Chapter

METHODS AND DISCOVERIES IN THE PURSUIT OF UNDERSTANDING THE GENETIC BASIS OF ADAPTATION TO HARSH ENVIRONMENTS IN *MIMULUS*

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ABSTRACT

The *Mimulus guttatus* D.C. species complex (Phrymaceae) is a model system for understanding the genetic basis of adaptation to variable environments. Recent studies in this system on the evolution of drought escape via shifts in flowering time as well as tolerance to serpentine, copper mine, and saline soils have provided new insights into the mechanisms of adaptation and speciation. Determining the genetic basis of plant adaptation to such harsh environmental conditions is of fundamental importance to biology and has many applied benefits. Here, we review research on adaptation to extreme habitats in *Mimulus* and describe how recent developments in high-throughput phenotyping and the use of genomic approaches are driving further advances in understanding the genetics of adaptation and speciation.

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INTRODUCTION

Identifying the genetic and physiological basis of adaptations to complex environmental conditions is a major challenge in ecological genomics. Wild plant species offer particularly attractive systems for addressing these questions, as they often exhibit local adaptation of populations to different habitats across their geographic ranges (Hereford, 2009; Leimu & Fischer, 2008). Environmental heterogeneity results in selective pressures that differ between habitats which can promote population differentiation and maintain genetic variation (Clausen, 1951; Gillespie & Turelli, 1989; Hedrick, 1986; Kawecki & Ebert, 2004; Levene, 1953). Over time, adaptation to different habitats can lead to reproductive isolation (RI), either directly through the evolution of traits involved in local adaptation or indirectly if reproductive isolating barriers hitchhike along with adaptations (Coyne & Orr, 2004; Rundle & Nosil, 2005; Schluter & Conte, 2009; Wright et al., 2013). Plants that live in habitats characterized by harsh abiotic conditions—for example, drought, toxic soils, salinity, and thermal extremes—often provide particularly vivid examples of how habitat-mediated divergent selection creates biological diversity. Plants that are able to tolerate harsh environments are well suited for investigating the genetic and physiological basis of adaptation because selection in these habitats can be quite strong and, in some cases, the abiotic stress is known and can be manipulated in lab and/or field studies (Brady et al., 2005; Macnair, 1987). Furthermore, plants have repeatedly adapted to many of these stressful habitat types, providing opportunities to investigate the degree of parallel trait evolution and whether it is due to parallel changes at the genetic level.

Plants that are able to thrive in harsh habitats provide well-known, classic examples of adaptation (Antonovics & Bradshaw, 1970; Bradshaw, 1991; Kruckeberg, 1951; Macnair, 1981). Numerous field and lab-based studies have demonstrated that populations are often locally adapted to harsh environmental conditions (Hereford, 2009; Leimu & Fischer, 2008; O'Dell & Rajakaruna, 2011). Recent molecular work has offered insights into potential mechanisms of adaptation to harsh habitats. For example, the flowering time pathway is well characterized (Kobayashi & Weigel, 2007) and differences in flowering time often contribute to adaptive escape from drought (Franks, 2011; Hall & Willis, 2006; Ludlow, 1989; McKay et al., 2003) and cold (Mendez-Vigo et al., 2011). There has also been significant progress in understanding the molecular basis of plant ion homeostasis and metal tolerance (Baxter et al., 2010; Clemens, 2001; Colangelo & Guerinot, 2006; Hanikenne et al., 2008), which are likely important mechanisms for coping with extreme soil habitats. Notably lacking, however, are studies that have identified naturally segregating variants controlling these traits and characterized the fitness effects of these variants in native habitats.

In order to understand plant adaptation to harsh environments, there is a vital need for studies that integrate the cellular and molecular control of traits with an understanding of the ecological context of such traits. To elucidate how selection is operating on specific traits, researchers should test the relationship between phenotypic variation and fitness using manipulative field experiments whenever feasible. The best test of local adaptation is the classical reciprocal transplant experiment (Clausen et al., 1940). The genetic basis of adaptive differences can then be characterized using Quantitative Trait Locus (QTL) mapping, association mapping, or genome scan studies. To confirm that loci identified via these approaches actually contribute to adaptation these loci should be tested for their fitness effects

in native field habitats, as elegantly demonstrated by several recent studies (Ågren et al., 2013; Leinonen et al., 2013; Lexer et al., 2003; Lowry & Willis, 2010; Prasad et al., 2012; Verhoeven et al., 2008).

In this chapter, we discuss the genetic basis of adaptation to harsh environments in the *Mimulus guttatus* species complex (yellow monkeyflower) and the approaches that have enabled these studies. We begin by briefly summarizing what is currently known about species in the *M. guttatus* complex that are able to tolerate serpentine soils (Chapter 6), copper mine tailings (Chapter 14), saline habitats, or water-limited environments. We then focus on QTL mapping and population genomic approaches and what they have revealed about the genetic basis of adaptation to several of these habitats. As sequencing becomes faster and cheaper, phenotyping has become a limiting step for forward genetic studies. Here we describe both field and lab-based, high-throughput phenotyping methods that have been successfully used in *Mimulus* to assay tolerance to several different abiotic stresses.

MIMULUS GUTTATUS IS A MODEL FOR THE GENETICS OF ADAPTATION TO HARSH ENVIRONMENTS

The *Mimulus* genus contains approximately 160 species, which display an incredible degree of ecological variation including adaptation to numerous stressful habitat types (Table 1; Beardsley & Olmstead, 2002; Vickery, 1978; Wu et al., 2008). The center of diversity of the *M. guttatus* species complex is located in western North America. Members of the complex are broadly interfertile (Wu et al., 2008). The complex includes some species that have highly restricted ranges and are often associated with a specific marginal or harsh environment, including several edaphic endemic species (Macnair, 1989; Macnair & Gardner, 1998). Other species, in particular *M. guttatus,* are wide-ranging with populations occurring in countless different habitats (*calflora.org*; Vickery, 1964; Wu et al., 2008). Since the pioneering work of Robert Vickery (Clausen & Hiesey, 1958; Vickery, 1952), many of the studies on *Mimulus* have focused on elucidating the genetic basis of traits that contribute to RI and ecological divergence. With the development of genetic resources, *Mimulus* has become a model system for evolutionary and ecological genetics (Chapters 6, 9; Hellsten et al., 2013; Wu et al., 2008).

Mimulus guttatus, a focal member of the genus, combines incredible ecological diversity with the attributes of a true genetic model system. *Mimulus guttatus* is easily maintained in the laboratory with a short generation time (2-3 months), small size, high fecundity (100-400 seeds per cross), and reproductive flexibility (clonal propagation and self-fertile). The sequenced genome of *M. guttatus* (~430 Mbp) has been publicly available since 2010; the most recent annotated version (v2.0) is available on *www.phytozome.net* (Goodstein et al., 2012). In addition, there is extensive EST and RNA-seq data, over 1,000 highly polymorphic PCR gene-based markers, fingerprinted BAC libraries, and integrated genetic and physical maps (available on mimulusevolution.org; Wu et al., 2008). Gene-based (exon-primed intron-spanning) markers have been used successfully in widespread *M. guttatus* populations, as well as in distantly related species such as *M. aurantiacus, M. ringens,* and *M. primuloides* (Cooley et al., 2011; Griffin, 2010; Streisfeld et al., 2013). Finally, stable transformation protocols have been developed for *Mimulus* (Susič et al., 2014; Yuan et al., 2013) enabling

critical functional tests of candidate genes identified via forward genetic approaches. The wealth of genomic resources coupled with the ecological variability of the *M. guttatus* species complex make it a powerful system for studying adaptation to harsh environments.

Table 1. Common harsh environments of *Mimulus*.

Habitat	*Mimulus* species	Stressors	Reference
Coastal	*guttatus*	Soil salinity, wind, salt spray	Lowry et al., 2009
Copper mine tailings	*cupriphilus, guttatus*	Heavy metal (Cu) toxicity, early seasonal drought	Allen & Sheppard, 1971; Macnair 1981; 1989; Macnair & Christie, 1983; Wright et al., 2013
Geothermal soils	*guttatus*	High soil temperature, seasonal drought	Bunn & Zabinski, 2003; Delmer, 1974; Lekberg et al., 2012
Granite outcrops	*laciniatus*	Seasonal drought	Peterson et al., 2013
High elevation	*guttatus, laciniatus, mephiticus, primuloides, tilingii*	Cold temperatures, reduced growing season, seasonal drought, UV radiation	Douglas, 1981; Ferris et al., (*In press*).
Serpentine	*congdonii, douglasii, floribundus, glaucescens, guttatus, kellogii, layneae, mephiticus, nudatus, pardalis*	Low Ca:Mg and other nutrients, heavy metals, early seasonal drought	Consortium of California Herbaria, 2014; Macnair & Gardner, 1999; Nesom, 2012; Palm et al., 2012; Tilstone & Macnair, 1997; Hughes et al., 2001; Gardner & Macnair, 2000; Murren et al., 2006; Meindl et al., 2013.

NATURAL HISTORY OF ADAPTATIONS TO HARSH ENVIRONMENTS IN *MIMULUS*

Species in the *M. guttatus* complex have adapted to various abiotic stressors such as drought, high salinity, and soils with toxic metal concentrations and low essential nutrients. In

this section, we describe what is known about the natural history of adaptation to harsh environmental conditions in the *M. guttatus* species complex. This natural history lays the foundation for the remainder of the chapter, which describes efforts to understand the physiological and genetic bases of these adaptations.

Serpentine Adaptations

Serpentine soils, derived from the weathering of ultramafic rocks, are characterized by a unique suite of edaphic variables: extremely low levels of Ca and high levels of Mg; deficiency in the major macronutrients N, P, and K; high concentrations of heavy metals such as Ni, Co, and Cr; and low water holding capacity (Chapter 6; Alexander et al., 2007). Many plant species are unable to grow in serpentine habitats because they cannot tolerate the chemical and physical properties of these soils. However, several species within the *M. guttatus* complex have adapted to these harsh soils. The widespread *M. guttatus* can be found both on and off serpentine soils throughout much of its range while two closely related species, *M. nudatus* and *M. pardalis*, have restricted ranges and are found exclusively on serpentine soils (Gardner & Macnair, 2000; Hughes et al., 2001).

Reciprocal transplant and common garden studies show that *M. guttatus* is locally adapted to serpentine soils (Palm et al., 2012; Selby, 2014). When planted at serpentine field sites (Selby, 2014) or on serpentine soil in the lab (Palm et al., 2012; Selby, 2014), plants from non-serpentine populations died in the juvenile stage while serpentine populations had high survival. In contrast, a study by Meindl et al. (2013) found no survival differences between *M. guttatus* plants from serpentine and non-serpentine populations when planted on a mixture of native serpentine and potting soils. These contrasting results could be due to the different soil matrices that were used: full serpentine soil versus a mix of serpentine and potting soils. It is also possible that the non-serpentine populations investigated by Meindl et al. (2013) had a higher frequency of tolerance alleles segregating due to ongoing gene flow with nearby serpentine populations.

Hydroponic experiments are often conducted to determine the specific soil chemical variables that are important selective agents in serpentine habitats. Hydroponic studies using *M. guttatus* have revealed differential tolerance of serpentine and non-serpentine populations to low Ca:Mg ratio (Palm et al., 2012; Selby, 2014) and high Ni (A. Jeong, unpublished) growth environments. These results suggest that the low Ca and high Mg and Ni levels that characterize serpentine soils are likely driving local adaptation of *M. guttatus* populations to these habitats (but see Gardner & Macnair, 2000; Murren et al., 2006).

Adaptation to serpentine soils has also led to the evolution of new species within the *M. guttatus* complex, resulting in two serpentine endemic species: the outcrossing *M. nudatus* restricted to Napa and Lake Counties and the obligately selfing *M. pardalis* found in Calaveras and Tuolumne Counties. The serpentine endemics often grow sympatrically with *M. guttatus*, but inhabit drier microsites (Gardner & Macnair, 2000; Hughes et al., 2001). Accelerated development and flowering time are often selected for in rapidly drying sites as a means of drought escape (Franks, 2011; McKay et al., 2003). Differences in flowering time (Figure 1) likely contribute to RI between the serpentine endemics and *M. guttatus*. Self-fertilization in *M. pardalis* further contributes to RI with *M. guttatus*. In contrast, *M. nudatus* is outcrossing. However, pollinator constancy causes strong prezygotic isolation (RI = 0.947)

between *M. guttatus* and *M. nudatus*: *Dialictus* species preferentially visit *M. nudatus* flowers while honeybees preferentially visit *M. guttatus* (Gardner & Macnair, 2000; Lowry et al., 2008a). In addition to ecological causes of RI, postzygotic isolation in the form of hybrid seed lethality (RI = 0.958) is a strong barrier to gene flow between *M. nudatus* and *M. guttatus* (Gardner & Macnair, 2000; Macnair & Gardner, 1998; Lowry et al., 2008a).

Several other *Mimulus* species have also adapted to serpentine soils in western North America. *Mimulus glaucescens*, a member of the *M. guttatus* complex, and *M. primuloides*, a sister species to the complex, can both be found growing on and off of serpentine soils. Additionally, more distantly related species (*M. floribundus*, *M. layneae*, *M. douglasii*, *M. congdonii*, *M. kelloggii*, and *M. mephiticus*) have populations occurring both on and off serpentine soils. The repeated evolution of serpentine tolerance within the *Mimulus* genus provides a rich opportunity to explore whether shared or unique physiological and genetic mechanisms underlie serpentine adaptation in these different species.

Cu Mine Adaptations

Copper ore mining has resulted in high concentrations of heavy metals in surface soils and water (Chapters 14, 15) which exert strong selection on local plant populations (Bradshaw, 1991; Wu et al., 1975). Similar to serpentine soils, plant adaptations to mine tailings have occurred independently multiple times within species (Christie & Macnair, 1984; Macnair et al., 1989; Schat et al., 1996). However, in contrast to most serpentine habitats, mine tailings are often quite young and have only recently been colonized. *M. guttatus* has adapted to copper contaminated sites in western North America within the last 150 years. Populations of *M. guttatus* grow on copper mine tailings at multiple sites near Copperopolis, CA in the foothills of the Sierra Nevada (Allen & Sheppard, 1971), on the Bingham mine near Salt Lake City, UT (Christie & Macnair, 1984), and at mine sites in Shasta and El Dorado counties in northern CA (R. O'Dell & K. Wright, unpublished data). These mine populations of *M. guttatus* are located in close geographic proximity to populations living on uncontaminated soils, creating the potential for migration and hybridization (Allen & Sheppard, 1971; Macnair et al., 1993). Lab-based, hydroponic studies have demonstrated that populations of *M. guttatus* from Cu-contaminated soils are more tolerant of elevated Cu levels than plants from uncontaminated sites (Macnair & Christie, 1983). A survey of populations near Copperopolis found that Cu tolerance is nearly fixed in four mine populations (99.77%, N=2796), at intermediate frequency (12-45%; N=197) in three uncontaminated but adjacent sites, and at low frequency (0-2%; N=1118; 12 populations) in the majority of uncontaminated sites in the region (Macnair et al., 1993). These results suggest strong selection for tolerance in Cu contaminated habitats and little or no selection against tolerant plants in uncontaminated soils. Reciprocal transplant experiments show that genotypes from mine populations have greater fitness than off-mine genotypes in the Cu-contaminated habitat (K. Wright, unpublished data), providing further evidence that *M. guttatus* is locally adapted to Cu mine tailings.

Adaptation to the Cu mine environment has potentially resulted in a speciation event within the *M. guttatus* complex. *Mimulus cupriphilus* is a recently derived, morphologically distinct, and highly selfing species found only on two small Cu mines near Copperopolis (Macnair & Gardner, 1998). Recent morphological-based taxonomic research has

hypothesized that *M. cupriphilus* may be derived from the serpentine endemic *M. pardalis* (Nesom, 2012). Reproductive isolation between *M. guttatus* and *M. cupriphilus* has not been investigated in the field, but greenhouse experiments reveal that *M. cupriphilus* flowers under shorter day-lengths (Friedman & Willis, 2013), which may contribute to RI with *M. guttatus* (Macnair & Gardner, 1998; K. Wright, unpublished results).

Coastal Habitat Adaptations

Perennial populations of *M. guttatus* grow along the Pacific coast of North America (from southern California to the far western islands of Alaska) where they must cope with both salt spray and saline soils. Coastal perennial *M. guttatus* is morphologically the largest member of the species complex and has previously been classified as a distinct ecotype (Lowry, 2012), variety (Pennell, 1947), and species (Heller, 1904; Nesom, 2012). A series of laboratory experiments have confirmed that coastal perennial plants have evolved a high level of salt tolerance compared to other *M. guttatus* populations (Lowry et al., 2008b, 2009) and are even able to live in sites directly splashed by ocean waves. In the field, a reciprocal transplant study revealed that plants from inland *M. guttatus* populations sustain a high level of leaf necrosis and subsequent mortality when transplanted to coastal habitats (Lowry et al., 2008b).

There are three major mechanisms by which plants evolve salt tolerance: 1) Plant exclusion of Na^+ ions; 2) osmotic stress tolerance; and 3) tissue tolerance to Na^+ ions (Chapter 4; Munns & Tester, 2008). While some plants have evolved mechanisms that exclude toxic Na^+ ions from entering their stem tissue (Boyce, 1954; Munns & Tester, 2008), such exclusion often results in a major osmotic gradient between the environment and plant cells which can cause osmotic stress. Many plants actually uptake Na^+ ions to come into osmotic balance with their environment. However, high levels of Na^+ can be toxic to leaf tissues (Munns et al., 2006; Rus et al., 2006). Therefore, some plants have evolved mechanisms of ion stress tolerance that either allow cells to tolerate higher concentrations of Na^+ ions or to exclude these ions. Such tissue tolerance is often mediated by the sequestration of Na^+ ions in the vacuole of leaf cells (Munns & Tester, 2008; Zhu, 2001).

Lowry et al. (2009) conducted a series of physiological experiments to determine which salt tolerance mechanism was involved in adaptation to coastal habitats in *M. guttatus*. Both coastal and inland inbred lines accumulate similar concentrations of Na^+ ions in their leaves when grown under saline hydroponic conditions, suggesting that salt tolerance is not mediated by whole plant exclusion of Na^+. However, coastal plants are far more tolerant of elevated leaf Na^+ levels, implicating tissue tolerance as the likely mechanism of salt tolerance in coastal plants. The exact mechanism of leaf tissue tolerance in *M. guttatus* is currently unknown.

Flowering Time Escape from Harsh Environmental Conditions

Reciprocal transplant experiments have demonstrated that natural selection often favors different flowering times in different environments (Ågren & Schemske, 2012; Anderson et

al., 2011; Hall & Willis, 2006; Leinonen et al., 2013; Verhoeven et al., 2008; Weinig et al., 2002).

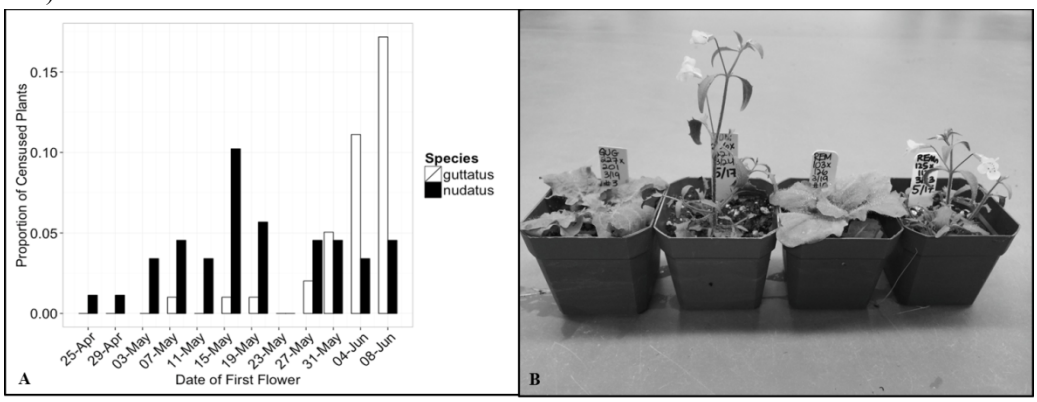

Figure 1. A) Under natural field conditions, a higher proportion of *M. nudatus* plants flower earlier in the season than *M. guttatus*. Data collected by J. Selby (unpublished) from multiple transects established in a single mixed population at the University of California McLaughlin Reserve in 2010. B) Two sympatric populations of *M. nudatus* and *M. guttatus* were grown under eight hour days. Only the *M. nudatus* plants flowered.

In the *M. guttatus* complex, differences in flowering time are often associated with differences in water availability between habitats. Adaptive differences in flowering time have been shown to occur between several early flowering species in the complex (*M. nudatus, M. pardalis, M. cupriphilus, M. laciniatus*, and *M. nasutus*) and sympatric *M. guttatus* populations (Friedman & Willis, 2013), as well as between annual and perennial populations of *M. guttatus* (Kiang & Hamrick, 1978; Lowry et al., 2008a; Martin & Willis 2007; Wu et al., 2010).

The developmental stage at which a plant will flower and the day-length required for flowering (critical photoperiod), differ between annual and perennial populations of *M. guttatus*. Annual populations of *M. guttatus* grow in habitats with very low soil moisture availability in the summer months, while perennial populations grow in streams and seeps with relatively high year-round soil moisture (Hall & Willis, 2006; Lowry, 2012; Lowry et al., 2008a). This difference in soil water availability results in divergent selection on both flowering time and critical photoperiod between annual and perennial populations (Friedman & Willis, 2013; Hall & Willis, 2006; Lowry & Willis, 2010; Lowry et al., 2008a). Populations of *M. guttatus* and closely related edaphic endemics—*M. cupriphilus, M. laciniatus, M. nudatus,* and *M. pardalis*—often occur sympatrically, but differ in microhabitat and critical photoperiod. While many populations of *M. guttatus* live in moist seeps and streams, the edaphic endemics often inhabit soils with low water-holding capacity that dry out earlier in the summer dry season. For example, *M. laciniatus* grows on moss patches in granitic outcrops (Sexton et al., 2011) that dry out rapidly after annual snow melts in the Sierra Nevada Mountains. *Mimulus nudatus* inhabits upland serpentine outcrops that dry out following the end of the California wet season. Most populations of *M. guttatus* require long days to flower; but sympatric populations of edaphic endemics flower under shorter day lengths possibly to avoid drought conditions in their rapidly drying microhabitats (Friedman & Willis, 2013). Both the serpentine endemic *M. nudatus* and the Cu mine endemic *M. cupriphilus* can flower under day lengths as short as 8 hours, while sympatric populations of

M. guttatus require over 11-13 hours (Figure 1; Friedman & Willis, 2013). These flowering time differences likely contribute to RI between the edaphic endemics and *M. guttatus*. Furthermore, *M. cupriphilus, M. laciniatus*, and *M. pardalis* are all self-fertilizing and these shifts of mating system may have evolved as forms of reproductive assurance (Franks, 2011; Ivey & Carr, 2012; Macnair & Gardner, 1998; Martin & Willis, 2007; Wu et al., 2010).

THE GENETIC ARCHITECTURE OF ADAPTATION TO HARSH ENVIRONMENTS

Much of the research aimed at understanding the genetic basis of adaptations to harsh environments in *Mimulus* has utilized quantitative trait locus (QTL) mapping approaches. However, recent technological advances provide new opportunities for using population genomics to determine the genetic basis of adaptive traits. These two approaches have different strengths and when used together they have the potential to provide a more complete picture of the genetics of adaptation than either approach in isolation.

Quantitative Trait Locus Mapping

Often, the first step to investigating the genetic basis of adaptation is to conduct QTL mapping in hybrids between divergent populations or ecotypes. Quantitative trait locus mapping has recently been criticized as an approach for understanding the genetic basis of adaptive traits based on its inability to detect small effect functional polymorphisms (Rockman, 2012) and disinterest by some evolutionary biologists in the "molecular details" of adaptation (Travisano & Shaw, 2013). Despite its detractors, QTL mapping has been crucial in identifying important loci that have advanced the understanding of fundamental evolutionary questions with regard to adaptation and speciation (reviewed in Bomblies, 2013; Coyne & Orr, 2004; Lee et al., 2014).

The genetic and genomic toolkit available for *Mimulus* has enabled numerous QTL mapping projects aimed at characterizing the genetic basis of adaptive phenotypic traits (Fishman et al., 2002; Hall et al., 2006; Lowry et al., 2009; 2013; Sweigart et al., 2006; Wright et al., 2013). These studies use traditional genotyping approaches (e.g., PCR-based markers) to investigate phenotypic variation segregating in a hybrid mapping population (F2, RIL, etc.). The availability of next-generation sequencing technologies has accelerated QTL mapping projects using a bulk segregant analysis (BSA) approach (Magwene et al., 2011; Michelmore et al., 1991). Bulk segregant analysis works through the selection of hybrid individuals from both tails of the phenotypic distribution for a particular trait. The DNA from hybrid individuals from each tail is pooled in equimolar concentrations and each pools is then sequenced. Allele frequencies at polymorphic sites across the genome are calculated for each pool. Allele frequencies at sites not associated with the phenotype should not differ between the pools, while allele frequencies at sites associated with the phenotype (QTLs) will diverge. Quantitative trait loci for photoperiod differences between *M. nasutus* and *M. guttatus*, as well as between annual and perennial populations of *M. guttatus*, have been efficiently and rapidly identified using BSA (Fishman et al., 2013; Friedman & Willis, 2013). These

techniques have also been used to map QTLs for salt tolerance, serpentine tolerance, flowering time differences, and leaf shape, not only in *M. guttatus* but also in other species such as *M. laciniatus* and *M. nudatus* (J. Selby and K. Ferris, unpublished). To confirm the presence of a QTL, individual F2s are genotyped at markers in regions of the genome that showed allele frequency differences in the BSA. Genotyping F2s individually also enables estimation of the effect size of QTLs. To ultimately identify the causal functional variant underlying these QTLs, additional fine mapping studies in larger mapping populations or association mapping/genome scan approaches are needed (*e.g.*, Yuan et al., 2013).

To investigate the genetic basis of adaption to the Cu mine habitat, Wright et al. (2013) conducted a QTL mapping experiment for Cu tolerance, measured using a lab-based hydroponic assay, and identified a single, large effect locus, *Tol1* (Wright et al., 2013). This experiment revealed strong genetic differentiation at markers in tight linkage with *Tol1*, consistent with the hypothesis that this locus was strongly selected during Cu mine colonization. Interestingly, adaptation to the mine environment has resulted in the development of a post-zygotic intrinsic reproductive isolating barrier (Macnair & Christie, 1983). The Copperopolis population of *M. guttatus* is fixed for an allele that results in F1 hybrid necrosis in crosses to plants from multiple off-mine populations (Christie & Macnair, 1984; Macnair & Christie, 1983). This incompatibility factor, *Nec1*, was fine mapped to a region in tight linkage (<1cM) with the major Cu tolerance locus, *Tol1* (Wright et al., 2013). The distribution of genetic variation between the Copperopolis population and adjacent off-mine populations suggests that strong selection on *Tol1* caused the hybrid incompatibility allele at *Nec1* to hitchhike to fixation at Copperopolis (Wright et al., 2013). This study demonstrates that natural selection on a locally adaptive trait can indirectly drive a hybrid incompatibility allele to high frequency due to tight genetic linkage.

Recent studies have also made major progress in mapping QTLs for variation in critical photoperiod between different populations and species of *Mimulus*. Differences in critical photoperiod for flowering between annual and perennial populations of *M. guttatus* are caused by two large effect QTLs (Friedman & Willis, 2013; Hall et al., 2006; Hall et al., 2010). Annual and perennial populations also differ in their vernalization requirements, and mapping studies have identified a mixture of large and small effect QTLs which contribute to these differences. The selfing species *M. nasutus* and the outcrossing species *M. guttatus* differ in the critical photoperiods at which they transition from vegetative to reproductive growth. These differences are controlled by two major effect QTLs, one of which co-localizes with one of the photoperiod QTLs identified between annual and perennial populations of *M. guttatus* (Fishman et al., 2013). One of the QTLs controlling critical photoperiod differences between *M. nasutus* and *M. guttatus* mapped near an ortholog of *Arabidopsis thaliana* FLOWERING LOCUS T/TERMINAL FLOWER 1, while the other mapped near an ortholog of the DELLA gene GIBBERELLIC ACID INSENSITIVE (Fishman et al., 2013). In all cases, these flowering time differences likely reflect adaptive divergence between habitats due to differences in the timing of low soil moisture availability.

Combining QTL and Field Experiments to Understand Adaptation

In order to identify QTLs that actually contribute to adaptive differences, mapping studies should be conducted in the field or QTLs that have been identified in laboratory-based studies should be tested for their fitness effects in native habitats. Reciprocal transplant studies that incorporate hybrid mapping populations (F2s, backcrosses, etc.) can identify adaptive traits which are strongly correlated with survival and fecundity. Hybrids can then be genotyped to identify the loci that contribute to fitness in the field. This approach has been used to map QTLs for the ability to survive on serpentine soils in the field in *M. guttatus* (Selby, 2014). Near isogenic lines (NILs), genetic lines that are identical with the exception of introgressed genetic regions of interest, can also be used to test whether specific traits and genetic loci are adaptive. For instance, a NIL may consist of a line that is identical to a local genotype except for a single foreign QTL. Near isogenic lines can be planted across environments and the fitness of each NIL can be measured to determine whether individual genetic loci are adaptive.

Lowry et al. (2009) identified three major QTLs that contribute to differences in salt spray tolerance in the laboratory between inland annual and coastal perennial *M. guttatus* populations. These salt tolerance QTLs were then evaluated for their effects on fitness in a field reciprocal transplant study using recombinant inbred lines (RILs). Interestingly, all three salt tolerance QTLs discovered in the lab had a significant effect on fitness in coastal habitats but no detectable effect on fitness in inland habitats. The fitness effect of the major Cu tolerance QTL, *Tol1*, was similarly investigated via reciprocal transplant of NILs possessing mine and off-mine alleles at *Tol1* (K. Wright, unpublished). The mine allele at *Tol1* significantly increased the probability a plant would survive to flower in the mine environment, but had no detectable effect on survival in the off-mine environment. This genotype by environment interaction in which a locus has significant fitness effects in one habitat but little or no fitness effects in a different habitat is called conditional neutrality (reviewed in Anderson et al., 2011; Kawecki & Ebert, 2004). Alleles with conditionally neutral effects are likely to reach high frequency in the population in which they are under strong selection, but have the opportunity to diffuse to other populations through gene flow.

Lowry & Willis (2010) conducted a reciprocal transplant experiment using NILs, in which a chromosomal inversion polymorphism was introgressed reciprocally into coastal perennial and inland annual *M. guttatus* genetic backgrounds. In contrast to the three salt tolerance loci that only had fitness effects in the coastal habitat, the inversion locus had contrasting fitness effects across habitats. In the inland habitat, the inland orientation of the inversion contributed to higher fitness by facilitating escape from seasonal drought via earlier flowering time. In the coastal habitat, the coastal perennial orientation of the inversion increased fitness by shifting the allocation of plant resources from flowering to growth and multi-season survival, which is advantageous in the coastal habitat because there is year-round soil moisture availability. This genotype by environment pattern of opposite fitness effects of a locus across habitats is called “antagonistic pleiotropy” (reviewed in Anderson et al., 2011; Kawecki & Ebert, 2004). Antagonistic pleiotropy will reduce gene flow between habitats at a particular locus because local alleles are advantageous over foreign ones in each environment.

Population Genomics

Recent technological advances have made whole genome population sequencing feasible for many systems, including *Mimulus* (Brandvain et al., 2014; Flagel et al., 2014; Hellsten et al., 2013). Such sequence data offer excellent opportunities to conduct population genomic analyses to identify loci involved in adaptation to harsh environments. Compared to QTL mapping, population genomic approaches can leverage numerous natural recombination events and therefore offer the potential to more precisely identify causative functional alleles. Large-scale population genomic studies have detected associations between nucleotide variation and climate across broad geographic spaces (Coop et al., 2009; Hancock et al., 2011; Lasky et al., 2014).

However, there are potential problems associated with population genomic studies. For example, the demographic history and population structure of sampled genomes can lead to false positive associations of alleles with environmental variables (Coop et al., 2010). Many methods have been developed to control for demography and population structure, but these can lead to false negatives if adaptive alleles are correlated spatially with population structure. Furthermore, it is very difficult to establish demographic history with confidence and virtually impossible to control for phenomena like "allelic surfing" (Excoffier & Ray, 2008). In addition, most population genomic studies have failed to account for the structure of the genome itself which can also lead to the discovery of false positive "outlier" loci. For example, intrinsic differences in recombination rate across the genome can skew the fixation index (F_{ST}) and other summary statistics by affecting local levels of nucleotide diversity (Cruickshank & Hahn, 2014; Lowry et al., 2013; Renaut et al., 2013). Finally, population genomics studies cannot distinguish between loci of major effect versus those that may be subtle modifiers.

Given the potential limitations of population genomic studies, careful consideration is advised in developing the sampling strategy of focal populations. For instance, sampling adjacent populations in habitats that differ sharply in environmental conditions will minimize the effects of population structure. Additionally, by sampling multiple independent pairs of adjacent populations in different habitats, researchers can more reliably identify alleles that are selected in each environment as well as test whether the same or different genes have been used by different populations in adapting to similar habitats. Investigating the genomic basis of adaptation to edaphic conditions is ideally suited to this experimental design because the environmental gradients between soil types are often discontinuous and multiple pairs of divergently adapted populations are often located within a single region. We further advocate that comparing results from genome resequencing studies with other lines of evidence (QTL mapping, functional molecular biology, and reciprocal transplant experiments) is the most thorough way to confidently identify loci involved in adaptation to environmental variation across space. We are currently conducting such experiments to investigate the genomic basis of adaptation to Cu and serpentine soils in *M. guttatus*. Finally, it should be noted that many analytical methods and technologies are still being developed which will likely improve the utility of population genomics.

High-Throughput Phenotyping

High-throughput, laboratory-based assays can assist in elucidating the function of QTLs that affect fitness in the field, in identifying specific traits that contribute to adaptive divergence, and in assisting fine scale genetic mapping to identify causal loci. However, development of robust laboratory-based assays that mimic the selective environment experienced by plants in native habitats is challenging. In this section, we discuss experimental designs we have used to study edaphic adaption and salt tolerance as well as highlight some of the challenges we encountered during the development of these assays.

Laboratory Edaphic Assays

The handful of QTL mapping studies that have evaluated the genetic architecture of serpentine adaptation (Bratteler et al., 2006; Burrell et al., 2012) have exclusively mapped QTLs that confer tolerance to a single soil chemical variable isolated in altered liquid nutrient feeds. We recognize the powerful insight that such hydroponic methods can provide; however, none of the QTLs that confer tolerance to an isolated soil chemical variable have been tested for their effects on plant fitness in native soils. The interactions between different ions (Brooks, 1987; Gabbrielli & Pandolfini, 1984) as well as the physical properties of the soils are likely to contribute to adaptation to serpentine habitats. To account for the full suite of selective factors associated with serpentine soils or mine tailings, mapping experiments should be conducted in native soils. For example, the genetic basis of serpentine tolerance in *M. guttatus* was originally investigated by planting F2s in the field and conducting a BSA on the survivors from serpentine and non-serpentine field sites. We have also grown F2 mapping populations on native serpentine soil in the lab. By planting seeds either in plug trays or on tissue-culture plates filled with serpentine soil, we are easily able to screen 1000s of F2s for juvenile survival which has enabled rapid fine-mapping of a major serpentine tolerance QTL in *M. guttatus* (Selby, 2014).

Mapping QTLs for hydroponic tolerance to isolated soil chemical variables and testing for co-localization of these QTLs with field fitness QTLs will enable researchers to begin to identify the mechanisms that contribute to fitness differences between populations. We have developed a high-throughput hydroponic platform to assay plant tolerance to individual soil chemical variables. Tolerance manifests as a differential response to a treatment medium. This differential response is typically observed by measuring a plant growth parameter (height, biomass, etc.) in both a treatment and a control solution. The ratio of plant size in treatment versus control solution is used as an index of tolerance to control for inherent size differences. This design requires that genetically identical individuals be grown in both treatment and control solutions. However, taking clones from large, robust plants that have already acclimated to a benign growth environment fails to mimic how plants would experience soils with altered nutrient profiles in the field. Therefore, we have developed a seedling assay based on the sequential testing method of Schat and ten Bookum (1992). Single genotypes are grown in increasingly severe treatment solutions and root growth rate is scored in each treatment level. For each individual, the treatment concentration that stops root growth, referred to as the "Effective Concentration 100%" (EC100), is scored. This strategy

controls for inherent differences in root growth rate without the requirement that individuals also be grown in a control solution.

We designed a growth platform that has enabled high-throughput hydroponic studies. Watertight boxes were constructed out of PVC foam board (11.5" x 5" x 7.5") with removable lids with holes (4 rows of 17) through which drinking straws are suspended into the solution (Figure 2). Seeds are sown on an inert rockwool medium stuffed into 200uL PCR tubes with the tips clipped off. The tubes are then placed into the holes in the lid of each box and seeds are allowed to germinate and grow in a nutrient solution (¼ strength Hoagland's, prepared as described by Epstein (1972) until most seedlings have roots protruding through the bottom of the rockwool (~7 days).

The position of root tips is then tracked for each plant by sliding a small dental rubber band around the straws. Every two days, the position of the root tip is marked and the treatment solution is changed. At the end ofa series of increasingly severe treatments, the distance between the rubber bands is measured, providing root growth rates in each treatment level from which EC100 can be calculated. We have successfully used this design to assay populations of *M. guttatus* for differences in tolerance to low Ca:Mg ratio, high Ni, and high Cu, and have also used this method to map QTLs for these tolerance differences (A. Jeong, J. Selby, & K. Wright, unpublished).

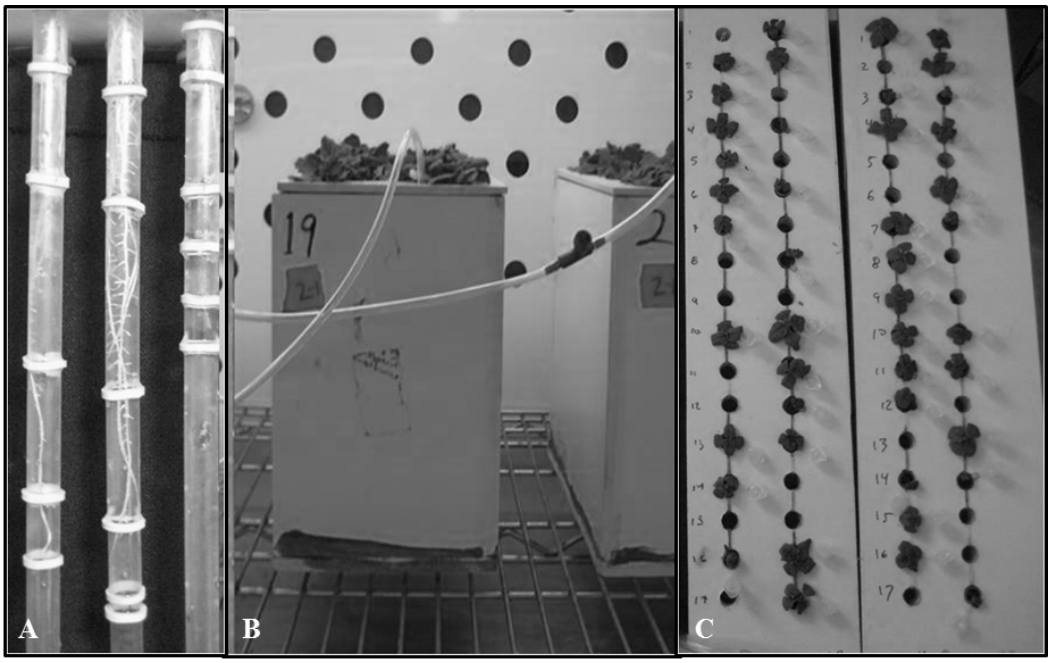

Figure 2. Photos of high-throughput hydroponic growth platform for root growth assays. A) Roots growing in straws with rubber bands marking positions of root tips in each treatment level. B) Front view of box at end of experiment with tubes connected to an air pump and rosettes of plants on top. C) Top of box showing *M. guttatus* seedlings at the start of the experiment.

Laboratory Salt Tolerance Assays

We have developed multiple methods for assessing the salt tolerance of individual plants for the purpose of genetic mapping in *M. guttatus*. Lowry et al. (2009) initially used a salt spray assay to map QTLs in RILs from a cross between inland annual and coastal perennial lines of *M. guttatus*. Plants were sprayed with 5mL of 500mM NaCl solution every other day. Plants were then scored for the day which they no longer had any green tissue (see Lowry et al., 2008b, 2009 for complete methods). This experiment identified three major salt tolerance QTLs, which were subsequently tested for their fitness effects in the field.

While the salt spray assay yielded the localization of three QTLs, it was difficult to conduct without introducing considerable random heterogeneity into the experiment due to some plants receiving more spray than others. A larger subsequent QTL mapping experiment was ultimately abandoned because the date of death for the salt spray assay was highly variable within inbred lines.

Following this setback, we sought a better methodology for assessing salt tolerance of individual plants. We first developed two types of assays with agar plates that contained various levels of NaCl in the media. One assay involved growing seedlings on plates and the other involved transferring hole punches from adult leaves to plates following the methods of Prasad et al. (2000). Both assays proved useful for detecting differences in tolerance between coastal perennial and inland annual populations. However, neither method was well suited for QTL mapping with large populations of hybrids. Assays involving plates with NaCl were generally plagued by large block effects between plates. Further, the transfer of seedlings to plates is problematic in general as it introduces a major shock effect since plants have no time to acclimate to the stress as they would in nature (Juenger et al., 2010; Munns & Tester, 2008)

We recently developed a hydroponic assay that is much more promising for future genetic studies of salt tolerance. This method involves growing plants in perlite with ½ strength Hoagland's solution as a nutrient media. The plants are initially grown for 2-4 weeks at a 0mM concentration of NaCl. The salinity treatment is then increased by 25mM increments each day to allow the plants to acclimate. Once the treatment solution reaches a concentration of 150mM NaCl it is no longer increased, but simply replaced every three days. Appropriate levels of calcium must be added to the solution once the NaCl treatments are initiated because sodium can interfere with the assimilation of calcium (Al-Harbi et al., 1995; Wakeel et al., 2009). Using this set-up, there are clear differences in date of death between coastal perennial and inland annual *M. guttatus* plants at the final treatment concentration of 150mM NaCl (D. Lowry, unpublished).

CONCLUSION

Mimulus has been established as a model system for investigating the genetic basis of adaptation to harsh environmental conditions. Significant progress has been made in understanding the genetic architecture of adaptive flowering time escape from seasonal drought, adaptation to toxic soils, and salt tolerance. The combination of classic reciprocal transplant experiments with modern molecular genetics has led to a deeper understanding of how individual loci contribute to adaptations across habitats (Hall et al., 2010; Lowry &

Willis, 2010). The detailed genetic dissection of tolerance to edaphic conditions has revealed new insights into the mechanisms by which natural selection can drive the formation of reproductive isolation (Wright et al., 2013). The importance of critical photoperiod in timing the initiation of flowering to avoid seasonally harsh conditions (Fishman et al., 2013; Friedman & Willis, 2013) has also been demonstrated and genetic dissection of these critical photoperiod differences is now underway in multiple *Mimulus* laboratories. While QTLs have been localized for many traits involved in adaptation to harsh environments, the actual genes that underlie these adaptations have thus far remained elusive. Fine genetic mapping of these QTLs has brought us closer to identifying the causal genes. However, these efforts can sometimes take over a decade to accomplish, even in model systems such as *Arabidopsis thaliana* (Des Marais et al., 2014). Further difficulties in identifying the causal locus can arise from genome assembly issues, as has been the case for identifying the major Cu tolerance locus in *M. guttatus* (Wright et al., 2013). Despite these challenges, we are optimistic that the combination of genetic mapping with new phenotyping methods and population genomic approaches will yield new insights into the evolution of adaptation to harsh environmental conditions. Beyond improving our understanding of evolutionary mechanisms, studies of the genetic basis of adaptation to harsh environmental conditions have many potential applied benefits. Many of the selective pressures present in these extreme habitats—water-limitation; toxic, nutrient poor soils; thermal extremes—are likely to increase as a result of climate change (see Chapters 7, 13), pollution, and other human-induced environmental impacts. Elucidating the genetic basis of adaptation to these stresses will have important applications for food security, restoration of polluted sites, and conservation of critical habitats that are the drivers of biological diversity.

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