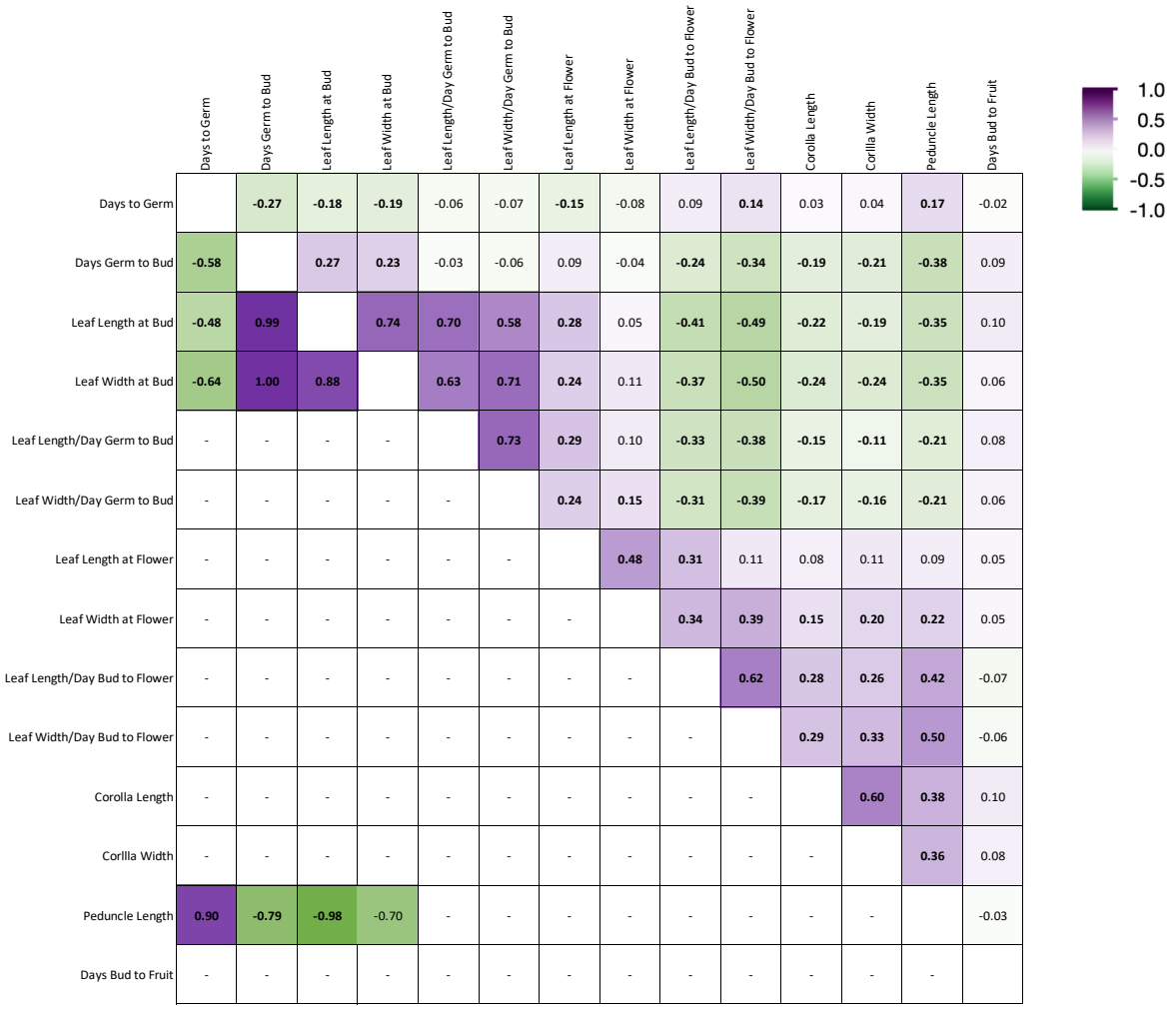


Figure S3.2: Dry weight basis soil moisture (θ_d) in each watering treatment over the first 38 days of the experiment. θ_d was calculated from five randomly selected flats within each of the 4 experimental conditions at eight time points. θ_d (error bars, *SE*) in well-watered flats remained constant over the course of the experiment, while dry-down flats experienced continually decreasing soil moisture as the experiment progressed. Soil moisture measurements were discontinued after day 38, as θ_d was no longer decreasing in the drought flats.



	Days to Germ	Days Germ to Bud	Leaf Length at Bud	Leaf Width at Bud	Leaf Length/Day Germ to Bud	Leaf Width/Day Germ to Bud	Leaf Length at Flower	Leaf Width at Flower	Leaf Length/Day Bud to Flower	Leaf Width/Day Bud to Flower	Corolla Length	Corlla Width	Peduncle Length	Days Bud to Fruit
Days to Germ		**-0.27**	**-0.18**	**-0.19**	-0.06	-0.07	**-0.15**	-0.08	0.09	**0.14**	0.03	0.04	**0.17**	-0.02
Days Germ to Bud	**-0.58**		**0.27**	**0.23**	-0.03	-0.06	0.09	-0.04	**-0.24**	**-0.34**	**-0.19**	**-0.21**	**-0.38**	0.09
Leaf Length at Bud	**-0.48**	**0.99**		**0.74**	**0.70**	**0.58**	**0.28**	0.05	**-0.41**	**-0.49**	**-0.22**	**-0.19**	**-0.35**	0.10
Leaf Width at Bud	**-0.64**	**1.00**	**0.88**		**0.63**	**0.71**	**0.24**	0.11	**-0.37**	**-0.50**	**-0.24**	**-0.24**	**-0.35**	0.06
Leaf Length/Day Germ to Bud	-	-	-	-		**0.73**	**0.29**	0.10	**-0.33**	**-0.38**	**-0.15**	**-0.11**	**-0.21**	0.08
Leaf Width/Day Germ to Bud	-	-	-	-	-		**0.24**	**0.15**	**-0.31**	**-0.39**	**-0.17**	**-0.16**	**-0.21**	0.06
Leaf Length at Flower	-	-	-	-	-	-		**0.48**	**0.31**	0.11	0.08	0.11	0.09	0.05
Leaf Width at Flower	-	-	-	-	-	-	-		**0.34**	**0.39**	**0.15**	**0.20**	**0.22**	0.05
Leaf Length/Day Bud to Flower	-	-	-	-	-	-	-	-		**0.62**	**0.28**	**0.26**	**0.42**	-0.07
Leaf Width/Day Bud to Flower	-	-	-	-	-	-	-	-	-		**0.29**	**0.33**	**0.50**	-0.06
Corolla Length	-	-	-	-	-	-	-	-	-	-		**0.60**	**0.38**	0.10
Corlla Width	-	-	-	-	-	-	-	-	-	-	-		**0.36**	0.08
Peduncle Length	**0.90**	**-0.79**	**-0.98**	-0.70	-	-	-	-	-	-	-	-		-0.03
Days Bud to Fruit	-	-	-	-	-	-	-	-	-	-	-	-	-	

Figure S3.3: Phenotypic correlations calculated as Kendall's Tau (**τ**, Kendall Rank Correlation Coefficient) above diagonal, genetic correlations below diagonal, under dry-down conditions. Significant correlations at the $\alpha = 0.05$ level are shown in bold. Negative correlations are shown in green, positive correlation are shown in purple. Genetic correlations are only estimated between pairs of traits which both have estimated $H^2 \geq 0.20$.

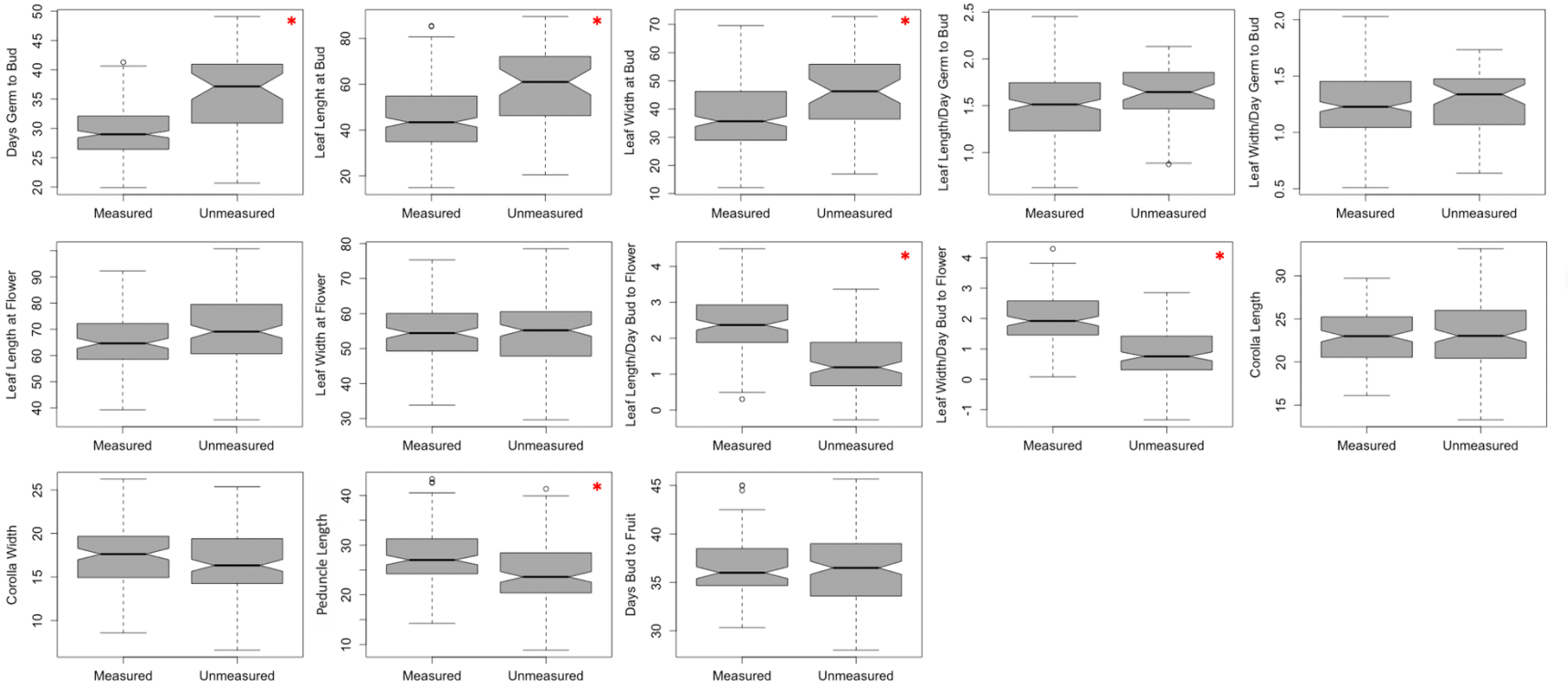
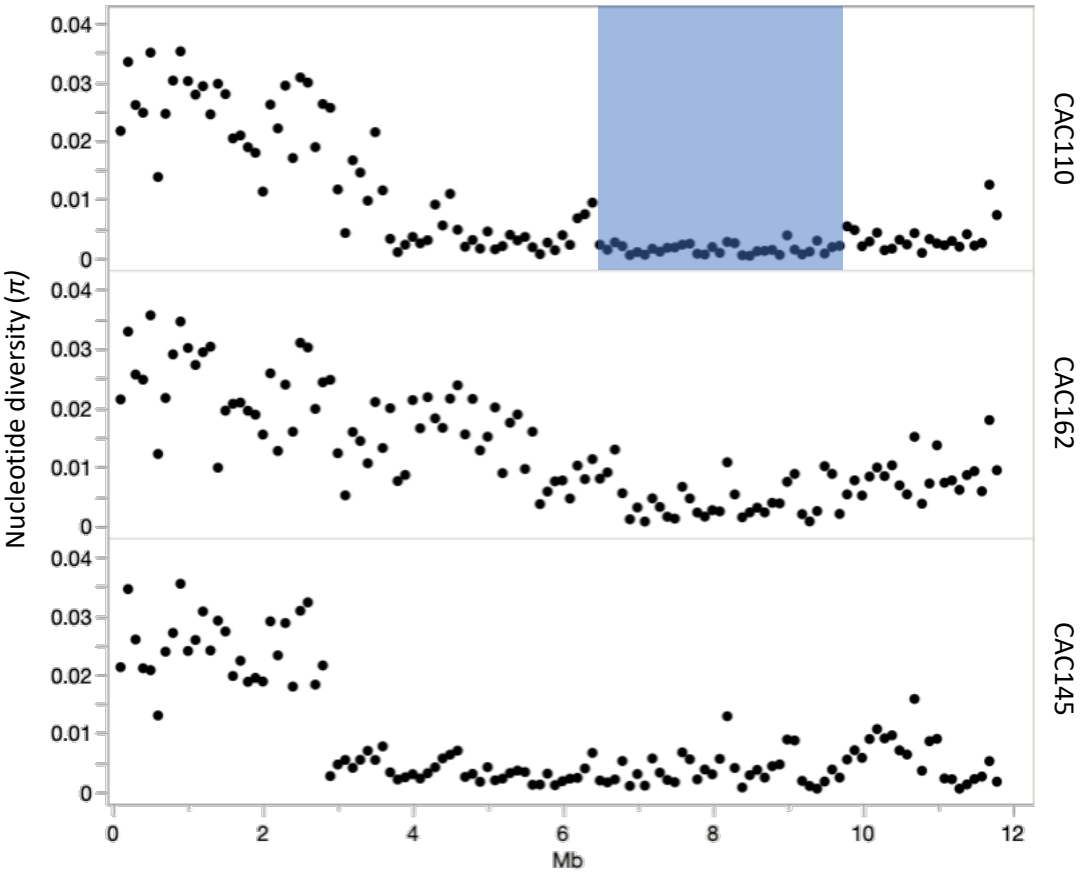


Figure S3.4: Boxplots of RIX set average values for each measured phenotype under well-watered conditions, split into two groups, RIX sets in which at least one individual survived to be measured under dry-down conditions (Measured), and RIX sets in which all individuals did not survive to be measured under dry-down conditions. Significant comparisons are marked with a red asterisk, significance was determined using a Bonferroni correction of $\alpha = 0.004$. Test statistics and p-values are shown in table S5

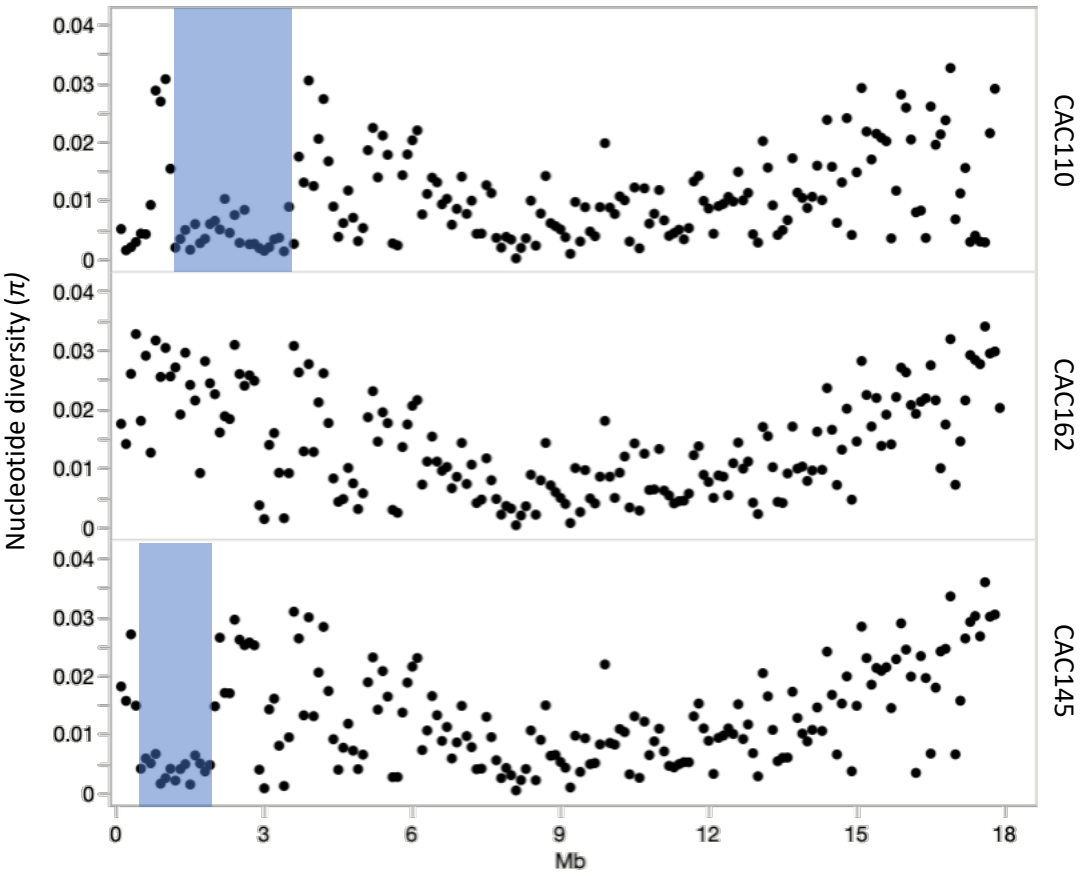
APPENDIX B

SUPPLEMENTARY TABLES AND FIGURES FROM CHAPTER IV

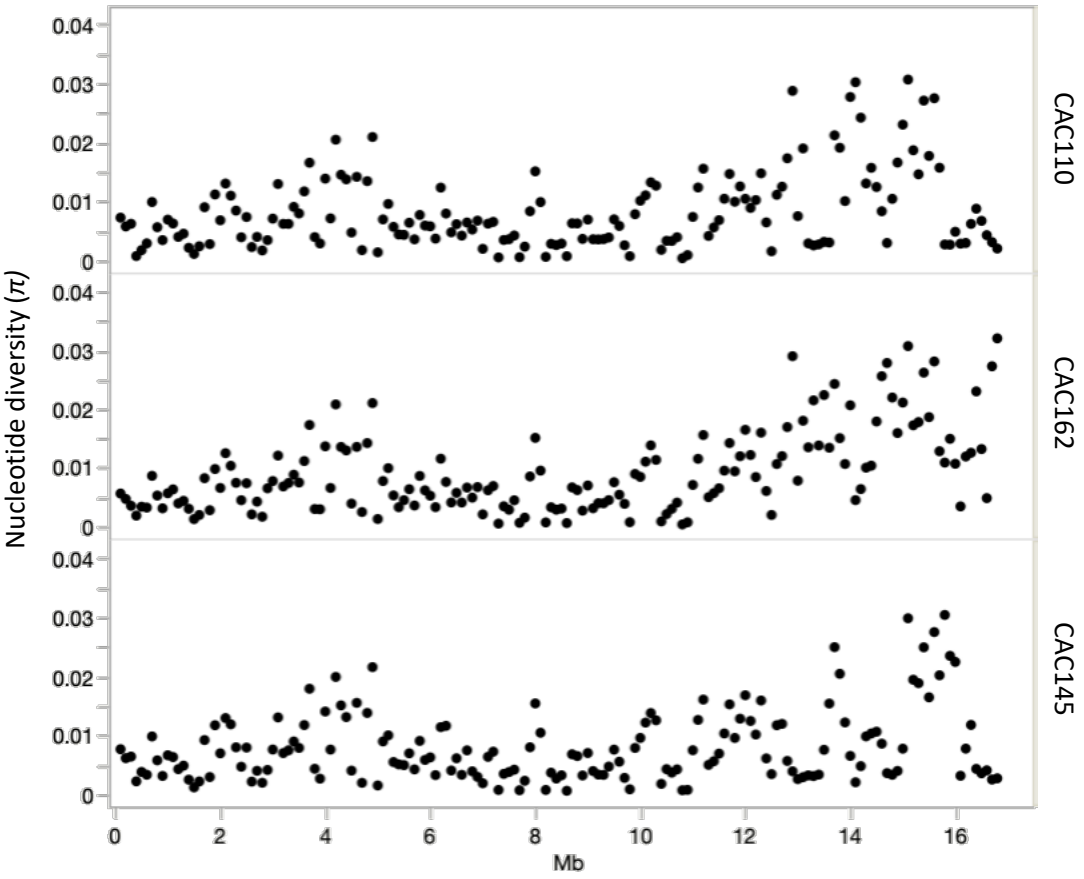
Chromosome 1



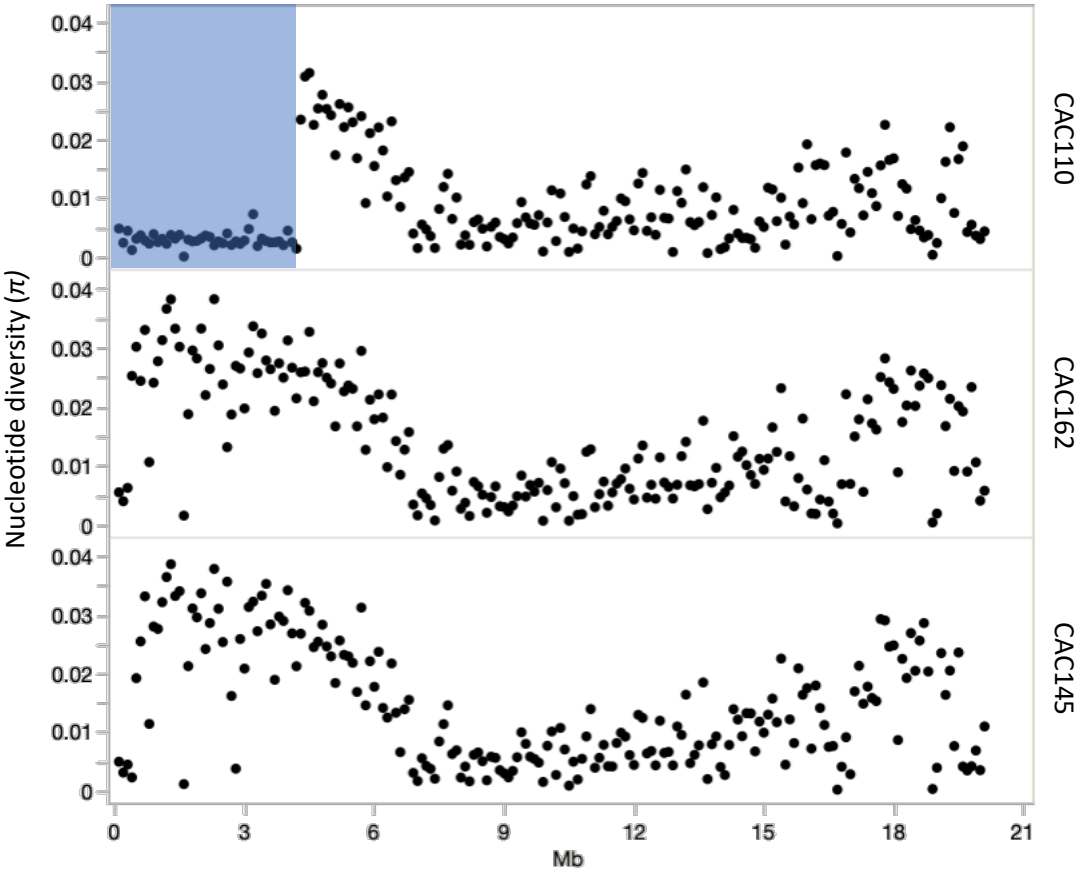
Chromosome 2



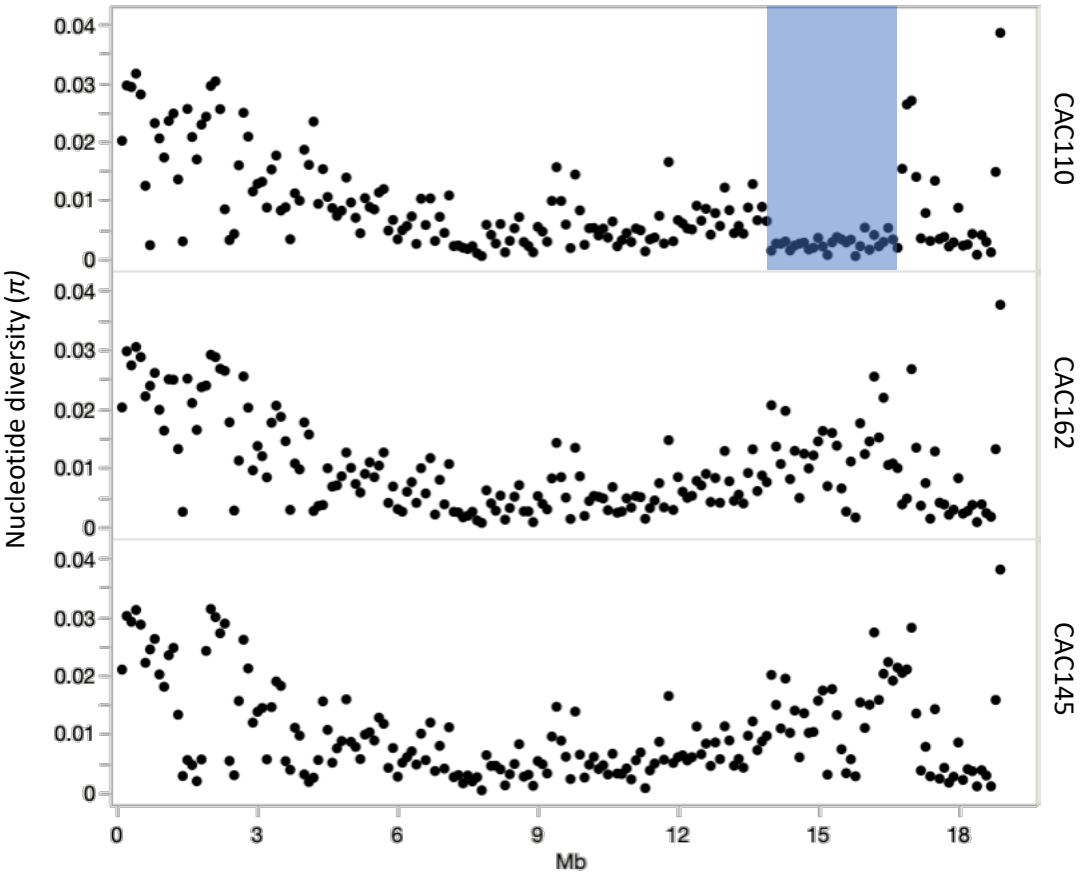
Chromosome 3



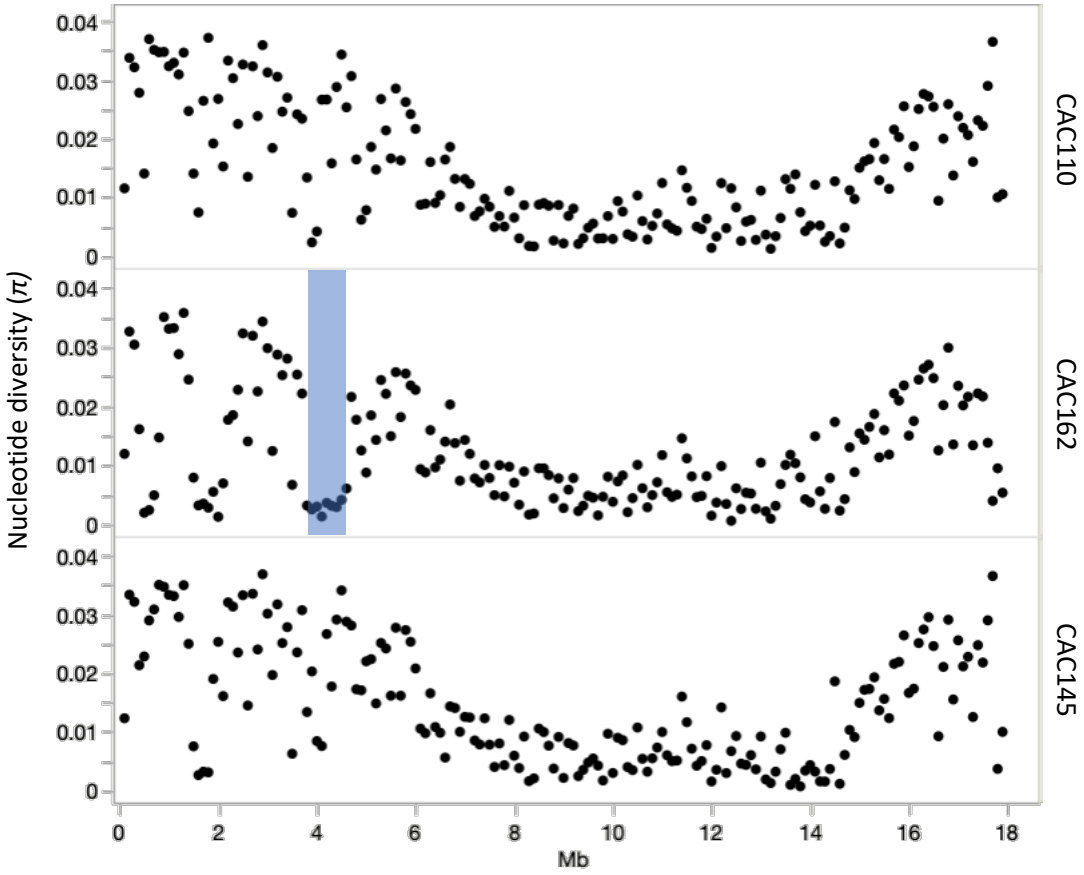
Chromosome 4



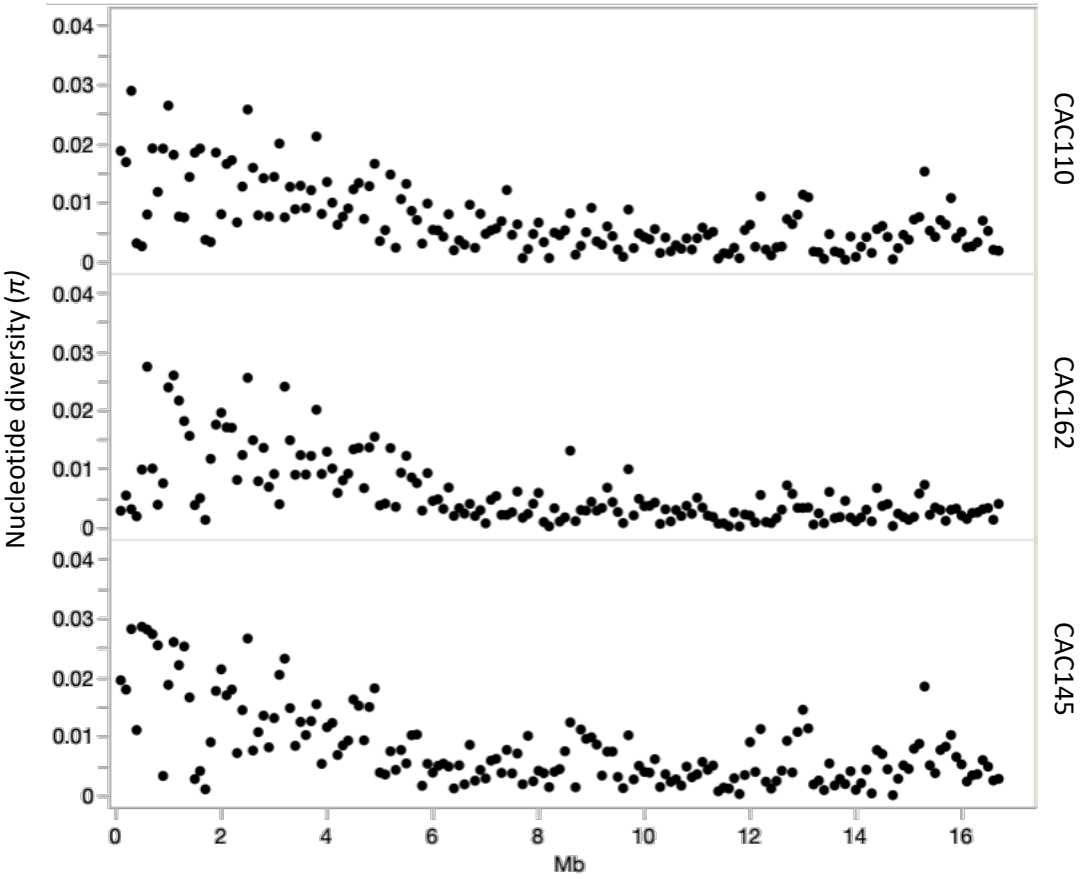
Chromosome 5



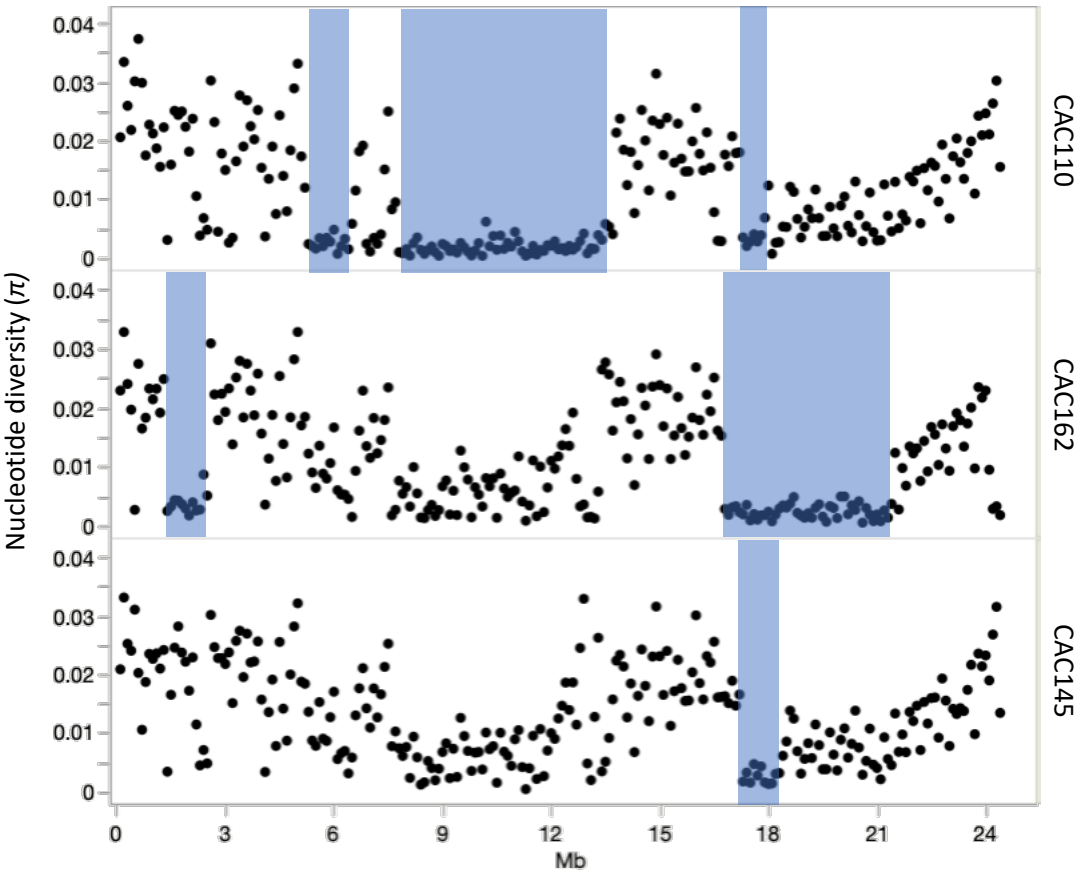
Chromosome 6



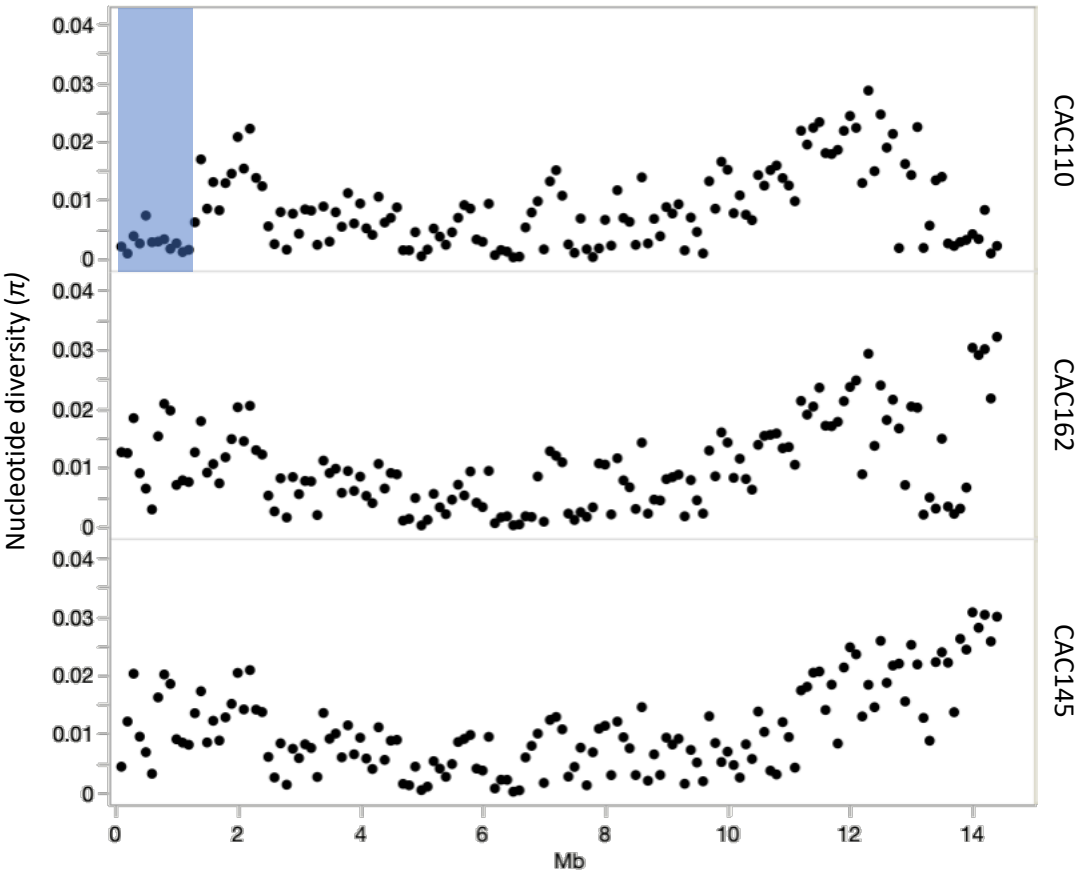
Chromosome 7



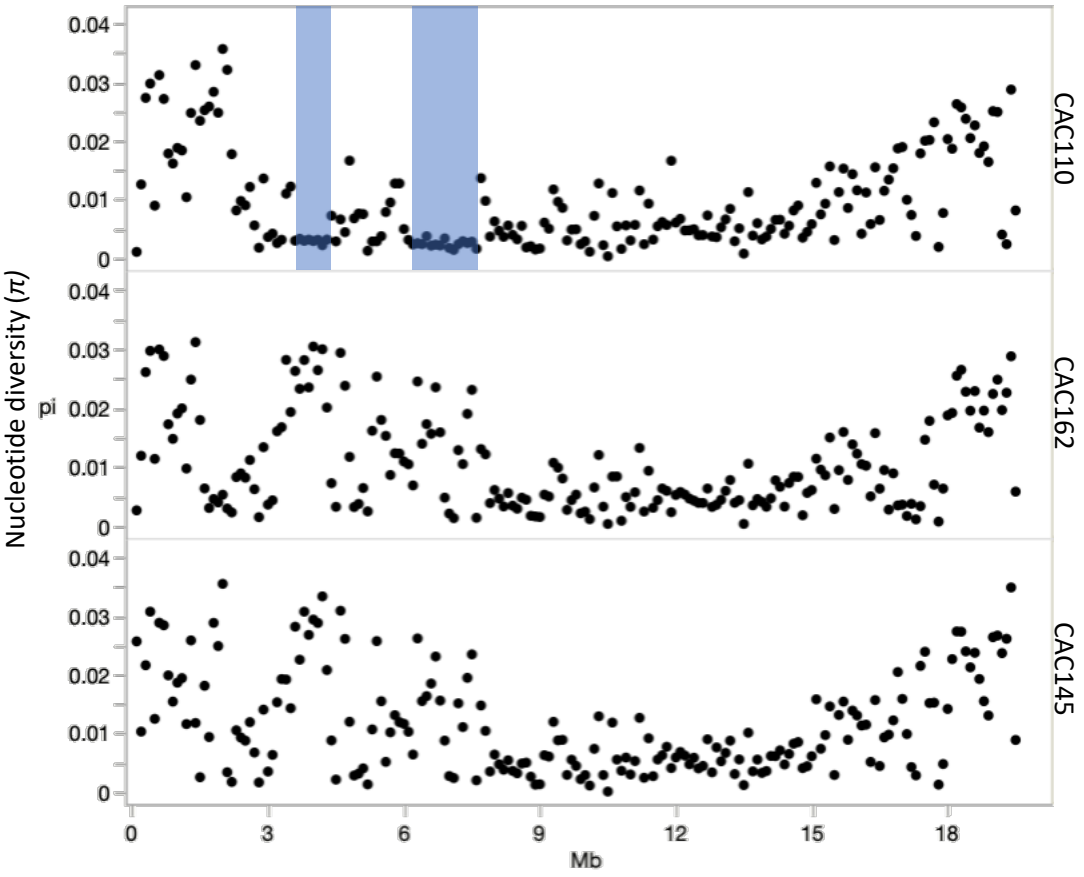
Chromosome 8



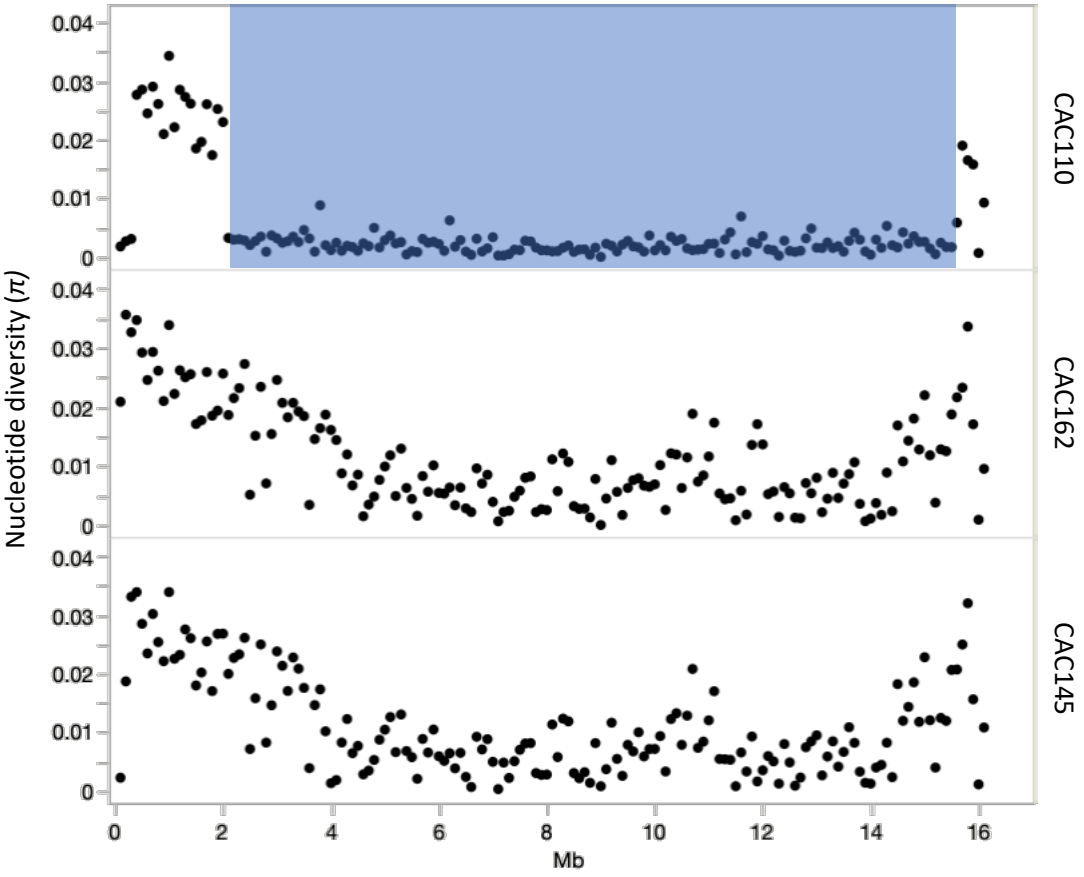
Chromosome 9



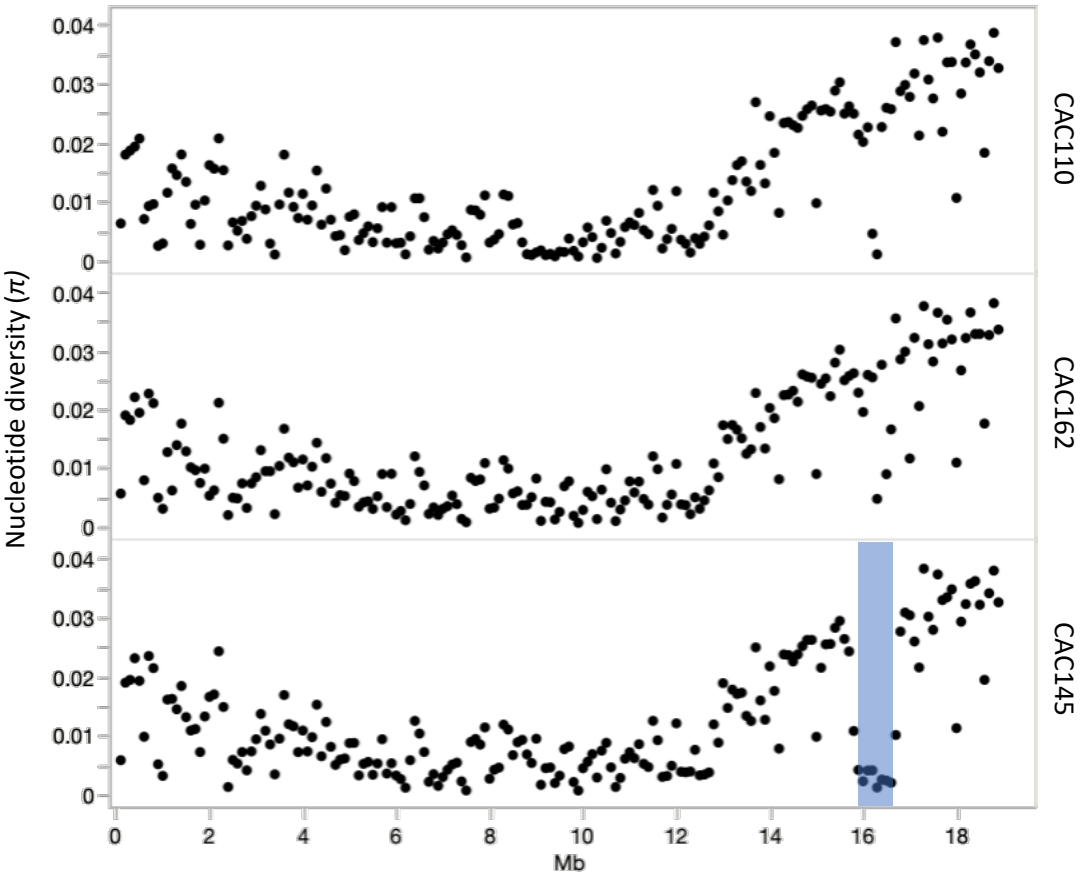
Chromosome 10



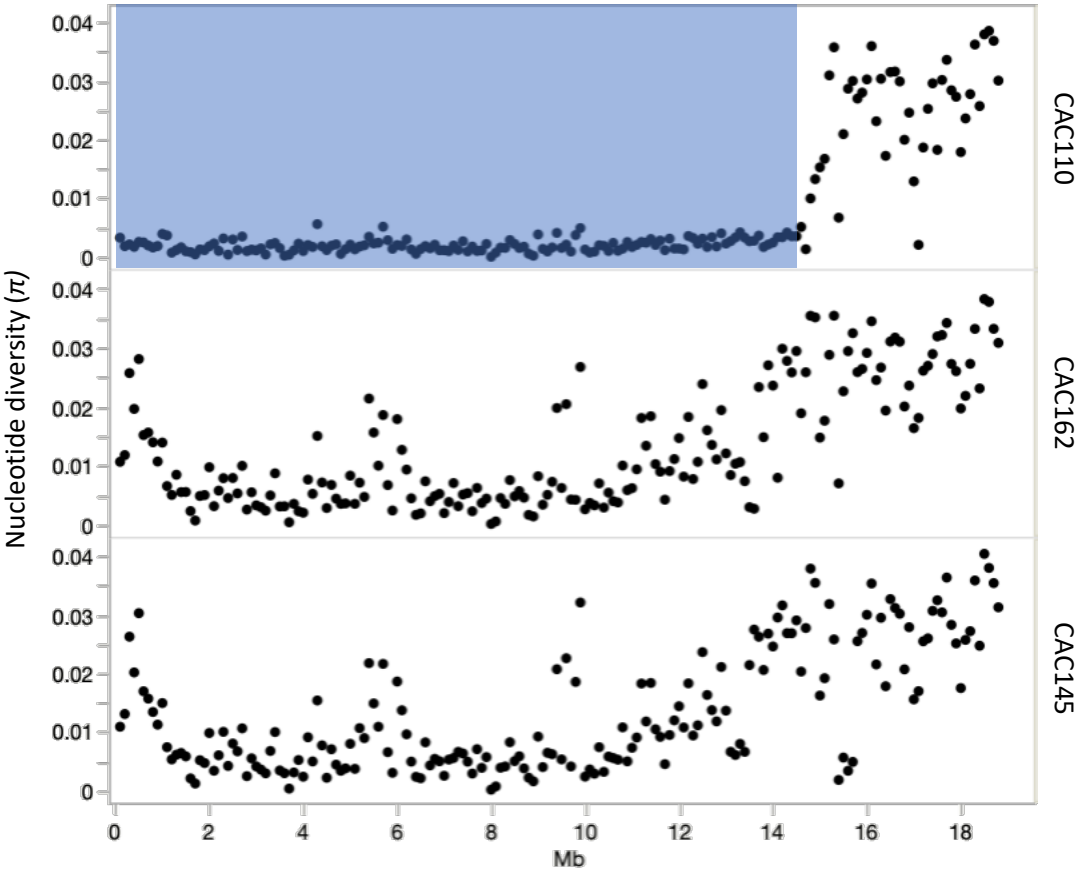
Chromosome 11



Chromosome 12



Chromosome 13



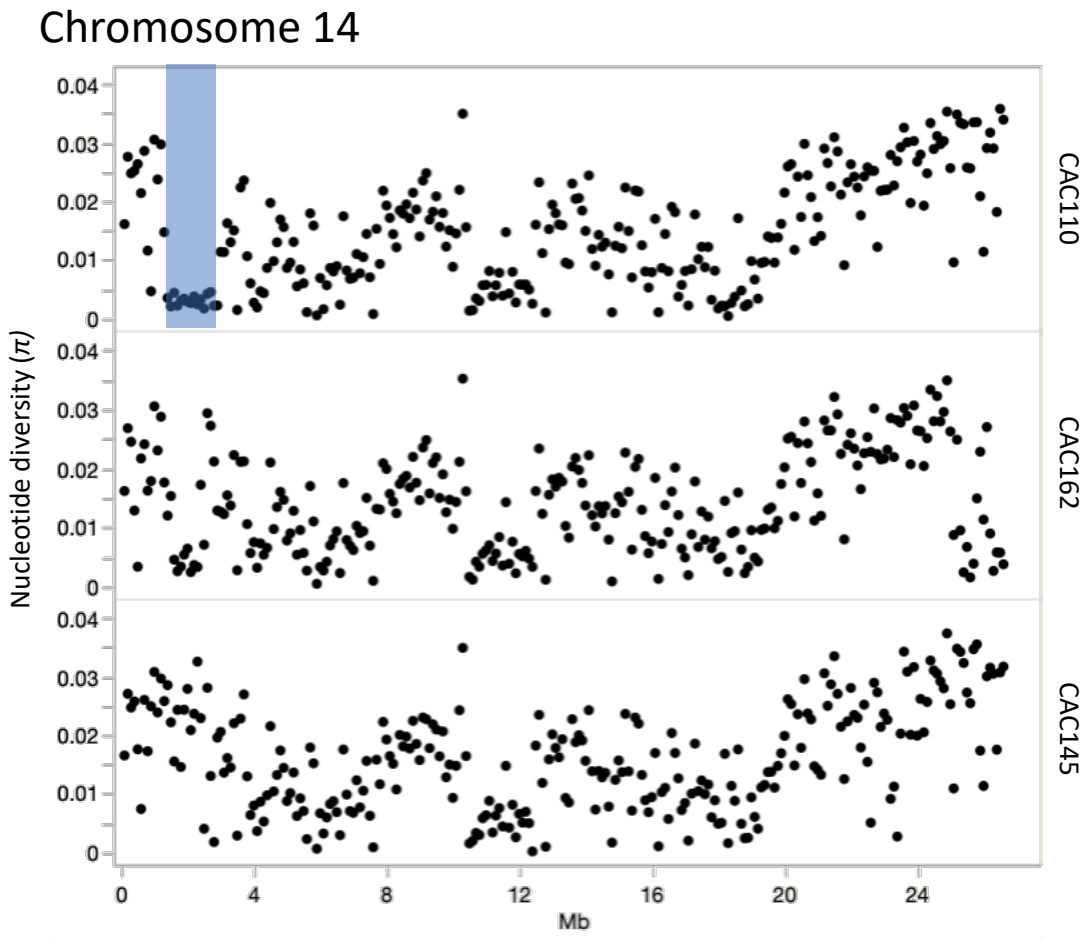
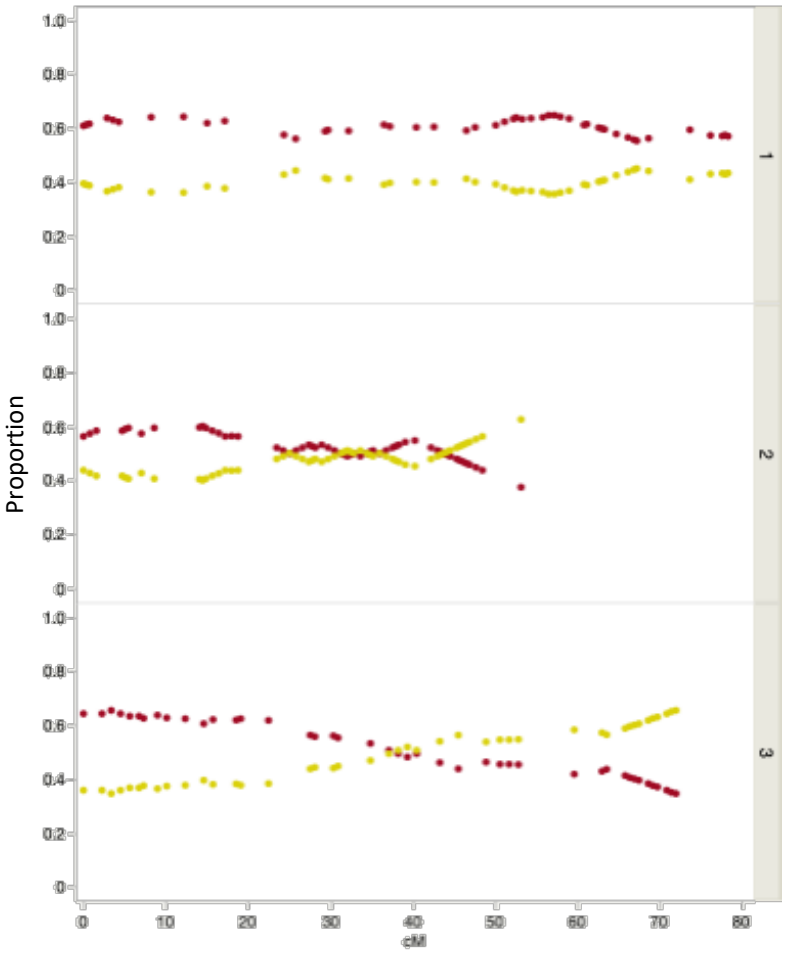
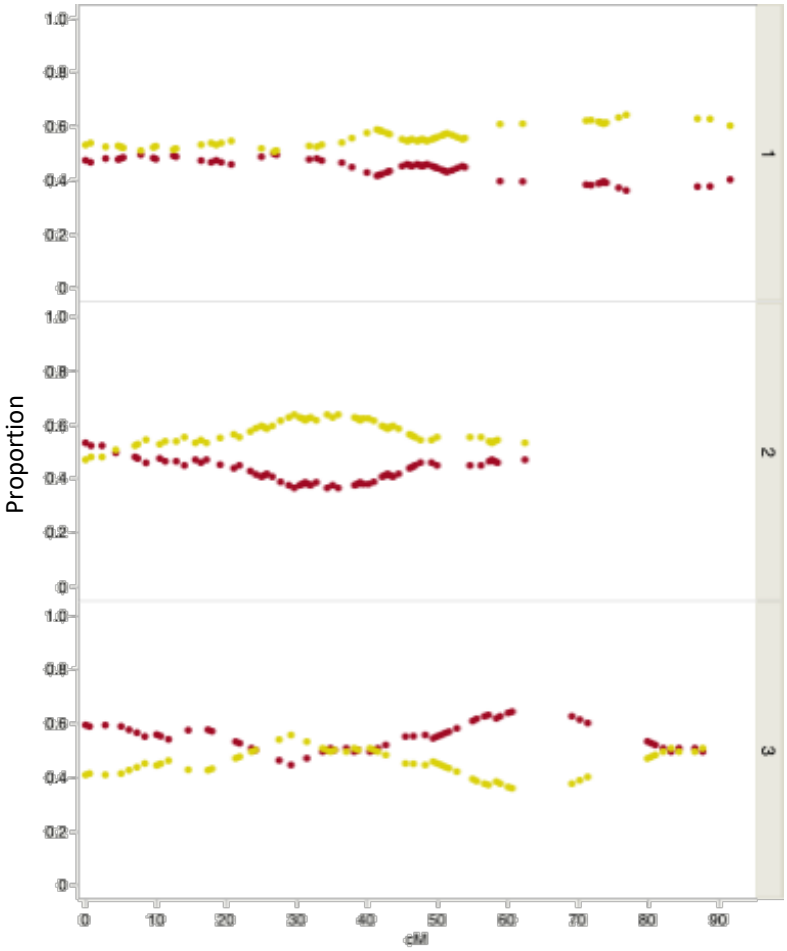


Figure S4.1 Nucleotide diversity in 100kb windows for each pair of WGS sequenced parental lines. Group 1(top): CAC9 and CAC110, group 2(center): CAC9 and CAC162, group3(bottom): CAC9 and CAC415 for each chromosome. Putative areas or introgression, areas which show abruptly decreased nucleotide diversity and multiple windows of $\pi > 0.05$, are highlighted in blue.

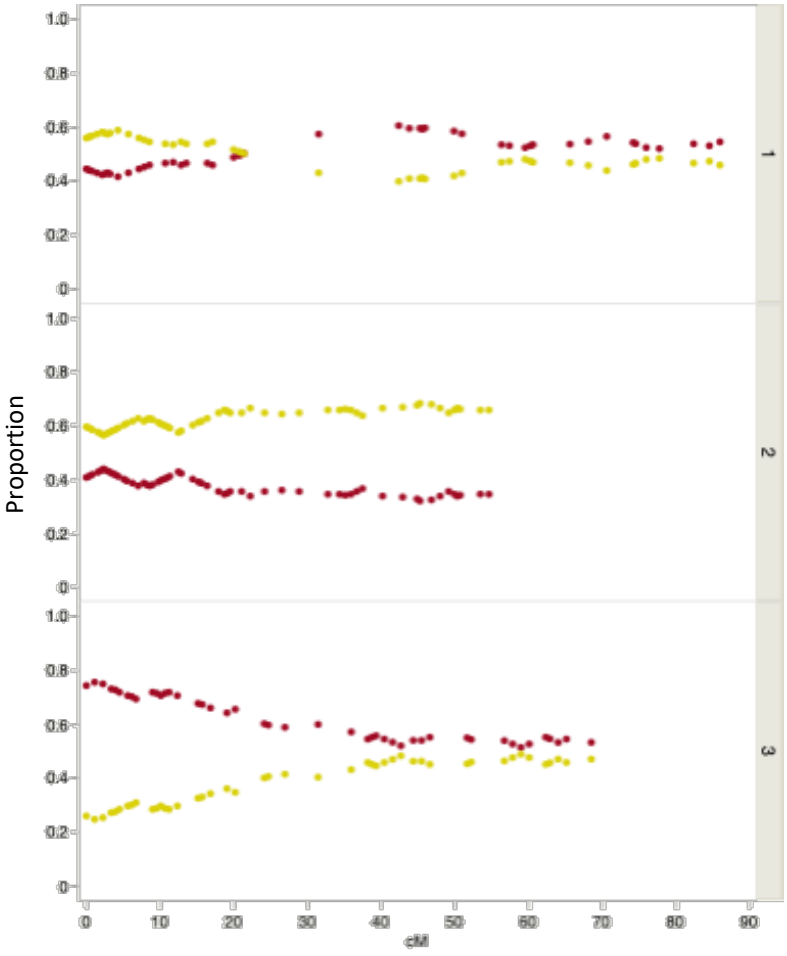
Chromosome 1



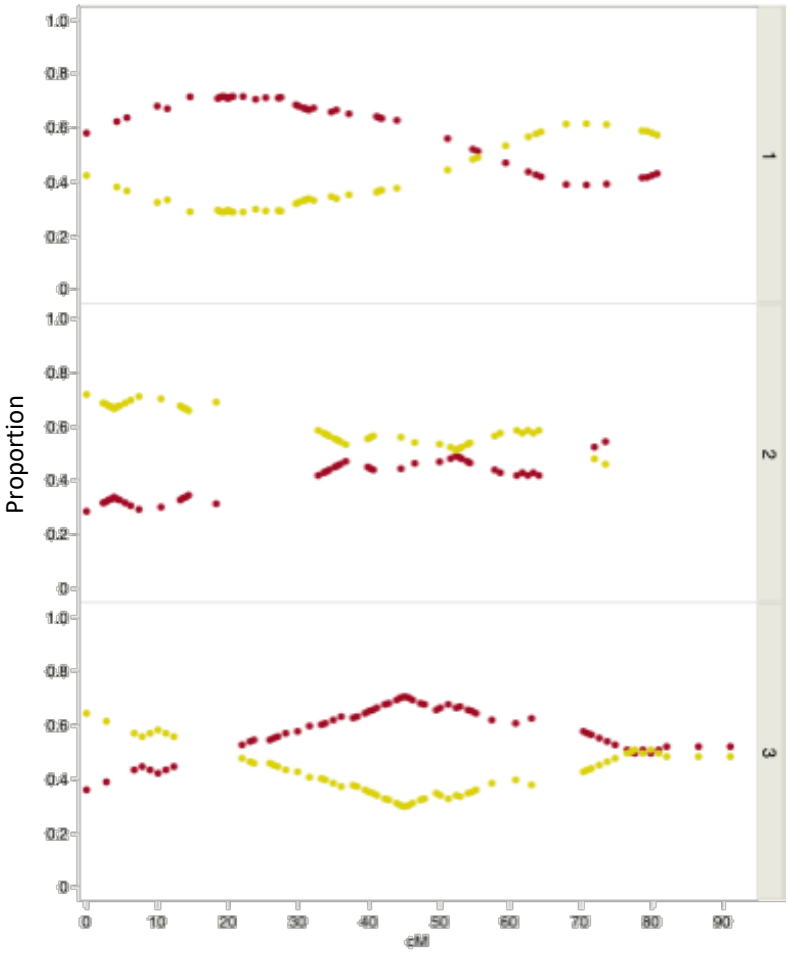
Chromosome 2



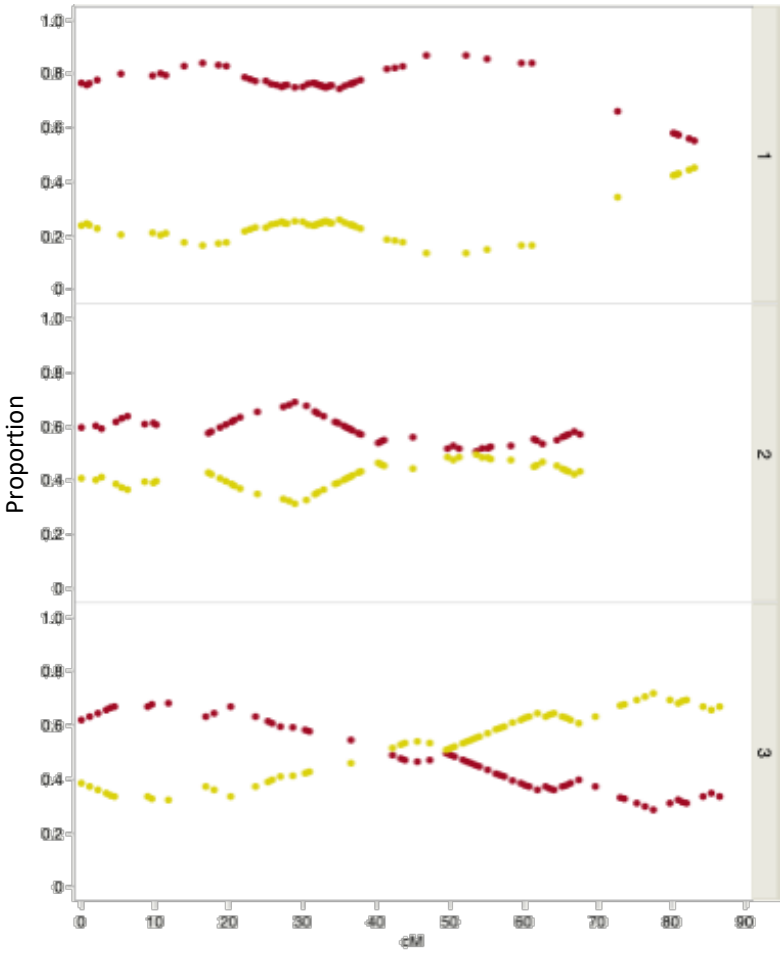
Chromosome 3



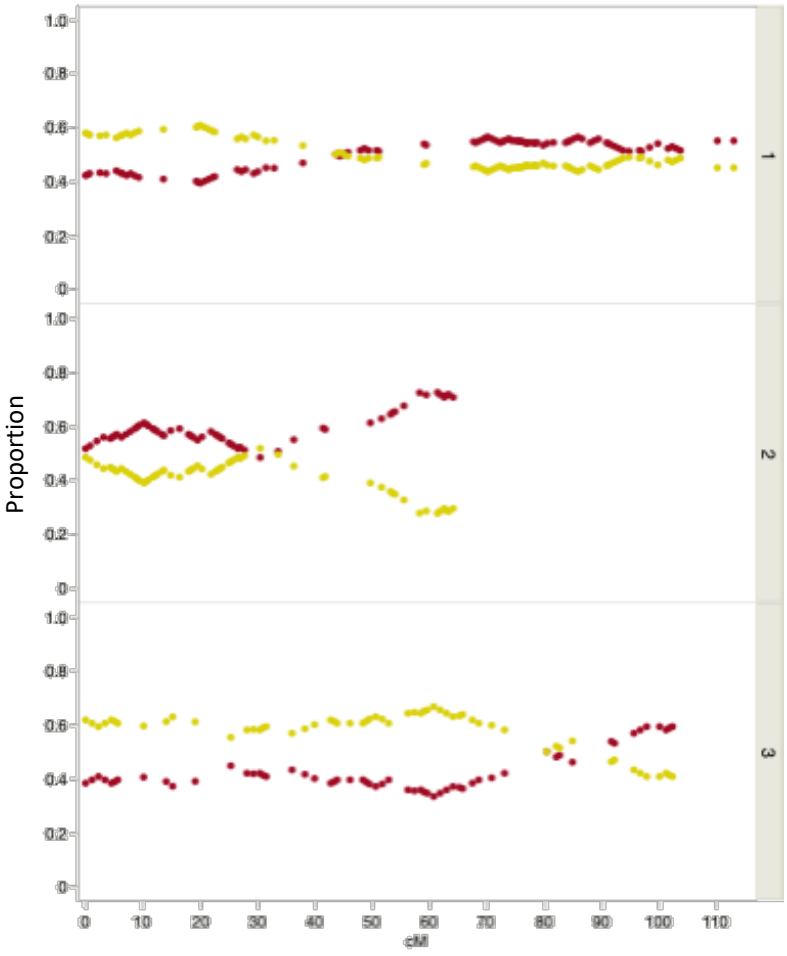
Chromosome 4



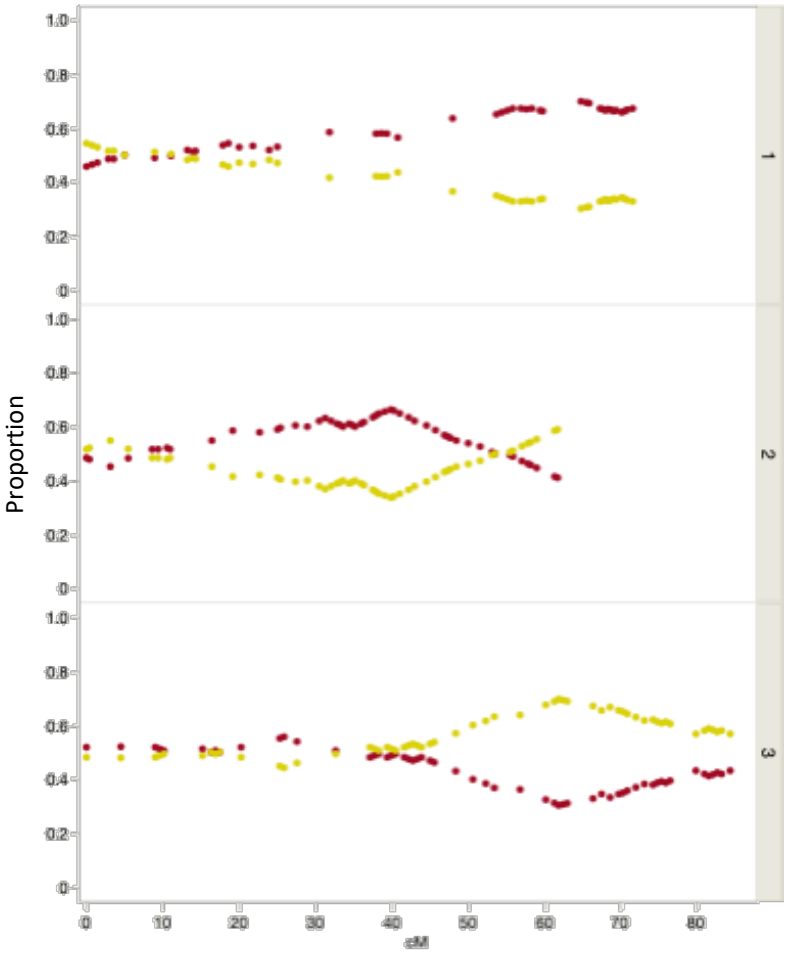
Chromosome 5



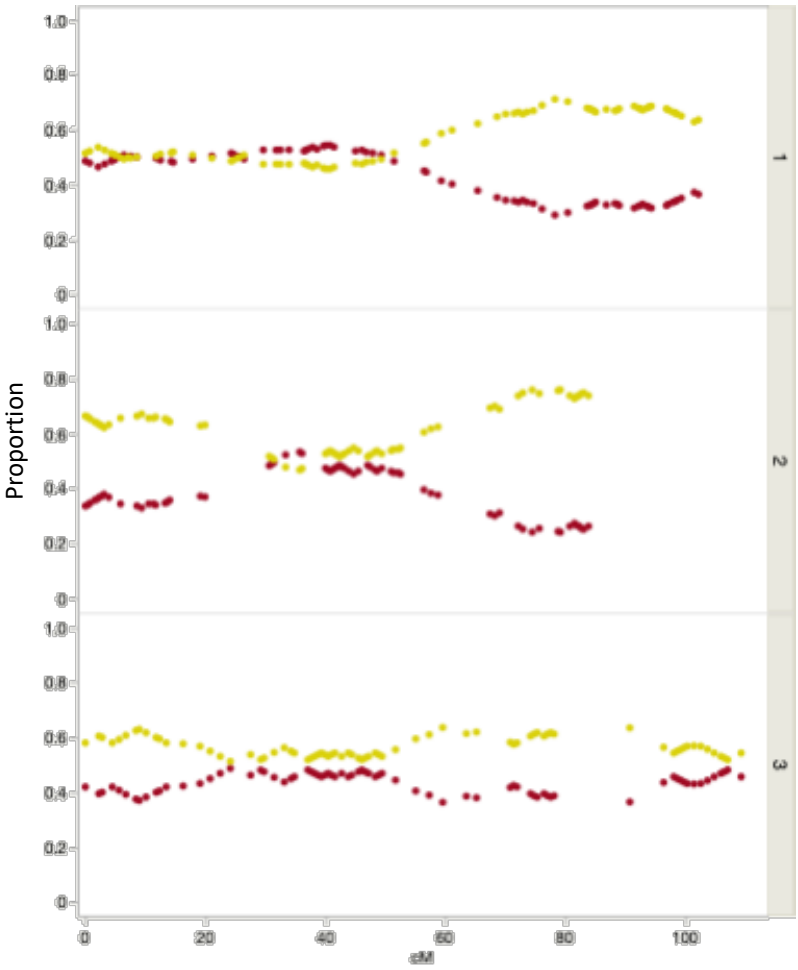
Chromosome 6



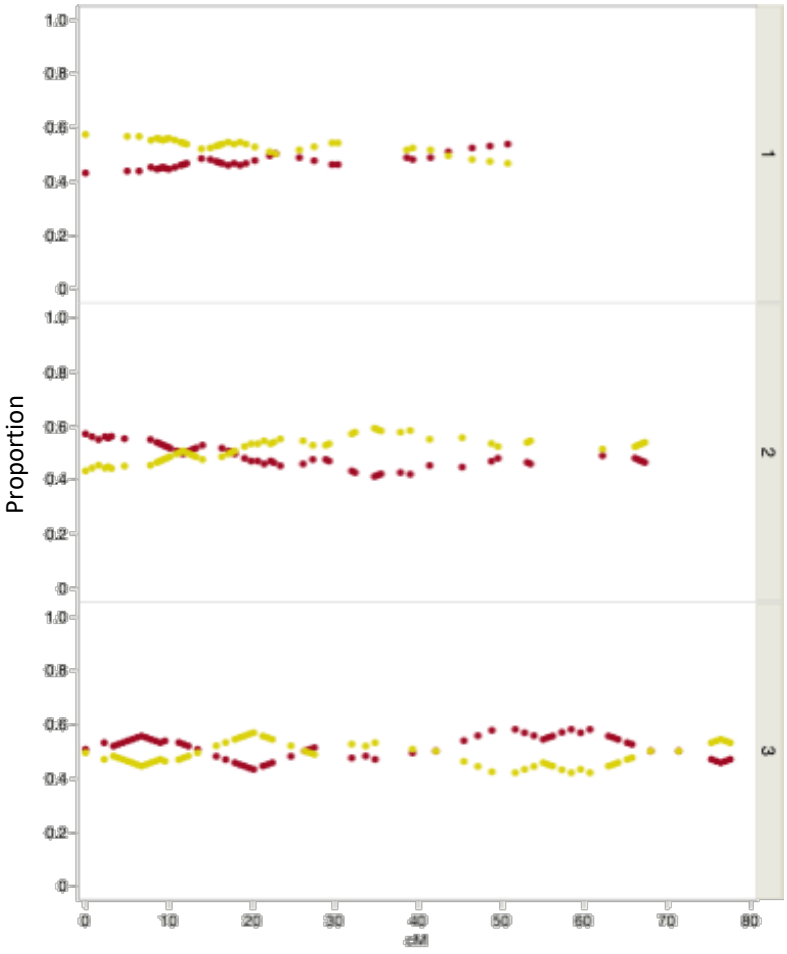
Chromosome 7



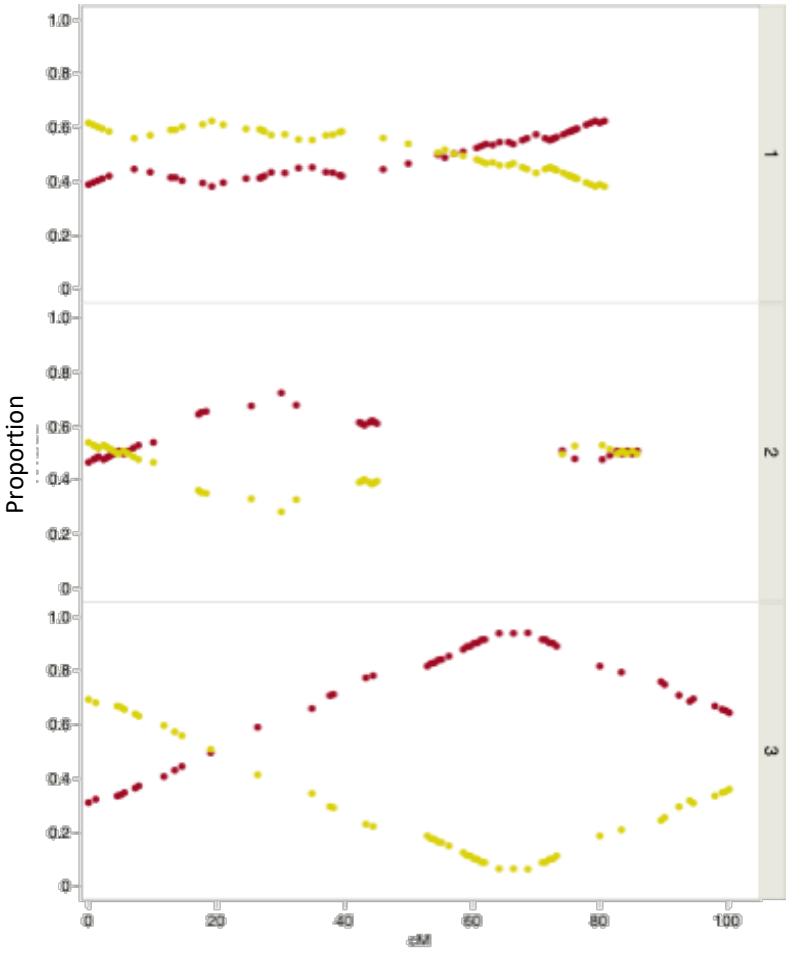
Chromosome 8



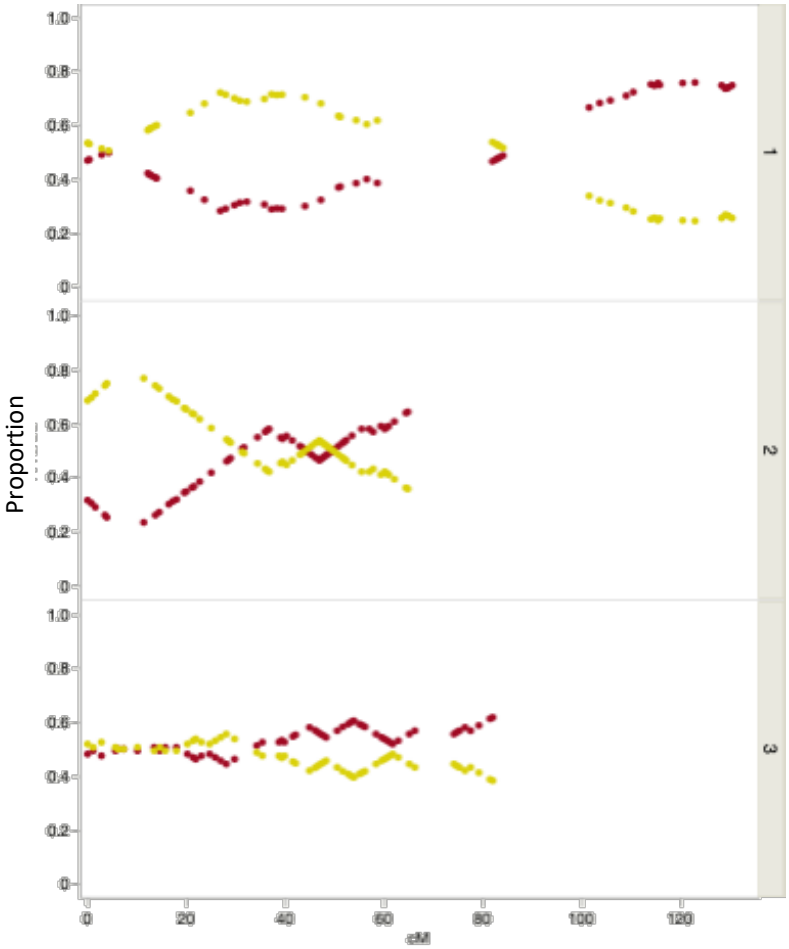
Chromosome 9



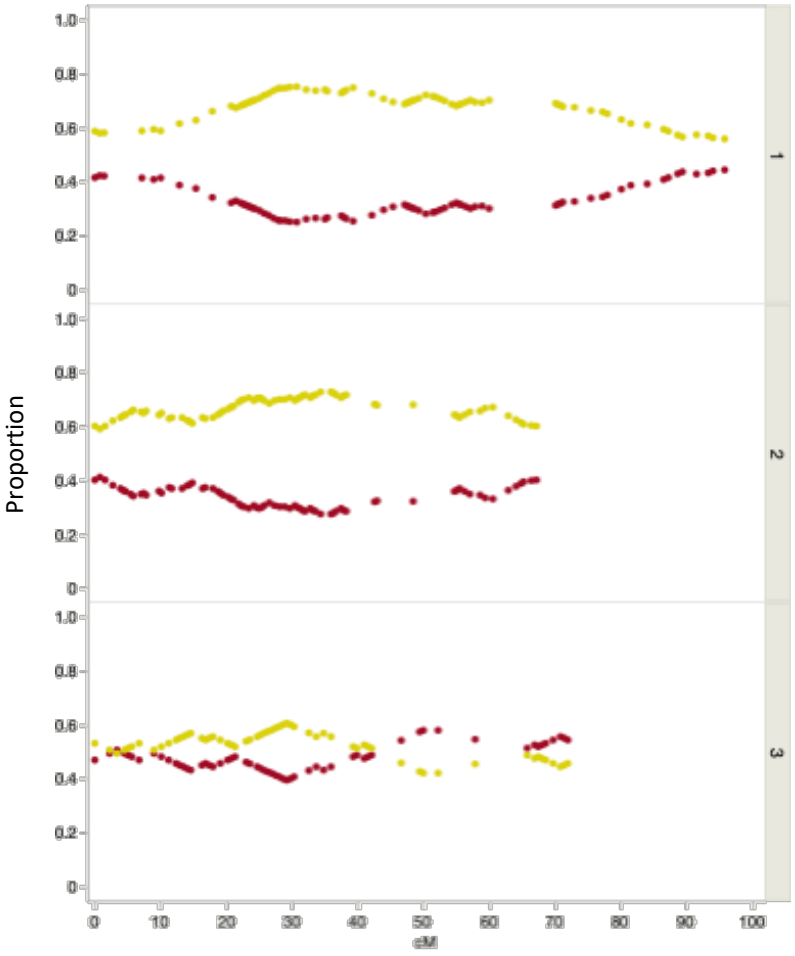
Chromosome 10



Chromosome 11



Chromosome 12



Chromosome 13

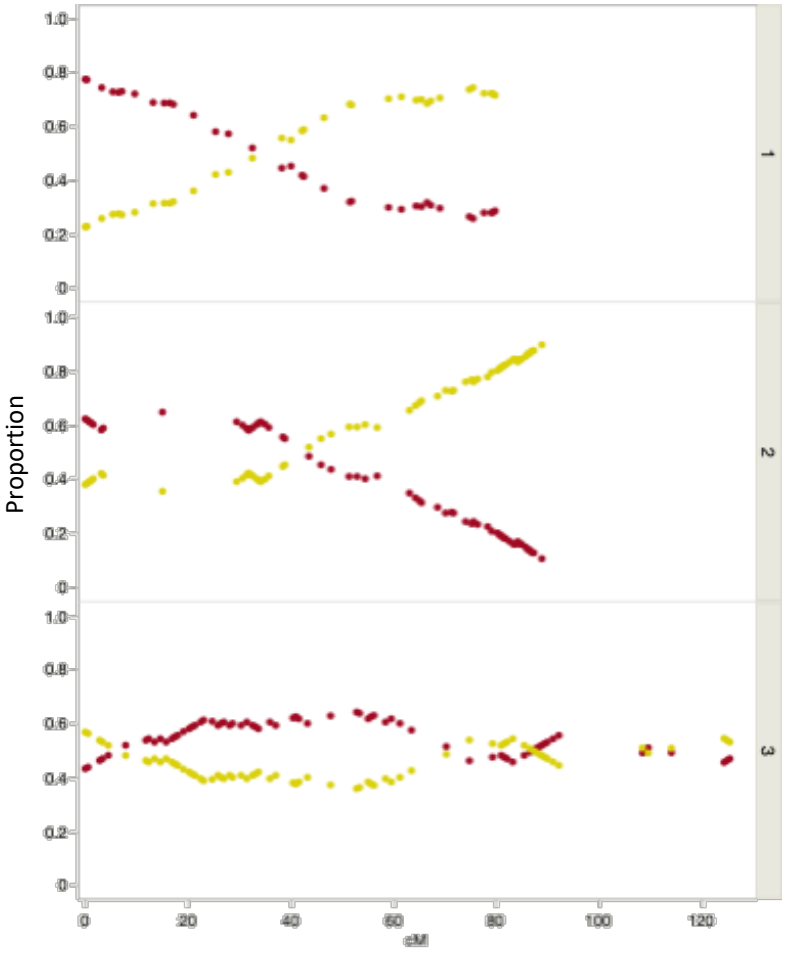


Figure S4.2 Genotype frequency at markers across the genome in each of the three RIL populations produced. RIL population 1 was derived from CAC9 x CAC110, 2 was derived from CAC9 x CAC162, and 3 was derived from CAC9 x CAC415. Frequency of *M. guttaus* alleles is shown in red, frequency of *M. nasutus* alleles are shown in yellow.