Integrating Genetics, Geography, and Local Adaptation to Understand Ecotype Formation In The Yellow Monkeyflower, *Mimulus guttatus*

by

David Bryant Lowry

University Program in Genetics and Genomics
Duke University

Date:_____
Approved:

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John Willis, Supervisor

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Mohamed Noor

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Mark Rausher

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William Morris

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
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ABSTRACT

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Abstract

Speciation is a constantly ongoing process whereby reproductive isolating baririers build up over time until groups of organisms can no longer exchange genes with each other. Adaptation is thought to play a major role in the formation of these barriers, although the genetic mechanisms and geographic mode underlying the spread of barriers due to adaptive evolution is poorly understood. Critically, speciation may occur in stages through the formation of intermediate partially reproductively isolated groups. The idea of such widespread ecotypes has been the subject of great controversy over the last century. Even so, we have relatively little understanding about whether widespread ecotypes exist, wheather they are reproductively isolated, and how adaptive alleles are distributed among partially isolated groups. In this dissertation, I examined these issues in widespread coastal perennial and inland annual ecotypes of the yellow monkeyflower, *Mimulus guttatus*. First, I determined that coastal and inland populations comprise distinct ecotypic groups. I then determined that these ecotypes are adapted to their respective habitats through genetically based flowering time and salt tolerance differences. I assessed the genetic architecture of these adaptations through quantitative trait loci (QTL) analysis and determined the geographic distribution of the underlying alleles through latitudinally replicated mapping populations. I quantified the contribution of these loci to adaptation in the field through the incorporation of advance generation hybrids in reciprocal transplant experiments. In the process, I discovered a widespread chromosomal inversion to be involved in the adaptive flowering time and annual/perennial life-history shift among the ecotypes. Overall, the results of this study suggest that widespread reproductively isolated ecotypes can form through the spread adaptive standing genetic variation between habitats and that chromosomal rearrangements can integral to this process.

Dedication

I would like to dedicate the dissertation to my parents who have nurtured my curiosity for the last three decades. My mother, Susan Lowry, is a saint who provides to her family and community far more than she receives thanks in return. My father, John Lowry, first introduced me to Carl Sagan's Cosmos and the thrill of intellectual debate while always insisting that my fortunes are a debt to those less fortunate in my collective society. During the course of graduate school, my parents supported this research through their inquisitiveness and by allowing me to use their home in California as my *ad hoc* field station.

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Acknowledgements

Graduate school is a lot of things: An intellectual journey, a breeding ground for deep thoughts, and yet it is people whom one spends time with during graduate school that will define how the experience appears through the cherry-blurred lens of nostalgic reverie. I am extremely grateful for the incredible people that have made my time in Durham and at Duke University so completely fulfilling.

I have been remarkably fortunate to have John Willis as my advisor and guide through the journey. John's ceaseless optimism is the fuel that stokes the fires of exploration in all who he mentors. However, I would be remiss if I did not acknowledge Carrie Wu as my *ad hoc* coadvisor. Carrie dedicated enormous time to the success of my dissertation research and our amazingly complementary writing styles resulted in three publications as well as the NSF grant that currently funds the Willis lab. I also owe much of the success of my research to Megan Hall, who pioneered the research on the genetics underlying the divergence of coastal perennial and inland annual ecological races of *Mimulus guttatus.*

Duke currently has the best collection of plant ecological and evolutionary geneticists in the world. The many members of the Willis, Rausher, and Mitchell-Olds labs all helped me constantly during my dissertation. My committee of Mohamed Noor, Mark Rausher, Greg Wray, and Bill Morris were extremely supportive. Seven undergraduate and high school students participated directly in my research enterprises and taught me numerous valuable lessons about mentorship. Anne Lacey, Jim Tunney, Jo Bernhardt, Andrea Lanahan, Carolyn Woolley, Clint King, Beverley Calhoun, John Mays, Michael Barnes, and Lisa Bukovnik were all vital to ensuring that administrative efficiency, keeping the plants alive, genotyping my samples, and providing an atmosphere of good cheer.

A cast of jolly compatriots, drinking buddies, and great friends supports every PhD dissertation. Kevin Wright journeyed with me on an 18,000-mile collecting trip in 2005, constantly calls my bluff, and is my partner in crime. Jenny Tung and David Garfield are my go-to people for a rousing debate on any subject. For the last decade, Sriram Kosuri has continuously introduced me to real scientific discoveries that sound more like science fiction. Dan Speiser and David Des Marais are really good friends, good scientists, and supported me through a rough patch in my life. "The Bros," composed of Max Wilmarth, Azul Couzens, Cotton Rockwood, and Katie Cochrane have been there for me for years and are my favorite people in the world.

The coffee houses of Durham, especially Shade Tree (RIP), Joe van Gogh, and Francescas, provided the atmosphere for transforming my disparate ideas into cohesive experimental design and rhetoric. This is not to mention that these coffee shops were the venue in which I would eventually meet my fiancée, Sheril Kirshenbaum. Sheril is my muse, my inspiration, and a damn good writer; I cannot imagine life without her.

Much of my dissertation research was conducted in the field. Numerous people and governmental agencies provided permission for my collections/research and housed me along my travels. In particular, I want to thank Scott and Kim Fraser as well as Peter Bradford, who were generous to offer their farmland as sites for my reciprocal transplant experiments. Kurt Merg and Peter Steel facilitated my research at the University of California Big Creek and Angelo reserves.

Finally, I want to recognize the people who made big differences in my career path before graduate school. My high school AP Biology teacher Joan Vreeburg introduced me to the excitement of molecular genetics. When in college at UC Berkeley, Ellen Simms mentored me like a graduate student. Tom Juenger, who was a postdoc at the time in the Simms lab, became my role model for how to balance a science career with the real world. John Maron first hired me as a plant ecology field assistant in 1998

and forever changed my career trajectory. Dr. Maron later provided me the opportunity "to live the dream" for the summer of 2004 in a Scotty trailer set in the middle of the gorgeous Blackfoot Valley of Montana. Long conversations with Cedar Brant, who shared that trailer under the summer sky, would profoundly impact my perceptions of life and prepare me mentally for the years of graduate school that lay ahead with the coming fall.

1. Introduction: A brief history of local adaptation and the stages in the formation of plant species

Interest in the mechanisms underlying the origin of species rapidly expanded in the last 15 years, especially in terms of how ecology and local adaptation contribute to this process (Reviewed in Schluter 2001, 2009; Coyne & Orr 2004; Rundle & Nosil 2005). Researchers in animal systems for the most part led the way forward during this renaissance period and thus, have been primarily responsible for framing the historical narrative on the evolution of ideas regarding the role of ecology in speciation (Schluter & Nagel 1995; Schluter 1996; Rundle et al. 2000; Schluter 2001; Nosil et al. 2005; Rundle & Nosil 2005; Nosil 2008). Most of these papers cite the early views of Dobzhansky (1951) and Mayr (1942, 1947) as a foundation for the idea of *ecological speciation*. Rarely cited are the plant evolutionary biologists during the mid-20th century (Turesson 1922a,b, 1925; Gregor 1930, 1936, 1938; Clausen et al. 1939, 1940, 1948; Stebbins 1950; Clausen 1951; Clausen & Hiesey 1958; Grant 1981) who accumulated a wealth of evidence for the role of ecological adaptations in the formation of reproductive isolating barriers through rigorous experimental studies in the field. While the narratives provided by animal evolutionary biologists have been extremely valuable, many contemporary workers have overlooked the history and some have even suggested that a consensus among botanists about the mechanisms of speciation was reached long ago.

Recently, Mallet (2008) demonstrated how obscure the historical controversy over the nature of plant species has become: "Ecological races and forms within species, as well as hybridization between species are all well known in plants, and these are the chief reasons why the biological species concept is not generally accepted by plant biologists" (p. 2975). While it is true that many plant evolutionary biologists have long accepted intermediate forms within a species as well as the commonality of reticulation,

this has by no means diminished their appreciation for reproductive isolating barriers in the formation of species. Clausen's (1951) classic book, "Stages in the evolution of plant species" clearly illustrates this view: "In the evolution of plant species there is a long series of stages of increasing evolutionary distinctness, starting with the local populations, continuing through ecological races, ecospecies, and groups of species of higher and higher order" (p. 177; Fig. 1) while stating that this process occurs because "two segments of what was formerly one species become separated by barriers to free interbreeding of one kind or another" (p. 87). To his credit, Mallet (2008) does acknowledge that some plant evolutionary biologists do accept the biological species concept. However, the idea of intermediate stages in the evolution of plant species has itself been a major controversy over the last century (Heywood 1959; Langlet 1971; Quinn 1978; Raven 1980; Levin 1993), and contemporary workers often overlook the existence of intermediates. Even "Speciation" (Coyne & Orr 2004) failed to cite Clausen (1951), which was one of the three most important books on plant speciation of the 20th century and the only book by Clausen, Keck, or Hiesey to directly address the question of speciation.

Consensus on plant speciation has never been reached at any point in history and has often been very contentious (Turesson 1922a,b; Faegri 1937; Clausen 1951; Heywood 1959; Ehrlich & Raven 1969; Langlet 1971; Quinn 1978; Levin 1993; Baum 2009; Sobel et al. 2010). Interestingly, much of this controversy is intimately tied to the usage of the term *ecotype* and whether or not widespread distinct ecotypes exist within plant species. Very often the views of plant evolutionary biologists were influenced by the system in which they worked. Those studying the distribution of variation among distinct habitats (Turesson 1922a; Stebbins 1950; Clausen & Hiesey 1958; Grant 1981) tended to support the existence of widespread ecotypes as a stage in the evolution of plant species, while those working in systems with gentle clines (Langlet 1971; Quinn

1978; Levin 1993) or adaptation to discrete edaphic conditions (Kruckeberg 1951; Antonovics & Bradshaw 1970), tended to ignore or dismiss the existence of widespread ecotypes.

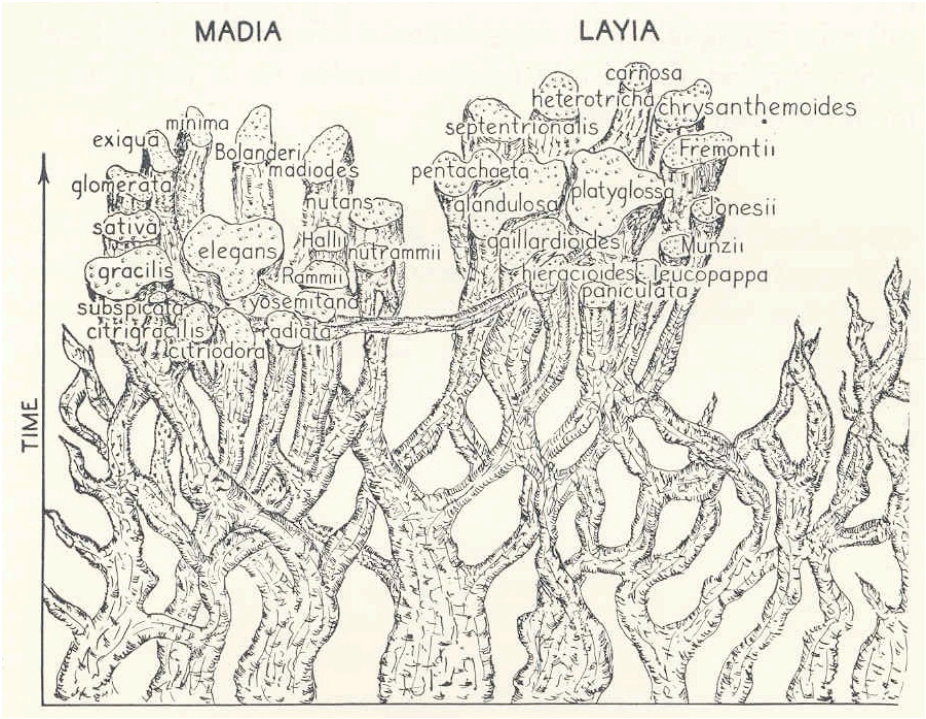

Figure 1: The interrelationship of two plant genera (*Madia* and *Layia*) through time as envisioned by Clausen (1951), based on a long series of experiments. The figure is a representation of Clausen's belief in how the evolution of speciation proceeds through the formation of reproductively isolated races, while involving multiple extinction and reticulation events.

The goal of this review is to provide a brief history of the development of my understanding regarding the role of local adaptation in the "stages in the evolution of plant species." While I do emphasize plant species, I have tried to make this review accessible and interesting to researchers on other organisms and highlight research from workers across taxonomic groups. After all, the greatest achievement of speciation studies over the last decade has been to demonstrate the role of ecology in the process across a broad swath of taxa (Schluter 2009).

1.1 The birth of ecotype

Even though Darwin (1859) soundly rejected the Platonic-Aristotelian essentialist view of the immutability of organisms, early plant evolutionary biologists were confounded about the nature of varieties and species in relation to adaptation to local environmental conditions. Much of this confusion resulted from an incomplete understanding of the mechanisms of heredity and a persistence of Lamarckian ideas well into the 20th century (Smocovitis 2006).

Around the turn of the century, Gaston Bonnier (1890, 1895, 1920) published a set of flawed studies (Hiesey 1940; Hagen 1984), which claimed that he converted many lowland varieties of plants into alpine varieties simply by transplanting them in the mountains. While this result contradicted the findings of numerous agriculture and silvicultural studies (reviewed in Langlet 1971) as well as the Austrian botanist Anton Kerner von Marilaun (1895), many evolutionary biologists of the early 20th-century were sympathetic to Bonnier's Lamarckian beliefs (Hall & Clements 1923; Hagen 1984). It was not until a set of experiments published by Gote Turesson in the 1920s that many plant evolutionary biologists began to accept that a large portion of the phenotypic differentiation among populations has a genetic basis (Turesson 1922a,b, 1925; Clausen 1951; Hagen 1984).

To test whether there was a genetic basis to population differences, Turesson (1922a, 1925) set up common garden experiments, where plants collected from across Sweden were grown under one set of environmental conditions. After conducting similar such experiments across multiple species, Turesson recognized a pattern: Plants collected from coastal habitats had a certain set of distinct traits in common, while plants from inland habitats had a different set of traits in common. In general, coastal forms are distinguished from inland forms by later flowering, prostrate growth, and

succulent leaves (Table 1). Upon further inspection, Turesson soon realized that populations growing in coastal sand dunes along also had distinct features from those growing along the coastal cliffs and bluffs.

Turesson was not the first to recognize the distinctness of coastal varieties from their inland conspecifices. Over two hundred years earlier, British naturalist John Ray conducted the first recorded transplantation study in the late-17th century. While crude by today's standards, Ray documented that when a coastal variety of *Geranium sanguineum* from the island of Walney on the Irish Sea was transplanted to multiple sites across England it maintained its coastal prostrate stature (Briggs & Walters 1997).

However, multiple factors set Turesson's research apart from previous experiments and as a result stimulated an explosion of interest in genecology research on local adaptation (Hagen 1984), much the same way that Schluter's (1996) term *ecological speciation* recently inspired a flurry of studies. The work Turesson conducted was extensive in that it simultaneously quantified similar patterns in multiple plant species (Table 1). He also set out "a provocative theoretical framework by formulating a set of theoretical units that could be used to discuss the ecological (later ecological-genetic) aspects of intraspecific variation" (Hagen 1984, p. 253). Turesson was a talented writer and skillfully argued his opinions in text. Early in his seminal paper (Turesson 1922a), he cautiously reported: "A few of the cliff individuals were excavated and brought home in order that the reversion to the inland form might be followed more closely. This reversion has not yet taken place, however" (p. 262). But after presenting overwhelming data from dozens of species showing clear parallel patterns of genetically-based differences corresponding to particular habitats he confidently states: "To take refuge in the Lamarckian view of the origin of the characteristics in question seems wholly futile" (p. 332).

Having confirmed genetically based differences among groups of plants growing in particular habitats, Turesson (1922b) coined the term *ecotype* as way to refer to a set of ecologically distinct populations. Ecotype is now a commonly used among biologists, although Turesson's vague definition led to multiple contemporary meanings as well as multiple predictions of the demise of its usage (Faegri 1937; Heywood 1959; Langlet 1971; Quinn 1978; Linhart & Grant 1996). In the same paper, he also employed the term *ecospecie*s (Turesson 1922b). To Turesson, ecospecies were a widespread group of ecotypes that had a common set of ecophysiological traits. For example, he considered multiple ecotypes growing along the coast of Sweden, whether from dunes, bluffs, or cliffs, as one ecospecies.

Turesson's work established foremost that phenotypic differences among populations derived from particular ecological regions were not due to chance. Instead, these genetic differences might actually be due to adaptations to specific sets of environmental factors. Jens Clausen, a Danish botanist, was one of the first supporters of Turesson ideas (Clausen 1922) and after moving to United States, would develop a research program that would prove widespread local adaptation of plant populations.

Table 1: Examples of trait divergence between coastal and inland ecotypes grown under common environmental conditions.

Species	Location	Coastal ecotype traits	Inland ecotype traits	Reference
Achillea borealis	Western North America	Compact short stature Late flowering	Tall stature Early Flowering	Clausen 1951, p. 43
Achillea lanulosa	Western North America	Compact short stature Late flowering	Tall stature Early Flowering	Clausen 1951, p. 43
Achillea millefolium	California	Compact short stature	Tall stature	Clausen 1951, p. 51
Agrosti stolonifera	Great Britain	Less salt retained on leaves	More salt retained on leaves	Ahmad & Wainwright 1976
Armeria vulgaris	Sweden/Norway	Late flowering Thick leaves	Early flowering Thin leaves	Turesson 1922
Geranium robertianum	Great Britain	Prostrate growth Red stems	Erect growth Green stems	Baker 1957
Gilia capitata	California	Short stem Late flowering	Long stem Early flowering	Grant 1950, 1952 Nagy & Rice 1997
Hemizonia multicaulis	California	Short central stem Short internodes No central leader	Long central stem Long internodes Central leader	Clausen & Hiesey 1958 p. 205
Hemizonia paniculata	California	Short central stem Short internodes	Long central stem Long internodes	Clausen & Hiesey 1958 p. 205
Hieracium umbellatum	Sweden	Prostrate growth Thick leaves	Erect growth Thin leaves	Turesson 1922a
Hordeum spontaneum	Israel	Late flowering Low relative growth rate Larger seeds	Early Flowering High relative growth rate Smaller seed	Verhoeven et al. 2008
Layia chrysanthemoides	California	Prostrate growth Late flowering No central leader Short internodes Smooth akenes	Erect growth Early Flowering Central leader Long internodes Hairy akenes	Clausen 1951, p 68-71
Layia platyglossa	California	Prostrate growth Late flowering No central leader Succulent	Erect growth Early Flowering Central leader Not succulent	Clausen 1951, p 61-68 Clausen et al. 1947
Matricaria indora	Sweden	Perennial Prostrate growth Isolatral leaves Thick fleshy leaves Short blunt leaves	Annual Erect growth Palisade cells only on lower side Thin leaves Long segmented leaves	Turesson 1922a
Melandrium rubrium	Sweden	Thick leaves	Thin leaves	Turesson 1925
Melandrium rubriun	Sweden	Succuelent leaves	Thin leaves	Turesson 1922a
Mimulus guttatus	Western North America	Prostrate growth Perennial Late flowering Large flowers Short internodes	Erect growth Both annual and perennial Early flowering Small flowers Long internodes	Vickery 1952 Clausen & Hiesey 1958 p. 214-217 Hall & Willis 2006 Lowry et al. 2008
Nigella arvensis	Israel	Prostrate growth Rounded lobed leaves	Erect growth Linear dissected leaves	Waisel 1959
Plantago major	Sweden	Robust growth form	Diminutive growth form	Turesson 1925
Rumex crispus	Great Britain	Late flowering Dense inflorescences Few flowering stems	Early flowerng Sparse inflorescences Many flowering stems	Akeroyd & Briggs 1983
Sedum maximum	Sweden	Short stature	Tall stature	Turesson 1922a
Silene cucubalus/maritama	Northwestern Europe	Prostrate growth Late Flowering	Erect Growth Early Flowering	Masden-Jones & Turrill 1928
Solanum dulcamara	Sweden	Thick fleshy leaves Hairy leaves	Thin leaves Smooth leaves	Turesson 1922a
Solidago sempervirens/rugosa	Eastern North America	Succulent leaves Large rosette leaves More disk florets per head Large seeds More auxin	Thin leaves Smaller rosette leaves Less disk florets per head Small seeds Less auxin	Goodwin 1937a,b,c
Spiraea ulmaria	Sweden	Short stature Thick leaves	Tall stature Thin leaves	Turesson 1925
Viola tricolor	Denmark	Prostrate growth Perennial Succulent leaves Purple stems No central leader Thick leaves	Erect growth Annual Thin leaves Green stems Central leader Thin leaves	Clausen 1951, p 55-57

1.2 Stages in the evolution of plant species

In the 1930s, Clausen assembled an interdisciplinary collaboration with William Hiesey and David Keck at the Carnegie Institution at Stanford University. The goal was to gain a comprehensive understanding of the genetics, ecology, cytology, physiology, and taxonomy of plant adaptations in California. This work built on a foundation established by Harvey Hall and Frederic Clements, who both pioneered "experimental taxonomy," at Carnegie before the arrival of Clausen in 1931 (Hagen 1984). Unlike Clausen, Clements was an avid Lamarckian and with Hall he contested Turesson's initial findings (Hall & Clements 1923).

Over a 20-year period, Clausen, Keck, and Hiesey examined the adaptive differentiation of numerous plant species through a heroic series of experiments (Clausen et al. 1940, 1947, 1948; Clausen 1951; Clausen & Hiesey 1958). Instead of setting up a single common garden as Turesson had done previously, Clausen, Keck, and Hiesey conducted reciprocal transplant experiments. Here, common garden sites were located in three ecoregions of California: Stanford, a low elevation site in the coastal hills; Mather, a mid-elevation (1400m) site in the Sierra Nevada foothills; Timberline, an alpine site (3000m) high in the Sierra Nevada Mountains. Plants from populations across California were transplanted into all three of the field sites. In this way, Clausen, Keck, and Hiesey were able to compare the morphological, physiological, and fitness responses of different populations across distinct environments.

Most importantly, the reciprocal transplant experiments allowed for direct tests of whether local populations were adapted to the habitats from which they derived. After Clausen, Keck, and Hiesey, the gold standard to test local adaptation would remain the reciprocal transplant experiment and the results of their studies would serve

as confirmation of many of the ideas developed during the modern synthesis (Hagen 1984).

While Clausen, Keck, and Hiesey accomplished a remarkably in depth analysis of adaptation in *Potentilla* and *Achillea*, the greatest strength of their research, like Turesson, was the repeatability of findings over a broad range of taxonomic groups. Across plant families they found the parallel evolution of coastal, inland, and alpine ecotypes. Instead of calling these widespread groups ecotypes or ecospecies, Clausen (1951) distinguished them as *ecological races*. Early in "Stages" Clausen (1951, p. 9) stated his belief that "the ecological race is now commonly recognized as a basic element in evolution."

Clausen, Keck, and Hiesey conducted numerous genetic crosses among different ecological races, in some of the first attempts to deconstruct the genetic basis of differentiation of races collected from the wild. Without modern QTL mapping (Lander & Botstein 1989), actual determination of the genetic architecture was not possible. However, Clausen, Keck, and Hiesey were able to determine the relative complexity of different traits based on how frequently parental phenotypes were recovered in hybrids. Crosses among populations within particular environmental regions were used to test whether ecological races were cohesive genetic groups. For example, they used genetic crosses to evaluate whether there was a common genetic basis to the prostrate late-flowering maritime populations of *Layia platyglossa*, which are found to grow in scattered colonies for nearly 500 kilometers along the California coast (Fig. 2). In a cross between a pair of these coastal populations separated by 225 kilometers, they found all of the F1s and 1400 F2s retained the late-flowering, succulent, prostrate growth without a central leader stem (Clausen et al. 1947). Since no inland phenotype was recovered in this cross, it was concluded that the maritime race may have arisen only once and then

spread along the coast of California or, as Clausen (1951) deemed more plausible, that the maritime race was ancestral to the inland populations of *L. platyglossa.*

Figure 2: Differences among coastal (left) and inland (right) ecotypes of *Layia platyglossa*. The F1 hybrid of the ecotypes is in the center. Hybrids between distant coastal populations all resulted in progeny with the prostrate stature (left).

While Clausen often emphasized the role of adaptation across the landscape in the formation of the initial stages of speciation, he also pointed out numerous situations where postzygotic barriers were the dominant form of reproductive isolation. Clausen, Keck, and Hiesey discovered many genetic incompatibilities at various taxanomic levels. Amazingly, to this day, their work combined with that of their student Vickery (1978), on the geographic distribution of incompatibilities remains remarkably unparalleled accomplishment for North American plants.

1.3 Controversy over stages in the evolution of species

When Turesson used the terms ecotype or ecospecies and Clausen, Keck, and Hiesey used the term ecological races, they were referring to their belief in the existence of a level of organization below the species level but above that of the individual population. However, this original concept of ecotype differed from the idea of

subspecies because the former was defined by their differential response to environmental conditions while the latter was based primarily on morphological differentiation (Stebbins 1950).

Animal evolutionary biologists of the same period also supported the idea of ecotype. For example, Dobzhansky (1951) defined races as "mendelian populations of a species, which differ in the frequencies of one or more genetic variants, gene alleles, or chromosomal structure" (p. 138) and noted that "most races are ecotypes in the Turesson's sense" (p. 147). For Dobzhansky, races were also stages in the process of speciation: "A race becomes more and more of a 'concrete entity' as the process goes on; what is essential about races is not their state of being but that of becoming. But when the separation of races is complete, we are dealing with races no longer, for what have emerged are separate species" (p. 177).

However, the concept of an intermediate in the stages of speciation was controversial from the beginning (Faegri 1937; Turrill 1946; Heywood 1959; Langlet 1971; Raven 1976). Several factors led plant evolutionary biologists to question the validity of terms like ecotype and ecological race. For example, during this period, the biological species concept (Dobzhansky 1935; Mayr 1942) gained wide acceptance. While the biological species concept created an experimentally verifiable mechanistic definition of species (Coyne & Orr 2004), it also created a conundrum of how to deal with intermediates in the process of speciation (Mallet 2008). The core problem is that while most biologists would agree that reproductively isolated species are preceded in time by partially reproductively isolated types, many have balked at classifying those intermediate types, even though this may be necessary if one is to study the stages over which species form. The plant experimental taxonomists (Turesson 1922a,b; Gregor et al. 1936; Clausen 1951), while scorned for creating a complex scheme of classification

(Faegri 1937; Turrill 1946; Ehrlich & Raven 1969; Langlet 1971), did so out of their perceived necessity to deal with the inherent gradual progression in formation of species.

While Mallet (2007, 2008a,b) has recently pointed to the existence of stages in the formation of species as reason to reject the biological species concept, it does not appear that experimental taxonomists had such an extreme interpretation. For example, the definitions that Gregor et al. (1936) provided for ecotype, ecospecies, and cenospecies were all based on reproductive isolation. Clausen et al. (1939) presented similar views on speciation occurring through reproductive isolating barriers. A decade later, Clausen (1951) fully fleshed out his theory of speciation based on the accumulation of isolating barriers over multiple stages. However, in contrast to the biological species concept, where speciation can be completed by any combination of barriers (Mayr 1942; Dobzhansky 1951; Coyne & Orr 2004), both Gregor et al. (1936) and Clausen (1951) appear to have seen the process being completed only through the eventual formation of complete postzygotic reproductive isolation (i.e. different cenospecies). Clausen (1951) further suggested the term *comparia* (p. 173) as a final stage to refer to groups of plants that can no longer ever form an F1 hybrid.

Many who rejected the biological species concept held even greater contempt for the term ecotype (Faegri 1937; Ehrlich & Raven 1969; Raven 1976). Classic taxonomists felt threatened both by the ecotype and biological species concept because both were predicated on the argument that experimentation was necessary for classification. Raven (1976) in particular hated the idea that experiments might be used as a method to classify groups of populations and declared: "The period of 1935-1960 in particular was marked by a 'conflict of categories' in which some workers attempted to substitute experimental criteria for morphological-ecological ones in plant classification, and we are not yet completely free of the effects of this confusing and naïve effort" (p. 288).

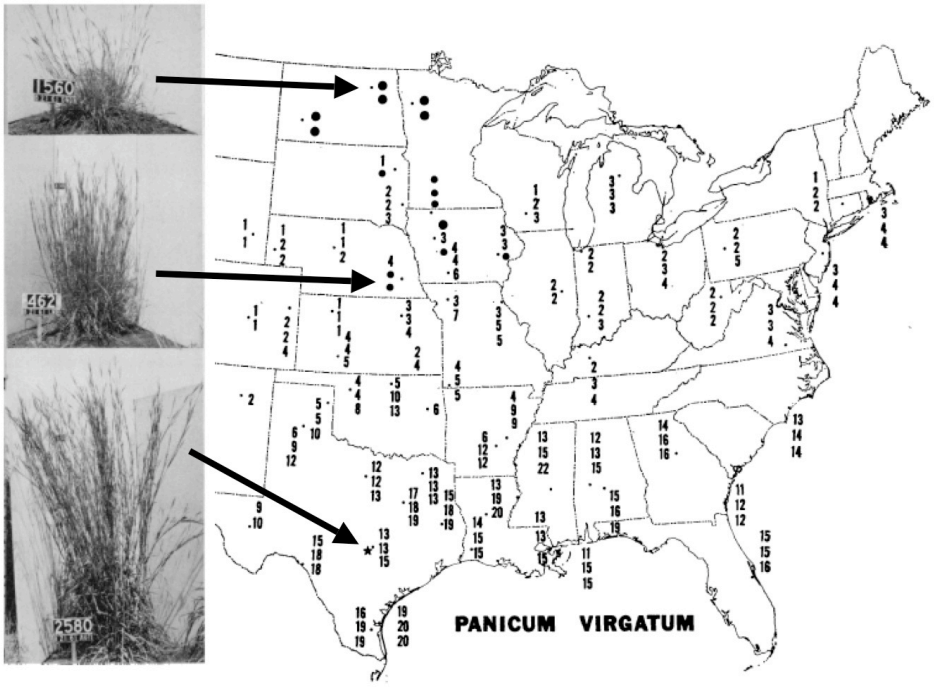

Figure 3: Clinal distribution of flowering time in *Panicum virgatum* collected across the eastern United States and transplanted to Austin, TX. First week of flowering is listed on the map with week 1 being April 27 – May 3; week 10 was June 29 –July 5; week 22 was September 21 – 27, 1961. Photos are of plants collected from Devil's Lake, ND (top), Lincoln, NB (middle), and Austin, TX (bottom). Figure is adapted from McMillan (1965a, 1969).

Most importantly, other plant evolutionary biologists found results inconsistent with the concept of ecotype. Over the second half of the 20th-century it became clear that the distribution of adaptive genetic variation within a plant species could range from a smooth distribution over a cline to extremely discrete. The classic works by Gregor (1930, 1938) on the distribution of phenotypic variation of *Plantago maritima* along the coastline of Great Britain, Langlet (1936, 1971) studies of the Scots Pine (*Pinus sylvestris*), and McMillan's (1959, 1965, 1967, 1969) studies of grasses across the Great Plains of North America (Fig. 3) contrasted with that of Clausen, Keck, and Hiesey. In

these three systems, phenotypic variation was not associated with distinct ecotypic groups, but rather distributed continuously along what Huxley (1938) would label as a *cline*. As a result of his research, Gregor (1944) suggested that *ecoclinal subspecies* should receive taxonomic recognition. In contrast, Langlet (1963, 1971) argued that all plant variation is distributed clinally and that Turesson had received far too much credit for what he believed to be the false idea of ecotype. While acknowledging that Clausen, Keck, and Hiesey were conducting research over much steeper ecological gradients (McMillian 1965b), McMillian (1969) preferred to think of species as being composed of "genetically-based variation that is habitat correlated."

At the opposite extreme, another set of researchers discovered adaptation of plants to extremely local edaphic conditions, such as mine-tailings (Antonovics & Bradshaw 1970) and serpentine outcrops (Kruckeberg 1951). Kruckeberg (1951) found the evidence for discrete serpentine-adapted ecotypes of *Achillea* within the range of ecological races previously documented by Clausen et al. (1948). Given this result Kruckeberg argued that "in light of the case of *Achillea borealis* where edaphic races appear to be superimposed upon climatic races…ecotype seems appropriate only when a single environmental factor is under scrutiny" (p. 415) since analysis under multiple environmental conditions "would render the term 'ecotype' synonymous with either a local population or a small segment of a population" (p. 416). He concludes: "Natural populations might best be visualized as consisting of a continous or discontinuous array of *ecotypic variation* in response to the sum total of environmental factors in an area" (p. 416).

Stebbins (1950), in his grand review "Variation and Evolution in Plant Species" recognized that widespread distinct ecotypes might evolve under some conditions but not others. For example, Stebbins contrasted the high habitat heterogeneity of California to other regions: "In species occupying an area like the eastern United States, which is

comparatively uniform in many climatic characteristics and where a single set of factors, such as temperature and length of the growing season, varies gradually and continuously, continuous or clinal ecotypic variation will be particularly prevalent" (p. 47). Stebbins also translated overlooked later writings of Turesson (1936), who according to Stebbins "pointed out, species with obligate crosspollination, particularly those like pines and other wind-pollinated trees of temperate regions, in which the pollen may be carried through the air for many miles, are most likely to show continuous variation" (p. 47). Stebbins concludes that: "clines and ecotypes are not mutually exclusive concepts" (p. 48).

Many were less balanced than Stebbins, and given the mounting evidence that functional genetic variation within plant species could be distributed continuously or extremely discretely, multiple reviews (Heywood 1959; Langlet 1963, 1971; Quinn 1978) dismissed the utility of the term ecotype. Quinn's (1978) main contention with ecotypes was rooted in his disbelief that widespread ecotypes could ever form since: 1) Quinn believed that near uniform environments were necessary for ecotype formation and argued that such environments are never geographically widespread; 2) gene flow is too low among plant populations to maintain the cohesiveness of widespread ecotypes or species. The second argument is at least partially rooted in the views of Ehrlich & Raven (1969), who upon reviewing the data showing patterns of restricted gene flow among populations argued that gene flow was insufficient to hold species together and thus, the biological species concept itself was flawed. Instead they concluded: "For sexual organisms it is the local interbreeding population and not the species that is clearly the evolutionary unit of importance" (p. 1231).

In response to the arguments that widespread ecotypes or species cannot persist due to low levels of cohesive gene flow, Grant (1981, p. 91) reasoned that "extensive interbreeding within the population system is not an essential property of biological

species; non-interbreeding with other population systems is." In other words, it does not make sense to make arguments about what holds a species together when reproductive isolating barriers, geography, or a combination of the two are strong enough to keep ecotypes and species from falling apart. Grant then goes on to write (p. 92), "Biological species represents a stage in divergence…and other stages of uncompleted speciation and secondary refusion of species also exist. Consequently the array of population systems at any given time consists of both biological species and semispecies." Thus, Grant, like Clausen (1951) was a supporter of stages in the formation of plant species. Even so, he did recognize that reproductive isolating barriers, if not complete biological speciation, could evolve at small geographic scales. After all, Grant championed *quantum speciation.*

As plant evolutionary biologists turned their attention away from genecology and toward microevolutionary processes as well as phylogenetics in the 1980s-1990s, interest in the role of ecotypes as stages in the formation of species would wane (Barrett 2001). By the 1990s, the debate on the existence of ecotypes appeared to be settled mostly because of apparent irrelevance. While use of the term ecotype persisted, it was associated with both regional variation and local populations (Briggs & Walters 1997). Linhart and Grant (1996), who conducted the most comprehensive review of local adaptation in the 1990s, suggested that "the cline versus ecotype controversy has not proved particularly useful and it has mostly faded" because "some characters can vary gradually, others discontinuously, depending on, for example, gene flow, intensity of selection, number of genes involved, and terrain configuration" (p. 241). Briggs & Walters (1997), in the final edition of "Plant Variation and Evolution," also found that variation could be distributed in different ways depending on the characteristics of a species and geographic features of its range pointing out that "with hindsight one can see in Turesson's own results the possibility that, in common species, variation patterns

were more complex than the ecotype concept implied" (p. 190). These views are quite balanced and appear to be perfectly valid, but do not provide any insight into how different geographic distribution of genetic variation might affect the process of speciation.

With few plant biologists of the 1990s dedicated to understanding the stages in the evolution of species, Levin (1993, 1995) filled the vacuum with the argument that speciation occurs almost exclusively on the level of the local population or meta-population. His arguments against geographic stages in the formation of species were almost exactly the same as those of Quinn (1978), in his disbelief of widespread uniform environmental conditions and doubts that sufficient gene flow within widespread ecotypes could occur to facilitate their conversion to species. Levin's (1993) viewpoints also have deep roots in peripatric founder effect speciation (Mayr 1954; Coyne 1994) and quantum speciation (Lewis 1962; Grant 1981). While having a long history, there is still very little evidence for founder effect mechanisms of speciation with other explanations almost always more parsimonious (Barton & Charlesworth 1984; Willis & Orr 1993; Coyne 1994; Coyne & Orr 2004; Gottlieb 2004). Arguments for founder effect, quantum, and local speciation are based on the assumption that underdominant chromosomal rearrangements are the most significant source of reproductive isolation among species and somehow massive rearrangements rapidly occur in bottlenecked populations. For example, Levin (1993) argued that widespread ecotypes could not be converted to good species because it would be difficult for underdominant rearrangements to spread. However, all recent reviews on plant speciation do not support a major role for the involvement of underdominant chromosomal rearrangements in speciation (Rieseberg 2001; Rieseberg & Willis 2007; Lexer & Widmer 2008; Lowry et al. 2008b; Bomblies 2010; Sobel et al. 2010). Even classic examples of

quantum speciation, such as *Layia discoidea*, do not appear to be associated with rapid chromosomal evolution (Gottlieb 2004; Baldwin 2005).

While disdain for the term ecotype has waxed and waned through the decades, studies in the last fifteen years on the role of ecology in speciation appear to support existence of ecotype as it was originally conceived. For example, Gregor et al. (1936) provided a definition for ecotype as "a population distinguished by morphological and physiological characters, most frequently of a quantitative nature; interfertile with other ecotypes and ecospecies, but prevented from exchanging genes by ecological barriers." Interestingly, Gregor's definition of ecotype is similar to the recently coined term *ecological speciation* (Schluter 1996), which was defined by Rundle & Nosil (2005) as "the process by which barriers to gene flow evolve between populations as a result of ecologically-based divergent selection." Ecological speciation does differ from Gregor's definition of ecotype, in that it includes postzygotic barriers that are driven by ecological selection. Even so, the concept of ecological speciation appears to be similar to the process by which ecotypes were originally thought to form. And while Clausen (1951) believed that "the most normal pattern of speciation…is a gradual separation in morphologic, ecologic, genetic, and cytologic characteristics" (p. 90) he did highlight "examples of pollinating systems in wild plants that may keep natural entities distinct morphologically even though there are no genetic barriers. Such internal genetic barriers are not needed to keep inheritances apart when selection by external pollinating agents is constantly at work" (p. 93). Thus, it appears that Clausen believed complete reproductive isolation could occur due strictly to ecological barriers.

Beyond renewed interest focused on the role of ecology in speciation, the recent expansion of genetic tools to numerous systems coupled with new methodologies to analyze geographic patterns of population structure (Pritchard et al. 2000; Dyer & Nason 2004; Novembre et al. 2008; Novembre & Stephens 2008) have revived interests

in understanding how genetic variation is partitioned within plant species (Nordborg et al. 2005; Lowry et al. 2008a; Song et al. 2009). The increase in molecular capacity led Baldwin (2006) to conclude: "Although infeasible during Clausen's life, resolving 'ecological races' that correspond to natural groups worthy of taxonomic recognition is now possible and desirable" (p. 87). No longer do ecotypes have to be defined solely by experimentation. Researchers can now readily use phylogeographic methods or population structure analyses to detect partially reproductively isolated groups within a species. Follow-up experiments can then be used to determine the mechanisms that underlie reproductive isolation.

Given all of the predictions of its demise (Heywood 1959; Langlet 1971; Quinn 1978), the term ecotype persists in the literature much in the same way it was defined by Turesson (1922b) and Gregor (1936). Ecotype is an intraspecific group of individuals that are partially reproductively isolated from other groups by ecological, ecophysiological, or ecogeographic barriers, which have evolved in response to adaptation to local environmental conditions. Basing this term on reproductive isolation means that it can correctly be used to describe various organizations of genetic variation all the way from the widespread climatic ecological races of Clausen, Keck, and Hiesey to a section of a population subdivided by a steep edaphic transition. Without sharp environmental transitions, partially reproductively isolated distinct ecotypes are unlikely to form (Stebbins 1950; Briggs & Walters 1997). This is particularly true for gentle clines of wind-pollinated species such as those studied by McMillan (1959), Langlet (1971) and Quinn (1978). Finally, the term ecotype should not be seen as threat to traditional taxonomy or conservation as conveyed by Ehrlich & Raven (1969) and politely suggested by Turrill (1946). Baum (2009) recently echoed this long-standing taxonomic discontent with classification based on the results of experimentation arguing, "that the concept of species be moved, once and for all, out of the realm of

mechanistic evolutionary biology and kept fully within systematics" (p. 85). However, it is obvious that taxonomy for the purpose of conservation would be impossible if classification had to be based on experimentation; doing so would be as foolhardy as Borges' cartographers from the short story "On the exactitude of science" (Davis 1946). The goal of experimentation is to determine the mechanisms by which reproductively isolated groups form, which is ultimately the heart of the species question. Hence, ecotype is a functional term to describe the distribution of adaptive genetic variation over space and recognition of the existence of partially reproductively isolated groups as stages in the formation of species.

1.4 Deconstructing local adaptation of widespread ecotypes

One of the major arguments against the role of widespread ecotypes in the formation of species is the disbelief that alleles involved in adaptation might be restricted between the habitats in which alternative ecotypes occur (Langlet 1971; Quinn 1978; Levin 1993). The fixation of adaptive alleles between ecotypes was proposed long ago. This is most clearly seen in the writings of Gregor et al. (1936), who immediately after defining ecotype (above) wrote: "Spatially widely separated ecotypes may exhibit characters determined by genes restricted to the geographical regions in which they occur." Decades later, we still have little understanding whether or not Gregor's hypothesis regarding the distribution of adaptive alleles between ecotypes is correct.

There are indeed many reasons why allelic variation might not be fixed between habitats. First, adaptive phenotypes frequently have a complex genetic basis and thus, small shifts in allele frequencies across multiple loci can contribute to major changes in trait divergences without fixation of adaptive alleles (Goldstein & Holsinger 1992; Brookfield 1997; Kelly 2006; Novembre & Rienzo 2009). Second, if gene flow does occur

between diverging ecotypes, fixation of adaptive alleles could be prevented by migrational load (Hendry 2009). However, one of the most overlooked reasons that alleles may not be fixed between ecotypes is that local adaptation may not be caused by trade-offs (i.e. antagonistic pleiotropy) at underlying loci (Fry et al. 1998; Verhoeven et al. 2004, 2008; Gardner & Latta 2006; Lowry et al. 2009).

Local adaptation is most often defined as pair-wise comparison between populations where local individuals outperform foreign individuals in both of their respective habitats (Clausen & Hiesey 1958; Linhart & Grant 1996; Kawecki & Ebert 2004; Mitchell-Olds et al. 2007; Hereford 2009). Many models, especially those evoking a cost of adaptation, assume that the overall pattern of local adaptation is also caused by local alleles at individual loci outperforming foreign alleles across habitats (Levene 1953; Hedrick 1986; Gillespie & Turelli 1989; Turelli & Barton 2004). While this makes sense from a theoretical perspective, an overall pattern of local adaptation could also result from the summation of loci that contribute to a fitness advantage in one habitat, but have no effect on fitness in alternative habitats (i.e. conditional neutrality). Indeed, while antagonistic pleiotropy has been identified across environments under laboratory conditions (Sari-Gorla et al. 1997; Jiang et al. 1999; Leips & Mackay 2000; Hawthorne & Via 2001), conditional neutrality appears to be far more common (Hayes et al. 1993; Lu et al. 1996; Alonso-Blanco et al. 1998; Fry et al. 1998; Courtois et al. 2000; Saranga et al. 2001; Teulat et al. 2001; Johnson & Gepts 2002; Xing et al. 2002; Weinig et al. 2003; Verhoeven et al. 2004, 2008; Gardner & Latta 2006). This high frequency of conditional neutrality could be an artifact of laboratory and agricultural experiments, which are largely conducted with the manipulation of one environmental factor. It is possible that antagonistic pleiotropy may be more common under natural field conditions where multiple factors are involved.

Given its importance to my understanding of adaptation, surprisingly few studies have actually examined individual loci in field reciprocal transplant studies (Table 2). As of 2010, I have only identified three such study systems (*Hordeum spontaneum* (Verhoeven et al. 2004, 2006), *Avena barbata* (Garner & Latta 2006; Latta 2009), *Mimulus guttatus* (Lowry et al. 2009; Hall et al. *in review*)) and have yet to find a compelling example of antagonistic pleiotropy across natural habitats. However, only one of those systems (*Mimulus*) evaluated the effects of loci across habitats in ecotypes that were unambiguously locally adapted (Verhoeven et al. 2004, 2008; Hall & Willis 2006; Lowry et al. 2008a, 2009; Latta 2009).

Table 2: Results of reciprocal transplant QTL studies. Fitness QTLs classified into three categories based on effects across habitats.

Species	Anatagonic pleiotropy	Conditional neutrality	Universal superiority	Colocalizing traits	Reference
Hordeum spontaneum	0	11	3	Flowering time Relative Growth rate	Verhoeven et al. 2004 Verhoeven et al. 2008
Mimulus guttatus	0	7	0	Salt tolerance Flowering time	Lowry et al. 2009 Hall et al. *in review*
Avena barbata	0	2	2	None	Gardner & Latta 2006 Latta 2009

If conditional neutrality is the dominant mechanism underlying local adaptation, then the implications would be wide ranging. Antagonistic pleiotropy is often thought to be involved in the maintenance of intraspecific variation through habitat-mediated balancing selection (Levene 1953; Gillespie & Turelli 1989; Mitchell-Olds et al. 2007) and an assumption behind interpretations of genomic scans (Nosil et al. 2009). Models of sympatric speciation also often rely on antagonistic pleiotropy (Kondrashov & Kondrashov 1999; Fry 2003; Gaverlets & Vose 2005, 2007) although Kawecki (1997) showed that ecological race formation could occur under conditional neutrality.

Unfortunately, the lack of field QTL studies means that I still have little idea as to what mechanisms underlie local adaptation of widespread ecotypes.

1.5 The role of chromosomal rearrangements

Chromosomal rearrangements have long been thought to play a role in formation of species. The role of underdominant rearrangements in causing reproductive isolation through hybrid sterility caused by the formation of aneuploid gametes in heterozygotes at meiosis was emphasized in the past (Stebbins 1958; White 1978; King 1993; Levin 1993). While experiments involving "the chromosome doubling test," suggest that a role for rearrangements in plant hybrid sterility (Stebbins 1958; Rieseberg 2001; Coyne & Orr 2004), recent studies have also found sterility to be caused by genic factors (Fishman & Willis 2001; Moyle & Graham 2005; Li et al. 2007; Bomblies 2009, 2010). Further, contemporary plant evolutionary biologists have argued that underdominant rearrangements may play a limited role in speciation because they only cause one type of isolating barrier, hybrid sterility (Lowry et al. 2008b; Lexer & Widmer 2008; Sobel et al. 2010). Further, models of speciation based on the spread of underdominant rearrangements are problematic because they cannot easily spread since heterozygotes are at a disadvantage (Coyne & Orr 2004; Hoffmann & Rieseberg 2008). Therefore, fixation of underdominant rearrangements is only likely in bottlenecked populations (Lande 1979, 1985; Levin 1993).

Recently, an emphasis has been placed on the role of suppression of recombination caused by rearrangements (Noor et al. 2001; Rieseberg 2001; Ortiz-Barrientos et al. 2002; Navarro & Barton 2003; Kirkpatrick & Barton 2006). By suppressing recombination inversions create long regions of linkage disequilibrium that can trap genetic changes, including adaptive genetic changes and loci that contribute to postzygotic isolation through Dobzhansky-Muller type interactions (Rieseberg 2001;

Noor et al. 2001; Hoffmann & Rieseberg 2008). Dobzhansky (1970) suggested a role for suppressed recombination in facilitating the coadaptation of epistatically acting loci. However, Kirkpatrick & Barton (2006) demonstrated with a recent theoretical model that even fully additive linked loci could influence the spread of an inversion that holds adaptive haplotypes together. Regardless of the mechanism, inversions are thought to contribute to adaptive evolution.

The best evidence for distribution of adaptive alleles being distributed by local adaptation comes from classic studies of the distribution of chromosomal rearrangement polymorphism in *Drospohilia* and other insect species. Dobzhansky's (1951) early work documented distributions of chromosomal inversions distributed clinally along the same altitudinal transect in California as that found for plant variation by Clausen, Keck, and Hiesey. Similar altitudinal patterns were also found around the same time in the Great Smoky Mountains (Stalker & Carson 1948). With such short generation times, changes in allele frequencies can change rapidly in *Drosophilia*. Interestingly, both traits and inversion polymorphisms have been observed to change in frequencies over the course of a season (Dubinin & Tiniakov 1945, 1946; Stalker & Carson 1949). Multiple recent studies have even linked the distribution of inversion polymorphism to human-induced global climate change, with chromosomal orientations found at high frequency in warm climates spreading to higher latitudes over time (Rodriguez-Trelles & Rodriguez 1998; Anderson et al. 2005; Umina et al. 2005; Balanya et al. 2006; Etges et al. 2006). As an explanation for the clinal distribution of rearrangements, laboratory experiments have found effects of inversions on stress tolerance such as desiccation, heat, and cold (reviewed in Hoffmann & Rieseberg 2008).

While clinal patterns of inversion polymorphism have also been observed in many plant species (Reviewed in Levin 2002), there is far less evidence for a role of rearrangement polymorphism in plant adaptation. Stebbins (1950) found no such

examples in his survey of the early literature. Lewis (1953) claimed that chromosomal rearrangements in *Clarkia lingulata* were holding together coadapted gene complexes that were intimately involved in quantum speciation from its parental species *C. biloba*. However, twenty years later, Lewis (1973) admitted that *C. lingulata* was no more adapted to its local habitat conditions than *C. biloba* (Gottlieb 2004). Recently, Hoffmann & Rieseberg (2008) found many examples of inversions being linked to potentially adaptive traits, but not one of these was from a plant species. Most importantly, field experiments have yet to be conducted in any biological system to determine the relative contribution of inversions to local adaptation and reproductive isolation.

Since inversions are thought to play a role in adaptive evolution they are also hypothesized to be involved in the formation of ecotypes (Stebbins 1950; Dobzhansky 1951; Kirkpatrick & Barton 2006). Coluzzi (1982) even coined the term *ecotypificaiton* to describe the process whereby the spread of an inversion contributes to the formation of ecotypes in peripheral populations. But is there any reason to predict that inversions might often be involved in the formation of distinct ecotypes? Perhaps, inversions could play a major role if they cause patterns of antagonistic pleiotropy at higher rates than for individual genes. As seen above, conditional neutrality appears to be a more common type of genotype x environment interaction than antagonistic pleiotropy. Inversions that facilitate the linkage of adaptive changes would be more likely to cause pleiotropic effects than individual genes, including antagonistic pleiotropy across habitats. If inversions were more likely to lead to antagonistic pleiotropy, they would also be more likely to be fixed among diverging ecotypes. Thus, the interrelationship of recombination, adaptation, and ecogeography could play a major role in the observed pattern that sister species frequently differ by many chromosomal rearrangements.

1.6 Resurrecting "Stages": Coastal and inland ecotypes of Mimulus guttatus

In chapter 2 of this thesis, I use a combination of population genetic analysis, common garden experiments, and reciprocal transplant experiments to test whether or not coastal perennial populations fit the predictions of Clausen (1951) for the existence of widespread ecotypes. The combined evidence of morphological structure (Fig. 8), population genetic structure (Fig. 9), and quantification of reproductive isolating barriers (Table 10) suggests that a widespread distinct coastal perennial ecotype does exist. However, it should be noted that variation is also distributed along a cline within the coastal ecotype of *Mimulus guttatus* (Fig. 4, 5a), just as was found by Gregor (1930, 1938) for the coastal species *Plantago maritima*. Clausen, Keck, & Hiesey (1948) recognized similar genetic variation with the coastal climatic race of *Achillea borealis*, finding plants collected from north of the San Francisco Bay to be shorter in stature than those from farther south in California (Fig. 5b). These observations are very relevant to the arguments of Langlet (1971), Quinn (1978), and Levin (1993) because it suggests that: 1) widespread distinct ecotypes do exist within species, 2) clines can occur within these widespread ecotypes, and thus, 3) widespread uniformity of all environmental factors is not necessary for the formation and maintenance of distinct ecotypes.

In chapter 3, I evaluate the role of antagonistic pleiotropy in local adaptation. To address this question, I conducted experiments to further deconstruct the physiological and genetic mechanism of high salt tolerance of the coastal ecotype. First, I conducted a physiological study to determine why the coastal ecotype is more salt tolerant. The results of this experiment suggest that cell-based leaf tissue tolerance is the key coastal adaptation. I then used QTL mapping to identify loci involved with tissue tolerance to salt spray. Finally, I reanalyzed data from a previous reciprocal transplant experiment that incorporated genotyped recombinant inbred lines (RILs). This allowed

me to determine the effects of three salt spray QTLs across coastal and inland habitats. Interestingly, all three QTLs had significant effects on local adaptation in coastal habitat and not inland habitat, a pattern consistent with conditional neutrality and not antagonistic pleiotropy.

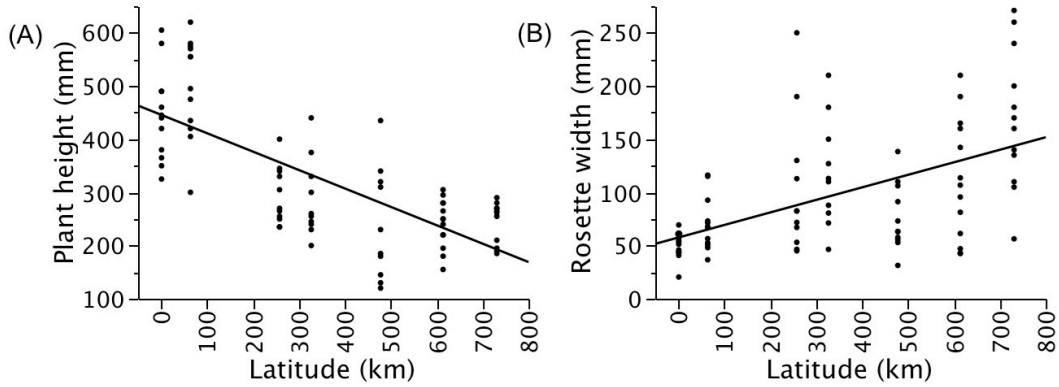

Figure 4: Clinal distribution of traits within the widespread coastal ecotype of *Mimulus guttatus*. (A) Plant height ($R^2 = 0.519$, $P < 0.0001$), (B) Rosette width ($R^2 = 0.266$, $P < 0.0001$). Latitude in kilometers north of San Luis Obispo, CA.

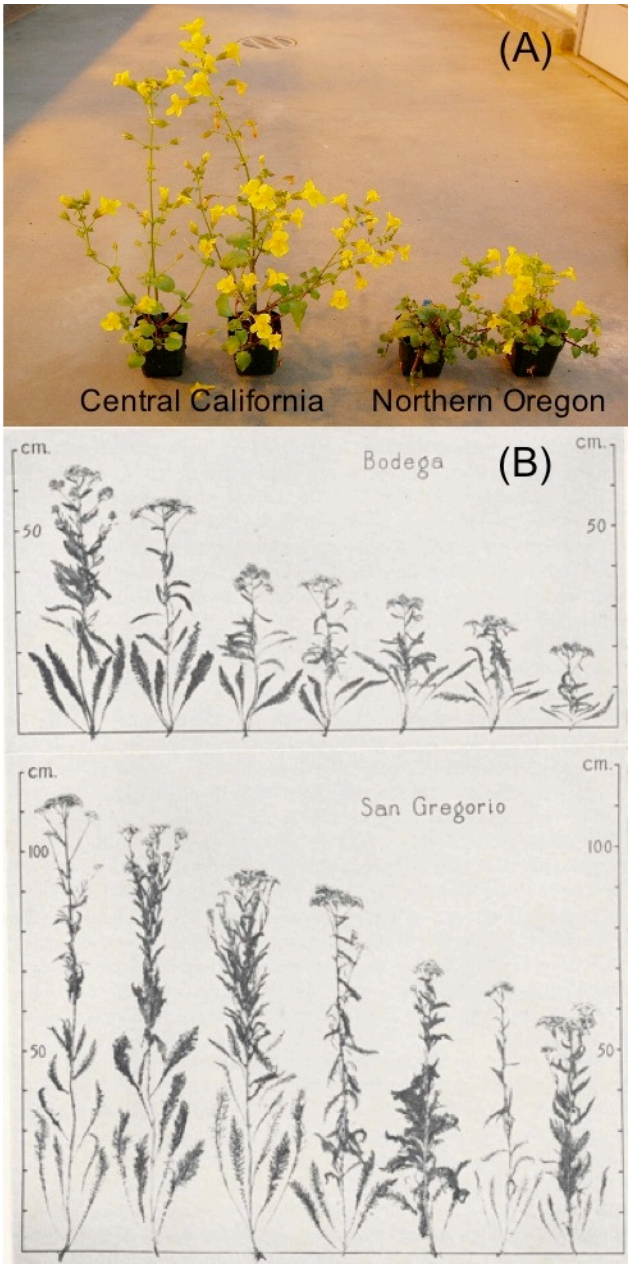

Figure 5: Genetic variation within coastal ecotypes. Differences in morphology of (A) *Mimulus guttatus* 10 days after date of first flower for plants grown at the Duke University greenhouses and (B) *Achellia borealis* grown in a common garden at Stanford (from Clausen et al. 1948). In both species, plants derived from populations north of the San Francisco Bay (Oregon, Bodega) were shorter in stature than those of southern populations (Central California, San Gregorio).

While Chapter 2 provides evidence for the existence of a widespread coastal perennial ecotype of *M. guttatus*, it does not necessarily follow that the same adaptive

genetic variation is responsible for divergence between coastal and inland ecotypes across their range. As mentioned above, Clausen et al. (1947) did use genetic crosses within the coastal ecotype of *Layia* to argue that its morphological distinctness from the inland ecotype was due to the same set of genes. However, Clausen (1951) interpreted this result as evidence that the coastal ecotype was the ancestral form. In chapter 4 of this thesis, I used replicated QTL analysis to demonstrate that the same locus is responsible for the genetic divergence of the coastal perennial ecotype from the inland ecotype over their range. Further examination revealed that this divergence QTL colocalizes with a widespread chromosomal inversion that appears to be fixed between coastal perennial and inland annual populations. I then demonstrate that the inversion contributes to local adaptation and the annual/perennial life-history divergence between the ecotypes. However, the inversion only accounts for about 20% of the local adaptation of inland annual habitat. Thus, adaptive divergence between the distinct ecotypes is due to polygenic natural variation. This result also conflicts with Levin's perspective on species formation, where the role of polygenetic variation in the formation of species was dismissed. Further, the coastal perennial orientation of the chromosomal orientation was found in inland perennial populations, which suggests 1) that this chromosomal inversion polymorphism is a source of widespread standing genetic variation and 2) when combined with the population genetic data from chapter 2, which found that the coastal populations are derived from the core variation of *M. guttatus*, it appears that the perennial inversion orientation may have been involved as a seed for the formation coastal ecotype. If this were indeed true, then coastal ecotype would at least partially be the product of standing genetic variation and thus, not derived by a mechanism consistent with local speciation.

While conducting QTL analysis in conjunction with reciprocal transplant studies is a challenging and time-consuming enterprise, it is necessary to understand the actual

nature of how local adaptation operates, how ecotypes form, and to deconstruct the stages in the process of speciation. With regard to questions such as the population genetics of local adaptations and the role of antagonistic pleiotropy versus conditional neutrality, we find ourselves curiously in the same position that Clausen (1951, p. 12) did with his research: "Theoretical calculations on the supposed genetic effects of local isolation have been undertaken by Sewell Wright and others, but there are few investigations on record from which one can judge whether or not the facts fit the theories." On page 13, opposite this quote, David Keck is pictured hunched over in the hot California sun out on an exposed alkaline flat collecting seeds for future experiments. This is the only picture of either Clausen, Keck, or Hiesey to appear in their six volumes of collected works and may be a subtle way of suggesting that if we really want to understand local adaptation and the formation of species we need to conduct our research in the field.

2. Ecological reproductive isolation of coastal and inland races of *Mimulus guttatus*

2.1 Introduction

It is commonly observed that recently diverged sister species tend to inhabit environments with different ecological characteristics, and possess habitat-specific adaptations (Mayr 1947; Clausen 1951; Schemske 2000; Coyne and Orr 2004; Angert 2006; Nakazato et al. 2008). What is not always clear is the extent to which such habitat differentiation contributes to the process of speciation because adaptation to different habitats can influence several potential prezygotic and postzygotic reproductive isolating barriers (Via et al. 2000; Ogden and Thorpe 2002; Rundle 2002; McKinnon et al. 2004; Nosil 2007; for reviews see Schluter 2001; Coyne and Orr 2004; Lexer and Fay 2005; Rundle and Nosil 2005; Hendry et al. 2007). For example, if the different habitats exist only in disparate geographic regions, then the habitat-related adaptations may affect the overall distribution and range limits of sister species, such that the species rarely if ever come into contact with each other (Schemske 2000; Coyne and Orr 2004; Angert and Schemske 2005). Such environmental imposition of an allopatric distribution on sister species is often termed ecogeographic isolation (Mayr 1947; Clausen 1951; Schemske 2000; Ramsey et al. 2003; Husband and Sabara 2004; Kay 2006). If environmental variation exists on a finer spatial scale, then habitat adaptation can cause reproductive isolation between species if immigrants have low viability or fertility (e.g., Nosil et al. 2005). The strength of immigrant inviability and ecogeographic isolation can be tested through reciprocal transplant experiments (Coyne and Orr 2004; Nosil et al. 2005). Indeed, numerous reciprocal transplant experiments conducted over the last 75 years support the role of local adaptation in restricting gene flow between species (Turesson 1922a; Clausen and Hiesey 1958; Linhart and Grant

1996; Wang et al. 1997; Nagy and Rice 1997; Angert and Schemske 2005; Hall and Willis 2006; Rieseberg and Willis 2007).

In addition to ecogeographic isolation and immigrant inviability, adaptation to alternative habitats may contribute to other components of reproductive isolation. It is well known, for example that in plants, sister species or even "ecotypes" adapted to different habitats often evolve differences in flowering time (Clausen 1951; Clausen and Hiesey 1958; Grant 1981). These differences in the timing of reproduction can cause temporal isolation, potentially a strong premating barrier between species (Coyne and Orr 2004; Martin and Willis 2007; Martin et al. 2007; Pascarella 2007; Yang et al. 2007). Even if populations or species adapted to different habitats occasionally interbreed, hybrids may be maladapted to either parental habitat, resulting in extrinsic postzygotic isolation (Wang et al. 1997; Hatfield and Schluter 1999; Rundle and Whitlock 2001; Rundle 2002; Forister 2005; Campbell and Waser 2007). Because of these complex and multifaceted relationships between habitat adaptation and reproductive isolation, the importance of habitat adaptation relative to other potential prezygotic and postzygotic barriers has rarely been quantified (but see Nosil et al. 2005; Nosil 2007; Lowry et al. 2008b). Furthermore, the specific ecological factors driving ecological reproductive isolation are often unknown. The geographic scale at which different stages of speciation occur has remained poorly understood, especially because geographically widespread population genetic analysis is rarely conducted in conjunction with the quantification of reproductive isolating barriers (Levin 1993; Coyne and Orr 2004; Rundle and Nosil 2005).

In this study, I address these issues by examining the extent of genetic divergence, habitat adaptations, population structure, and reproductive isolation between coastal perennial and inland annual forms of the wildflower *Mimulus guttatus*. *Mimulus guttatus* (yellow monkeyflower; Phrymaceae, historically Scrophulariaceae, order Lamiales) is a

highly variable species within the even more diverse *M. guttatus* species complex (Vickery 1978), which is distributed over western North America. Different populations often appear adapted to a multitude of elevational, climatic, and edaphic habitats (Vickery 1978; Wu et al. 2008). Throughout the lower elevation inland meadows, seeps, and rocky outcrops, from the Pacific coastal mountain range of the United States and Canada east to the Rockies, *M. guttatus* populations are typically composed of spindly spring flowering annual plants (Vickery 1952; Clausen and Hiesey 1958; Wu et al. 2008). In adjacent fog-bound cliffs, sand dunes, and coastal terrace that occur within a couple of kilometers of the Pacific Ocean, *M. guttatus* populations are composed of large, robust, late summer flowering perennial plants with compressed internodes (Vickery 1952; Clausen and Hiesey 1958; Hitchcock and Cronquist 1973; Hall and Willis 2006). Based on field collections, the coastal perennial populations have frequently been assigned a separate taxonomic status: *M. guttatus var. grandis* Greene (Hitchcock and Cronquist 1973) and *M. guttatus ssp. litoralis* Pennell (Abrams and Ferris 1951). However, common garden experiments are needed to demonstrate that the morphological distinctness of coastal populations is not due to phenotypic plasticity. Inland perennial forms of *M. guttatus* are also found around permanently moist springs, creeks, and lakes throughout western North America (Vickery 1952; Clausen and Hiesey 1958), but they are not included in the present study.

A recent reciprocal transplant between a pair of coast and inland *M. guttatus* populations from Oregon provides evidence of strong local adaptation to their respective habitats (Hall and Willis 2006). The inland habitat of the coast ranges of California and Oregon experiences a very long (~6 month), hot summer drought, in which moist areas (seeps, creeks, arroyos) that contain annual *M. guttatus* populations completely dry out. Coastal habitat also receives little or no rainfall during summer

months, but persistent fog and cooler temperatures along the coast maintain year-round soil moisture and reduce the rate of plant transpiration (Corbin et al. 2005). However, coastal plants must contend with ocean salt spray, which is a major stress that is known to restrict the distribution of many plant species (Boyce 1954; Barbour 1978; Humphreys 1982; Wilson and Skyes 1999).

Here I report on a series of experiments to understand the evolution of reproductive isolation between coast and inland populations of *M. guttatus*. Using molecular genetic markers and common garden experiments, I ask whether multiple, broadly sampled coast and inland populations constitute two distinct morphological and genetically structured groups. Using additional reciprocal transplant experiments, I determine the extent to which coast and inland populations are locally adapted to their respective habitats. Finally, I combine results from field and greenhouse experiments to estimate the strength of multiple prezygotic and postzygotic barriers between coast and inland populations, and determine the role of adaptations to specific environmental factors in limiting hybridization and introgressive gene flow.

2.2 Materials and Methods

2.2.1 Field collections

In the spring of 2005, plants were collected from 14 latitudinal pairs of coast and inland populations (two extra inland populations, 30 populations total, 20–30 haphazardly collected plants per population) distributed from central California to northern Oregon (Fig. 6, Table 5). Inland populations used in this study were located 4.8–31.4 (mean = 16.7) kilometers from the Pacific Ocean. Coastal plants were all collected within 500 m of the ocean. Collected plants were shipped overnight to Durham, NC, where they were potted, raised to flowering, and self-pollinated in the Duke University greenhouses. Selfed seeds and tissue for DNA extraction were collected

from these plants. Tissue was placed into 96-well Costar Plates and immediately deposited into an −80°C freezer.

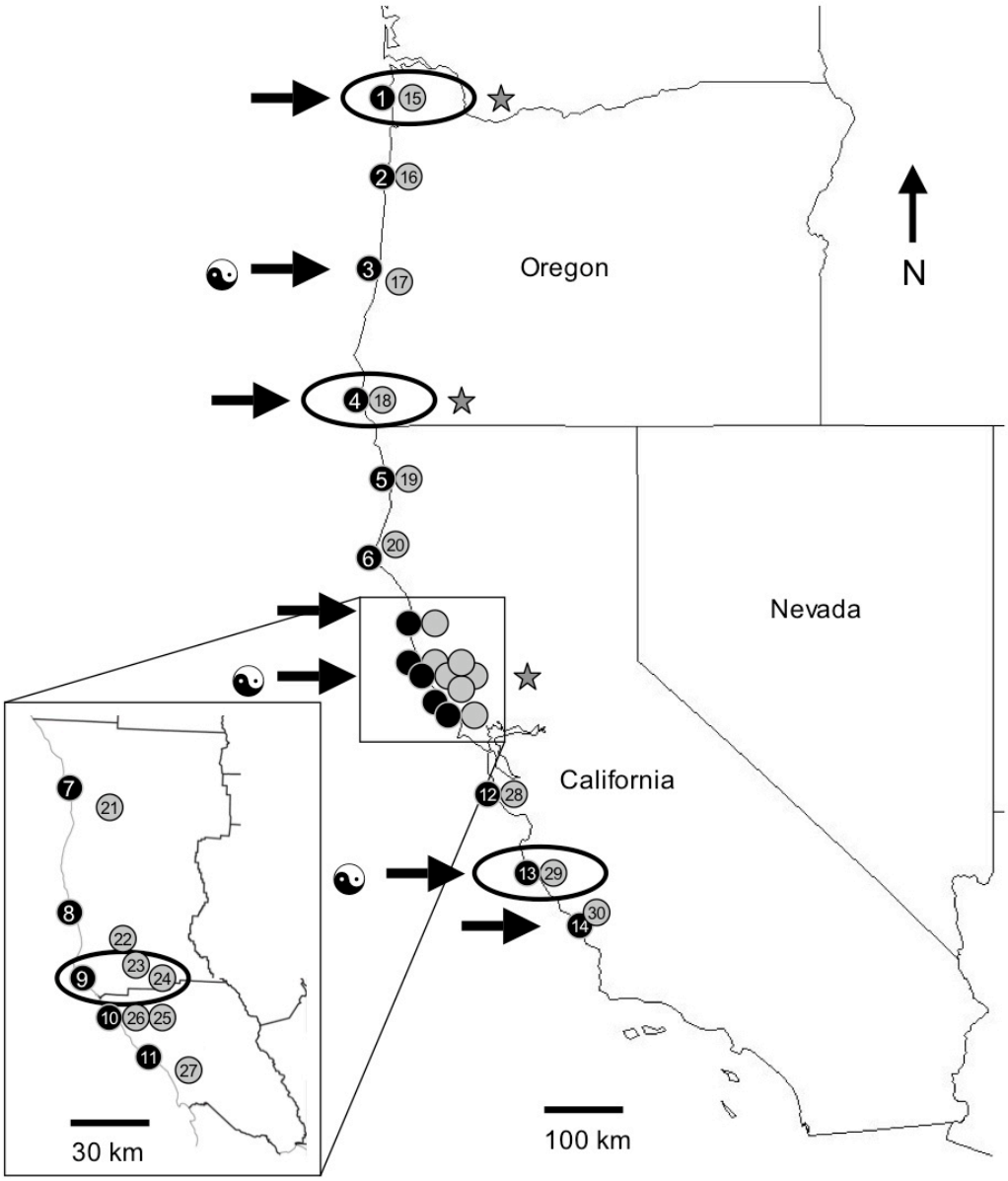

Figure 6: Map of western United States showing locations of coast (black) and inland (gray) populations used in this study. Arrows indicate coast/inland population pairs used in common garden greenhouse experiment. Yin-yang symbol indicates locations of reciprocal transplant experiments in this study (California) and that of Hall & Willis (2006) in Oregon. Stars represent population pairs used to test for salt tolerance differences. Ovals denote population pairs used for analysis of intrinsic postzygotic isolation. Numbers correspond to populations listed in Table 5.

2.2.2 Morphological population structure

To determine the extent of genetically based phenotypic divergence between coastal and inland populations; a common garden greenhouse experiment was conducted using six pairs of coast and inland populations and one extra coast population, for a total of 13 populations. Replicates within each population consisted of 12 selfed individuals, each descended from separately collected maternal plants. Some seeds failed to germinate, resulting in less than 12 replicates for some populations.

Seeds were sown individually in Fafard-4P soil in 4 in. square pots and were stratified in a dark room at 4°C for one week. Pots were then moved to the Duke University greenhouses for seed germination and subsequent growth. Greenhouse conditions consisted of 18-h days at 21°C with supplemental high-pressure sodium lights and 6-h nights at 16°C. Relative humidity was maintained at 30%. The location of all plants was fully randomized on the greenhouse bench.

For each plant I recorded the number of days from seed germination to first flowering and at the same time measured 12 morphological traits using digital calipers (first and second internode length, first internode length, first and second leaf width and length, leaf thickness, maximal corolla width and length, plant height, and rosette diameter). I also counted the number of flowers 10 days after the first flower opened. To determine whether there is overall multivariate morphological divergence between coast and inland populations, a multivariate analysis of variance (MANOVA) was implemented with the 13 morphological traits and flowering time as the dependent variables and habitat (coast or inland) as the independent variable. To visually assess morphological divergence between coast and inland populations, principle components analysis was conducted using the 14 traits, and subsequently PC1 was plotted against

PC2. To determine how morphological variation is partitioned between coast/inland habitats (fixed effect), among populations within habitats (random effect), and among individuals (error) within populations, a REML mixed-model nested analysis of variance (ANOVA) was conducted individually for each of the first two PCs and the 14 measured traits. To estimate the percent of variation partitioned among the hierarchical levels, the REML-nested ANOVA was rerun treating habitat as a random effect. All morphological analyses were implemented in JMP 6.0 (SAS Institute, 2005, Cary, NC).

2.2.3 Molecular genetic population structure

To determine if coast and inland populations are differentiated at loci located throughout the nuclear genome, molecular population genetic analysis was conducted with all 30 collected populations. Genomic DNA was extracted from plant tissue using a modified hexadecyl trimethyl-ammonium bromide chloroform extraction protocol (Kelly and Willis 1998). I used a total of 10 codominant markers for population genetic analysis, including six microsatellites (Kelly and Willis 1998) and four markers that reveal length polymorphisms in the introns of single copy nuclear genes (as described in Fishman and Willis 2005; Sweigart et al. 2006). Primer sequences are available in Table 3. All PCR products were subjected to capillary electrophoresis and fragment analysis on an ABI 3730×l DNA Analyzer. Size of the amplified fragments was scored automatically by the program GENEMARKER (SoftGenetics, 2005, State College, PA) and was confirmed by eye. The chromosomal location of all loci has been established by ongoing mapping studies. Although some markers were located on the same linkage group, they were never closer than 36.5 cM apart from each other (MgSTS423 and AAT230). Linkage disequilibrium is highly

Table 3: Primers used in this study for population genetic analysis.

		Population genetic primers	
Primer name	Linkage Group	Forward	Reverse
MgSTS278	8	ACGTCAGCCCTTTGTACACC	ACTCAGTTGTGCCAGTCACC
MgSTS332	10	GTGGTGTGCAATTCATTATCC	AAATTCATCACTGGACATTTCG
MgSTS423	6	TCTGATCTCTCGAACCTCTCG	ATCTAGCTCGCACCAACTCC
MgSTS474	3	TCGTCAGCATGCAAGTTAGC	CAATACGACGCCCAAAGG
AAT217	8	CCACAGAGAGGATTGGGTGT	TGAGCAGCTAAAAATGGAGG
AAT225	1	ATTCCGACTGGTTTCATTCA	CTTCCGACTAATCAGTAGAACAACA
AAT230	6	AATTTCACGTGCCAATCTGA	CCCTGGGTTAGCACTTAGCA
AAT240	13	CCCCTTTTAACCACTATATAATAACC	AGTGTGTGGGATTGAAAAGAA
AAT278	10	TGAGACTGTTTGGTGTGCAG	GGAAGAAGACGATAGGGCTG
AAT356	11	CAGCAACGGCCTCACTAATG	GGCGGAACCAGAATTTTATG

improbable at such large distances, but analysis with FSTAT 2.9.3.2 (Goudet 2001) was carried out to confirm that this is the case.

For all populations, I calculated the observed number of alleles (N_a), private alleles restricted to coast or inland habitat (P_a), observed heterozygosity (H_O), expected heterozygosity (H_S), total gene diversity (H_T), allelic richness (R_S), level of inbreeding (F_{IS}), and overall genetic differentiation (F_{ST}). Genetic differentiation among all pairs of populations was also quantified as pairwise F_{ST} (Weir and Cockerham 1984). All summary statistics were calculated with FSTAT (Goudet 2001). In addition, FSTAT was implemented for 1000 permutations to test for significant differences in H_S, H_O, F_{ST}, and F_{IS} between coast and inland populations.

To test for population structure between coast and inland populations I employed three different methods. Analysis of molecular variation (AMOVA, Excoffier et al. 1992) was implemented to determine how genetic variation is partitioned between coast/inland habitats, among populations within habitats, and among individuals within populations. AMOVA was implemented in the program Arlequin 3.11 (Excoffier et al. 2005).

The program STRUCTURE is a multilocus model-based clustering method that assigns individuals to a predefined number of populations (K) and detects admixed/migrant individuals (Pritchard et al. 2000). STRUCTURE was run for three iterations for K-values from 2 to 35, using the admixture model, independent allele frequencies, $\lambda = 1$, with a 200,000 burnin and 200,000 MCMC. I reran STRUCTURE 50 times at K= 2 and combined the result of those runs with the program CLUMMP (Jakobsson and Rosenberg 2007) and visualized this combined data with DISTRUCT (Rosenberg 2004).

The program POPULATION GRAPH uses graph theory techniques to determine the topological relationship among populations that may currently be exchanging genes

(Dyer and Nason 2004). This method is free of a priori assumptions about population geographic arrangements, unlike AMOVA, and works by simultaneously determining the high-dimensional covariance relationships among all populations using the genetic marker data. The program then determines the minimum set of edges (connections) that sufficiently explain the total among population covariance structure of all of the populations. The network of population connections can then be analyzed by various post hoc analyses. POPULATION GRAPH was implemented on the web (http://dyerlab.bio.vcu.edu/wiki/index.php/) using the population genetic dataset. A test for distinct clustering of coast and inland populations was conducted post hoc using the methods outlined by Dyer and Nason (2004).

2.2.4 Reciprocal transplant experiments

To test for local adaptation and reproductive isolation between coast and inland populations, reciprocal transplant experiments were conducted in California. Two sets of reciprocal transplants experiments, at different latitudes, were used to test the generality of trends and to compare to the results of a previous transplant experiment in Oregon (Hall and Willis 2006). The field sites for the experiment were located in Mendocino County (Experiment 1) and on the University of California, Big Creek Reserve in Monterey County (Experiment 2). The Mendocino coastal field site was located on seepy cliffs, just north of Manchester Beach state park (coast site 1, N39°00'29", W123°41'38"), whereas the Mendocino inland field site was located in hilly oak savanna habitat near Boonville, CA (inland site 1, N38°59'13", W123°21'03", 28.7 km from the ocean). The Big Creek coastal site was located near the reserve's bridge (coast site 2, N36°04'12", W 121°36'00"), and the inland Big Creek site was located at Shakemaker Meadow in mix grassland/chaparral habitat (inland site 2, N36°03'41",

W121°33'16", 3.5 km from the ocean). See Fig. 7 for location of field sites and seed source populations.

Plants growing at reciprocal transplant field sites might be adapted to highly local environmental factors instead of common features of inland or coastal habitats. To control for highly local adaptation and eliminate the possibility of genetic contamination of source populations, field sites away from seed source populations were used for field experiments (Fig. 7). Seeds for experiment 1 were derived from the SWB (coast) and LMC (inland) populations, whereas the seeds for experiment 2 were derived from the BCB (coast) and CAN (inland) populations (Fig. 7, Table 5 for location of these populations). Field sites in experiment 1 were located within existing *M. guttatus* populations. Field sites in experiment 2 were placed into habitat that appeared suitable for *M. guttatus* because UC Reserve restrictions forbid conducting transplant experiments within existing plant populations of the same species. All seeds were outbred and were derived from lines that had been grown in the greenhouse at least one full generation to reduce maternal effects. Four to six independent outbred full-sibling families were planted from each population, with the intention to capture much of the genetic variation within each population. At each field site, 100 inland parental plants, 100 coast parentals, and 100 F1s were planted. F1s were derived from crosses between coast and inland parentals. In sum, 300 plants were planted at each field site, 600 plants per reciprocal transplant experiment, and 1200 plants total.

Because of concern that *Mimulus* seeds would be displaced in the field, plants were transplanted at the seedling stage. To try to capture variation of germination conditions mediated by site-specific environmental factors, seeds were germinated in the soil of their eventual destination in December 2005. This timing allowed for the transplantation of individuals at the four-leaf stage, which was comparable to the developmental stage of native plants found in situ. Plants were grown in soil-filled flats

at the Bodega Marine Laboratory greenhouses until transplantation in January 2006. Rosette diameter, plant height, flowering time, leaf damage, and number of flowers produced were recorded for all plants every two to three weeks beginning on April 20, 2006 and continuing through December 27, 2006. Flowers and immature seedpods were removed from plants to reduce genetic contamination of local populations. This procedure prevented the collection of data on the number of seeds produced and thus, it was not possible to calculate full lifetime fitness.

2.2.5 Analysis of local adaptation and hybrid performance

To test for local adaptation and to analyze the performance of hybrids relative to native parental plants, data were analyzed with the program ASTER (Geyer et al. 2007). ASTER is a maximum-likelihood method for analysis of multiple fitness components. This method represents an improvement over previous methods (e.g., ANOVA) for the analysis of reciprocal transplant experiments because it accounts for dependencies among different components of fitness and properly incorporates variables that have different probability distributions (Geyer et al. 2007). For all analyses, ASTER was used to combine two dependent components of fitness, (1) survival to flowering and (2) number of flowers produced by plants that survived to flower. I fit multiple nested models to each set of data and compared these models using likelihood-ratio tests. The models listed here are the ones that best fit the data. A two-way analysis with ASTER was used to test for local adaptation of coast and inland populations. The model included genotype (coast or inland), family, and field site as independent variables. In this case, local adaptation is defined as a significant genotype × site interaction.

A separate analysis was conducted to assess extrinsic postzygotic isolation because this analysis involves the direct comparison of hybrids to local parentals. For

the model that best fits this data, genotype (local parental or hybrid) was the independent variable and performance (survival to flowering combined with number of flowers produced) was the dependent variable.

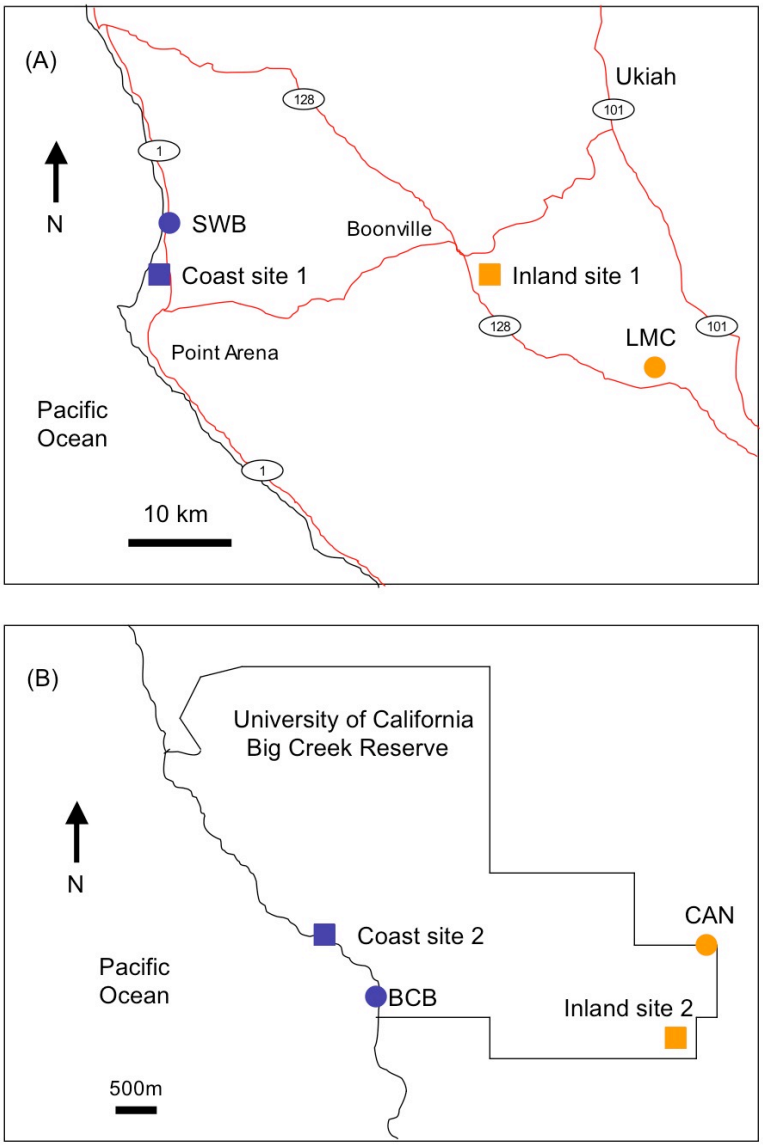

Figure 7: Map of location of field sites (square) and seed source populations (circle) used in the reciprocal transplant experiments. (A) Experiment 1 in Mendocino County, CA. (B) Experiment 2 at the University of California, Big Creek Reserve in Monterey County.

2.2.6 Salt spray tolerance

Oceanic salt spray is known to cause leaf necrosis (Boyce 1954). To determine if coast, inland, and F1 hybrid plants differed in salt spray tolerance in the field, I assessed percentage leaf damage during each visit to coastal field sites. Percentage leaf damage from April 26, 2006 was used for analysis of salt tolerance at coast site 1 because enough plants were still alive to make this comparison. Data were analyzed with a one-way ANOVA, where type of plant was the independent variable and percent salt damage was the dependent variable. Tukey-Kramer HSD tests were used for post hoc comparisons of means (JMP 6.0).

Greenhouse experiments were used to more directly determine salt spray tolerance of coast and inland populations. Plants from three latitudinal pairs of inland/coast populations were used in this experiment (LMC/SWB-California, RGR/OPB-Southern Oregon, SAM/OSW-Northern Oregon, 25 individuals per population). In addition, salt spray tolerance was assessed for 25 F1 hybrids of a cross between the LMC and SWB populations, which are the same populations used in reciprocal transplant experiment 1. Plants were grown under greenhouse conditions as described in the common garden experiment (above). All plants were sprayed with ~15 mL of 500 mM NaCl solution (approximately same concentration as ocean water) every other day starting four weeks after germination. Salt spray application leads to accumulation of salt directly on leaves as well as a build up in the soil, and in this way mimics conditions in the field at coastal sites. Percentage leaf damage and date of death (complete leaf necrosis) were recorded every other day. To compare the rate of accumulation of leaf damage between coast/inland habitats, among populations within habitats, and among individuals within populations, data were analyzed with a nested repeated-measures MANOVA. Here, damage at each time point was treated as a

separate dependent variable. Time till death was compared with a nested ANOVA, where population was nested within coast or inland type (JMP 6.0). Tukey-Kramer HSD tests were used for post hoc comparisons of mean time till death of LMC (inland) and SWB (coast) plants, as well as F1 hybrids between these two populations.

2.2.7 Intrinsic postzygotic isolation

To assess the level of intrinsic postzygotic isolation between coast and inland populations, crosses were made among four pairs of coast and inland populations from central California to northern Oregon (Fig. 6). Four to ten plants from each population were used in these crosses. Five different crosses were made for each coast/inland population pair, for a total of 20 crosses. All F1 hybrids were then intercrossed to produce F2 hybrids. To screen for hybrid lethality/inviability, 86 F1 hybrids representing all 20 crosses, as well as 613 F2s hybrids, were grown under common garden greenhouse conditions (above). Hybrid lethality and inviability were scored for all crosses. For purposes of this study, hybrid lethality is defined as death of plants at or before the four-leaf stage. Hybrid inviability is defined as dwarfing, necrosis, and the inability to survive to flower under standard greenhouse conditions (as described above).

2.2.8 Strength of reproductive isolating barrier

The strength of reproductive isolating barriers between coastal and inland population was calculated from the reciprocal transplant experiments using methods modified from previous studies (Ramsey et al. 2003; Kay 2006; Martin & Willis 2007; Lowry et al. 2008b). Potential for gene flow into inland habitat was calculated separately from potential gene flow into coastal habitat for reproductive isolating barriers that act asymmetrically. Data from both inland field sites were combined for these analyses, but not for coastal field sites (see Results).

To assess the potential for hybridization via pollen dispersal between habitats, I determined the approximate flowering phenologies of experimental plants growing in their native habitat by conducting censuses every 2-3 weeks and recording the total number of flowers produced since the previous census. In order to calculate a crude, conservative measure of reproductive isolation due to less than complete flowering time overlap, I assumed that pollinators disperse pollen at random among flowers within and between the two habitats despite allopatry, that the abundances of native flowers at the two habitats are equal during any flowering overlap period, and that all flowers produce equal seeds and pollen grains. With these admittedly unrealistic assumptions, the frequency of hybrids expected with complete flowering overlap is 0.5 at each site. Let the proportion of coastal flowers that were open during the overlap period be F_C and that of inland flowers be F_I. Given the above assumptions, the expected frequency of hybrids produced as seeds at the coastal site, q_C, is equal to $F_C/2$, and that for seeds produced at the inland site is $q_I = F_I/2$. Following the reasoning outlined in Martin and Willis (2007), the reproductive isolation at the coastal site due solely to flowering phenology differences between plants in allopatric habitats is $RI_{FA,C} = 1 - \left[q_C/(1-q_C)\right]$, with an analogous formula for isolation at the inland site.

Gene flow can also occur between species through seed dispersal. However, seeds that disperse between habitats must germinate, survive, and flower in the non-native habitat in order for hybridization to potentially occur. Thus, habitat mediated selection against immigrants, which may prevent survival and limit reproduction, can act as an impediment to gene flow (Nosil et al. 2005). I calculated the strength of reproductive isolation due to selection against immigrants as $RI_I = 1 - \overline{w}_i/\overline{w}_n$, where $\overline{w}_i$ is the mean number of flowers produced by immigrant individuals, and $\overline{w}_n$ is the mean number of flowers produced by individual plants native to the habitat of the field site.

For immigrants that survive to flower, hybridization cannot occur if immigrants flower at different times than native plants. To determine if flowering time differences restricts hybridization between immigrants and native plants in artificial sympatry, I calculated the probability that an individual immigrant flowered at the same time as any members of the native population and estimated this form of temporal reproductive isolation between immigrants and natives in sympatry, say at the coastal site, as $RI_{FS,C} = 1 - F_{M,I}$ where $F_{M,I}$ is the probability that a migrant from the inland population flowers while any native plants are flowering at the coastal site.

Postzygotic isolation can occur through intrinsic genetic incompatibilities. To determine the strength of intrinsic postzygotic isolation, the mean seedling to adulthood viability of F1 hybrids, $\bar{v}_{F1}$, was compared to the mean viability of parental populations. I did not include data from the F2 generation in my calculation as it is unclear what the relative frequency of F2 versus backcross hybrids would be in a natural situation. Because I was interested here in intrinsic barriers, I focused on viability in the relatively benign environment of my greenhouse experiments. Because all parental individuals survived from seedling to adulthood in these experiments, I calculated intrinsic postzygotic reproductive isolation was then quantified as $RI_{IP} = 1 - \bar{v}_{F1}/\bar{v}_P$.

Postzygotic isolation could also occur, even where there is no intrinsic postzygotic isolation, if the hybrids are less fit than native parental plants due to extrinsic ecologically based inviability or sterility (Hatfield & Schluter 1999). The strength of extrinsic postzygotic reproductive isolation was calculated as $RI_{EP} = 1 - \bar{w}_h/\bar{w}_n$, where $\bar{w}_h$ is the mean lifetime number of flowers produced by F1 hybrids in the field, and $\bar{w}_n$ is the mean lifetime number of flowers produced by native parental plants.

2.3 Results

2.3.1 Morphological population structure

Striking morphological differences were found between coast and inland populations in the common garden greenhouse experiment. Joint analysis of 13 morphological traits and flowering time showed substantial quantitative genetic divergence between coast and inland habitats (MANOVA, $F_{13,83} = 61.20$, $P < 0.0001$). In general, the coastal populations flowered later, had thicker stems, shorter internodes, and larger flowers than inland populations (Fig. 8). Plotting of PC1 and PC2 showed clear differences between coast and inland individuals (Fig. 8c). Most of the variation in flowering time (71.9%) and PC1 (88.6%), but not PC2 (6.1%), was partitioned between groups (coast versus inland) in the REML nested ANOVA (Table 4). It should be noted that I excluded the tetraploid (see below) inland SLO population for all morphological analysis.

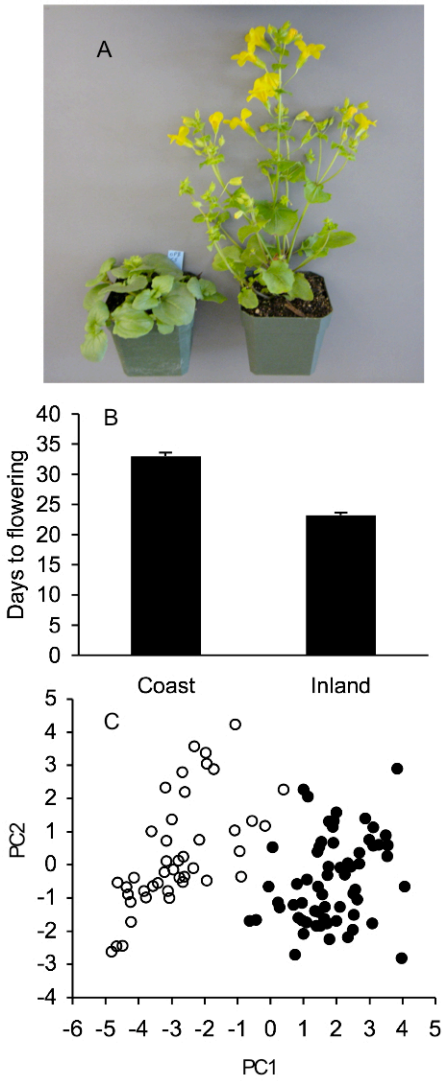

Figure 8: Morphological divergence of coast and inland populations. (A) Morphological differences between coast (left) and inland (right) populations grown in a common garden greenhouse environment. (B) Flowering time was significantly different between coast and inland races ($F = 49.55$, $P < 0.0001$). See Table 4 for analysis of all traits. Error bars denote one standard error. (C) Principle components analysis (PC1 plotted against PC2) of individuals from coast (closed circle) and inland populations (open circle) using morphological data (14 traits, N = 12 populations, 107 individuals). Data for tetraploid SLO population removed from this analysis.

Table 4: Results of REML mixed-model nested ANOVA of first two principle components, flowering time, and 13 measured morphological traits for plants from coast (N=7) and inland (N=4) populations. Plants were grown in a common garden greenhouse environment, so observed differences should be genetically based. Estimates for the partition of variation (%var) among habitats, populations within habitats, and among individuals within populations (error) estimated by treating all hierarchical levels as random effects. * P < 0.05, ** P < 0.01, *** P < 0.001

			Source of variation						
			Habitats		Populations			Individuals(Error)	
Trait	Coast Mean (SE)	Inland Mean (SE)	F-ratio	%var	χ^2	Var comp	%var	Var comp	%var
PC1	1.86 (0.13)	-2.87 (0.20)	181.24 ***	88.6	4.7 *	0.22	1.7	1.23	9.7
PC2	-0.14 (0.16)	0.36 (0.25)	1.58	6.1	63.6 ***	1.42	57.6	0.89	36.3
Flowering Time (days)	33.00 (0.57)	23.25 (0.40)	49.55 ***	71.9	15.0 ***	4.31	6.5	14.27	21.6
Stem Thickness 1 (mm)†	6.23 (0.13)	2.99 (0.17)	63.11 ***	78.2	18.5 ***	0.40	5.8	1.08	15.9
Internode length (mm)	23.28 (2.10)	59.49 (4.21)	9.88 *	48.4	70.8 ***	355.38	29.7	262.34	21.9
Leaf length 1 (mm)§	57.12 (1.60)	39.00 (1.86)	14.32 **	45.1	20.3 ***	56.27	15.9	138.07	39.0
Leaf width 1 (mm)§	33.01 (0.98)	27.50 (1.36)	3.24	11.6	18.5 ***	22.74	23.7	61.86	64.7
Leaf thickness (mm)	0.439 (0.01)	0.401 (0.02)	0.55	0.0	53.5 ***	0.01	49.9	0.01	50.1
Corolla length (mm)	37.17 (0.59)	25.80 (0.69)	80.27 ***	71.1	3.3 *	2.45	2.7	23.91	26.2
Corolla Width (mm)	31.38 (0.51)	20.55 (0.62)	67.46 ***	73.3	7.7 **	3.31	4.2	17.5	22.4
Number of Flowers	27.37 (1.60)	33.40 (2.96)	0.75	0.0	24.5 ***	98.53	31.8	27.33	68.2
Stem thickness 2 (mm)††	6.25 (0.14)	1.99 (0.11)	137.53 ***	88.0	16.1 ***	0.299	2.9	0.946	9.1
Leaf length 2 (mm)§§	71.47 (2.14)	25.43 (1.83)	85.30 ***	79.4	8.8 **	50.52	3.8	225.09	16.8
Leaf width 2 (mm)§§	45.88 (0.94)	24.06 (1.14)	127.08 ***	78.5	2.1	4.90	1.6	59.91	19.9
Rosette diamete (mm)	100.3 (6.62)	29.11 (2.88)	14.31 **	50.1	36.5 ***	913.01	19.0	1488.2	31.0
Height (mm)	326.40 (14.0)	279.21 (15.8)	0.83	0.0	67.3 ***	8334.27	55.9	6451.20	44.1

† Thickness of the first internode

†† Thickness of the second internode

§ First true leaf

§§ Second true leaf

2.3.2 Molecular genetic population structure

All ten codominant markers were highly polymorphic and successfully amplified alleles in all 30 populations. No linkage disequilibrium was detected among any of the ten loci using FSTAT 2.9.3.2 (Goudet 2001). Two populations (SLO, CAN) had aberrant molecular signatures and were removed from subsequent analyses. All individuals (N = 16) of the CAN population were completely homozygous for one allele at each of the ten loci. Plants of the SLO population had more than two alleles at multiple loci. Follow up analysis with flow cytometry revealed that plants of the SLO population are tetraploid (personal communication, J. Modliszewski).

Genetic divergence was high in all pair-wise population comparisons (mean pair-wise F_{ST} = 0.48, Table 7). Genetic variation was greater within inland populations than within coastal populations of *M. guttatus*. For all ten loci, observed heterozygosity (H_O) and expected heterozygosity (H_S) were significantly greater for the inland populations than the coastal populations, while genetic divergence (F_{ST}) was significantly greater for coastal populations than inland populations (FSTAT: N = 28 populations, 1000 permutations, $P < 0.0001$ for all tests, Fig. 11a; see Table 6 for locus-specific summary statistics). In contrast, the level of inbreeding (F_{IS}) did not differ (P = 0.486) between coast and inland populations (Fig. 11a). The inland populations collectively harbored far more habitat restricted private alleles than coastal populations (Fig. 11b; Table 6). Across all loci, 64.13% of the alleles found in inland populations were restricted to the inland habitat, whereas only 17.57% of the alleles found in coastal populations were

private (Fig. 11b, N = 28 populations, 2 habitats, 10 loci, 39.34, P< 0.0001).

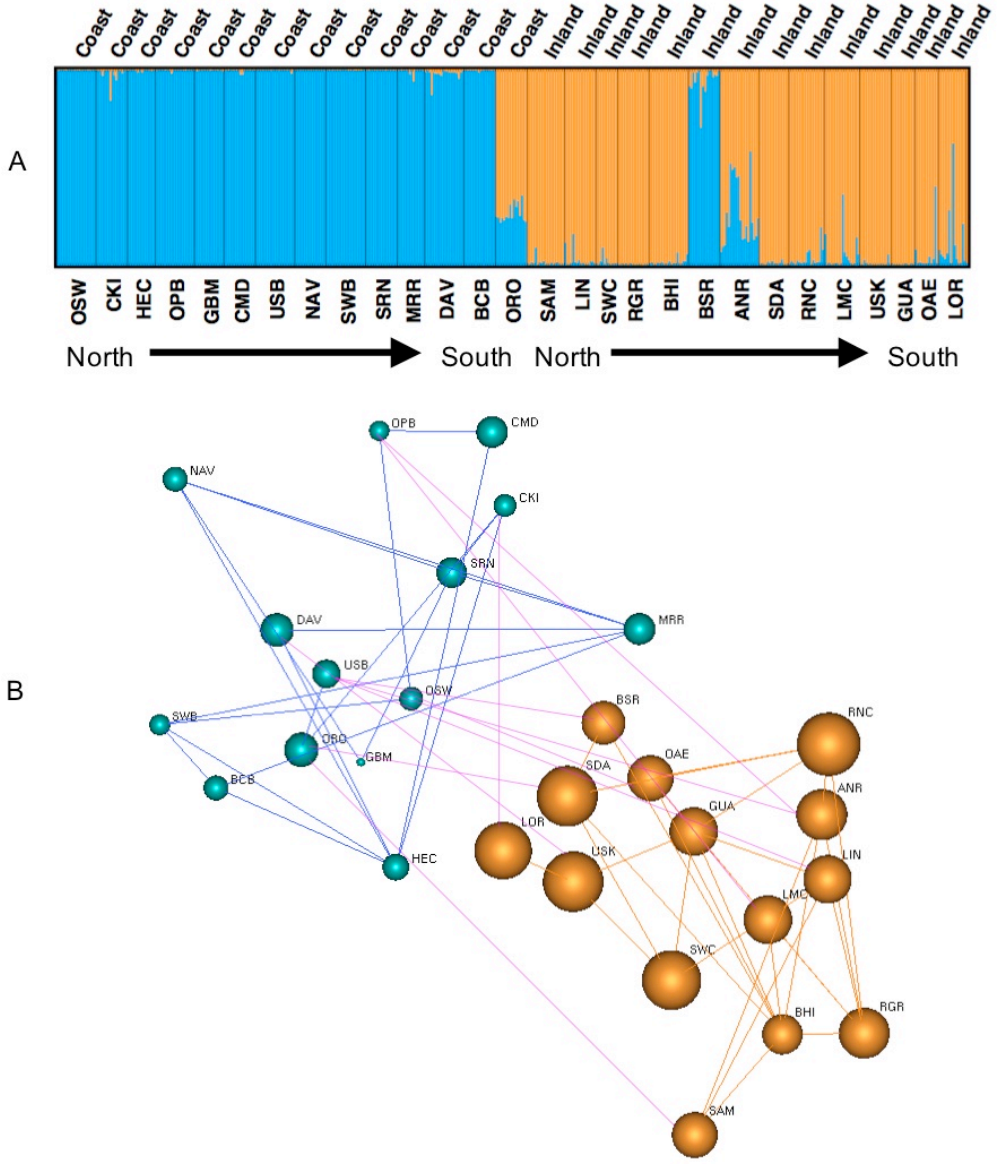

Figure 9: Genetic population structure of coast and inland populations. (A) Analysis with the program STRUCTURE (Pritchard et al. 2000) run 50 times at K = 2. Results of multiple runs combined with CLUMMP (Jakobsson & Rosenberg 2007) and visualized with DISTRUCT (Rosenberg 2004). (B) Analysis with the program POPULATION GRAPH (Dyer & Nason 2004). There were significantly more edges within coast (blue lines) and inland (orange lines) groups than between (pink lines) groups (P < 0.0001). The diameter of each sphere is equal to the expected heterozygosity (H_S) of the population, which was calculated in FSTAT (Goudet 2001). For both analyses: N = 14 coast populations, 14 inland populations, 479 individuals, 10 codominant loci.

We found strong evidence for genetic divergence between coast and inland populations with all three analyses employed. Analysis of molecular variation (AMOVA) detected structure (1023 permutations, $F_{CT} = 0.0845$, $P < 0.001$) between the coast and inland habitats. Even so, only 8% of the genetic variation was partitioned between habitats, while 39% of the variation was among populations within habitats, and the remaining 52% was among individuals within habitats (Table 8).

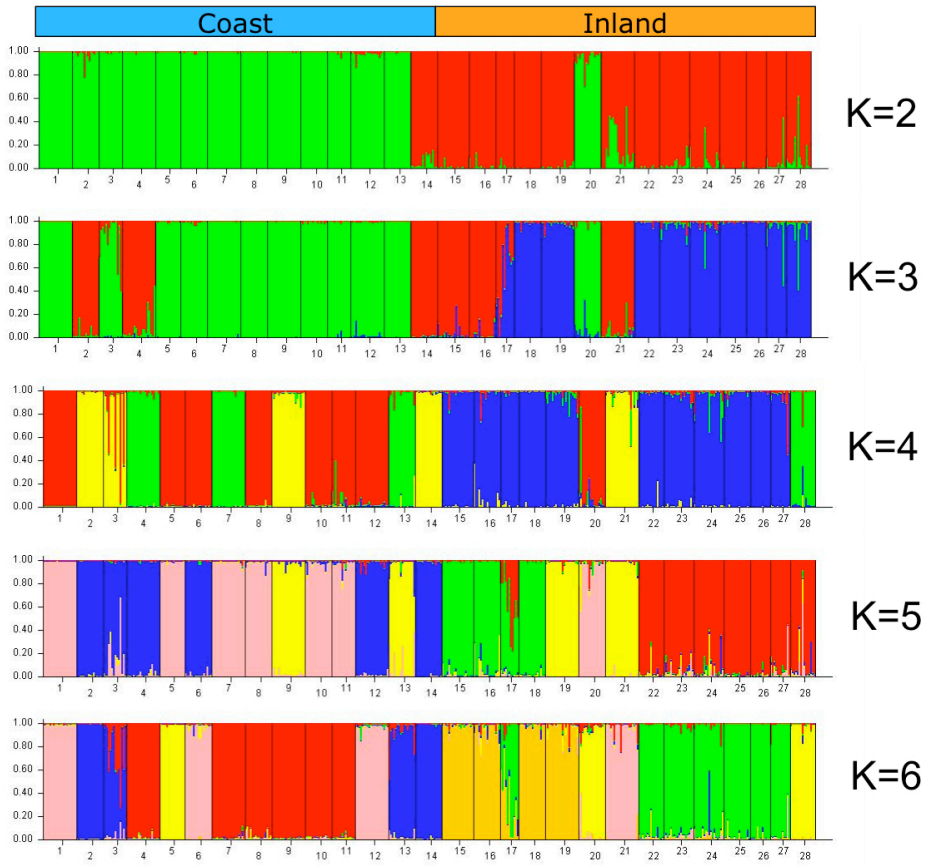

Figure 10: The program STRUCTURE run on K-values from 2-6. N = 14 coast populations, 14 inland populations, 479 individuals, 10 codominant loci. Numbers correspond to populations in Figure 6 and Table 5.

Table 5: Coast and inland populations of *M. guttatus* used in this study. For each population longitude and latitude, the number of samples (N), the expected heterozygosity (H_S), mean number of alleles per locus (N_a), mean allelic richness (R_S), and mean inbreeding coefficient (F_{IS}) are listed. Ten codominant markers were used to calculate statistics in FSTAT 2.9.3.2 (Goudet 2001). "Num" corresponds to the label of populations in Figure 6.

Race	Pop ID	Num	Location	Latitude (N)	Longitude (W)	N	N_a	H_S	R_S	F_{IS}
M. guttatus	OSW	1	Oswald West SP, Tillamook Co., OR	45° 45′ 665″	123° 57′ 994″	20	2	0.263	1.80	0.236
(Coast)	CKI	2	Cape Kiwanda SP, Tillamook Co., OR	45° 14′ 528″	123° 58′ 121″	16	1.9	0.252	1.75	0.246
	HEC	3	Heceta Lighthouse, Lane Co., OR	44° 08′ 104″	124° 07′ 368″	14	2.1	0.297	1.88	0.308
	OPB	4	Otter Point SP, Curry Co., OR	42° 27′ 841″	124° 25′ 375″	20	2.6	0.227	2.05	0.056
	GBM	5	Gold Bluffs Marsh, Humbolt Co., CA	41° 22′ 718″	124° 04′ 175″	15	1.6	0.096	1.36	0.283
	CMD	6	Cape Mendocino, Humbolt Co., CA	40° 24′ 554″	124° 23′ 523″	16	2.6	0.350	2.16	0.382
	USB	7	Usal Beach, Mendocino Co., CA	39° 49′ 931″	123° 50′ 957″	20	2.6	0.320	2.14	0.275
	NAV	8	Navarro River, Mendocino Co., CA	39° 11′ 214″	123° 45′ 435″	16	2.3	0.277	2.04	0.008
	SWB	9	Irish Beach, Mendocino Co., CA	39° 02′ 159″	123° 41′ 428″	20	1.8	0.234	1.61	0.260
	SRN	10	Sea Ranch, Sonoma Co., CA	38° 44′ 130″	123° 29′ 390″	16	2.1	0.339	2.00	0.436
	MRR	11	Russian River, Sonoma Co., CA	38° 27′ 384″	123° 08′ 452″	14	2.6	0.353	2.28	0.283
	DAV	12	Davenport Beach, Santa Cruz Co., CA	37° 01′ 499″	122° 13′ 050″	20	2.6	0.374	2.20	0.155
	BCB	13	Big Creek Reserve, Monterey Co., CA	36° 03′ 771″	121° 35′ 532″	16	2.5	0.277	2.10	0.431
	ORO	14	Montana de Oro, San Luis Obisbo Co., CA	35° 16′ 401″	120° 53′ 347″	16	3	0.384	2.41	-0.028
M. guttatus	SAM	15	Saddle Mountain SP, Clatstop Co., OR	45° 57′ 555″	123° 40′ 779″	19	5.5	0.505	3.72	-0.016
(Inland)	LIN	16	Little Nestuca River, Tillamook Co., OR	45° 08′ 156″	123° 53′ 783″	16	5	0.534	3.60	0.300
	SWC	17	Mapleton, Lane Co., OR	43° 57′ 568″	123° 54′ 147″	11	4.2	0.655	3.77	0.169
	RGR	18	Rouge River, Curry Co., OR	42° 29′ 355″	124° 12′ 504″	16	4.3	0.561	3.30	0.377
	BHI	19	Bald Hills, Humbolt Co., CA	41° 09′ 295″	123° 53′ 378″	20	4	0.446	2.99	0.312
	BSR	20	Rio Dell, Humbolt Co., CA	40° 31′ 771″	124° 09′ 783″	16	4.1	0.482	3.07	0.165
	ANR	21	Angelo Reserve, Mendocino Co., CA	39° 44′ 212″	123° 37′ 863″	20	5.2	0.565	3.58	0.232
	SDA	22	Boonville, Mendocino Co, CA	39° 01′ 099″	123° 19′ 149″	14	4.9	0.672	3.81	0.359
	RNC	23	Rancheria Creek, Mendocino Co, CA	38° 54′ 681″	123° 14′ 706″	18	8.3	0.701	5.06	0.115
	LMC	24	Yorkville, Mendocino Co., CA	38° 51′ 839″	123° 05′ 035″	18	6.4	0.537	4.10	0.179
	USK	25	Skaggs-Springs, Sonoma Co., CA	38° 40′ 344″	123° 12′ 613″	16	6.3	0.668	4.47	0.114
	GUA	26	Gualala River, Sonoma Co., CA	38° 40′ 090″	123° 18′ 699″	12	4.3	0.536	3.43	0.008
	OAE	27	Occidental, Sonoma Co., CA	38° 24′ 676″	122° 57′ 583″	12	3.6	0.507	3.04	0.309
	LOR	28	Boulder Creek, Santa Cruz Co., CA	37° 06′ 510″	122° 07′ 080″	15	4.6	0.634	3.64	0.180
	CAN	29	Big Creek Reserve, Monterey Co., CA	36° 04′ 134″	121° 33′ 091″	16	1	0.000	1.00	-
	SLO	30	Morro Road, San Luis Obsibo Co., CA	35° 27′ 647″	120° 44′ 403″	-	-	-	-	-

There was a striking dearth of admixture among populations in my analyses with STRUCTURE, such that all individuals within a given population were assigned to the same cluster regardless of *K*-value. Most populations were correctly assigned (at $K = 2$) to the habitat from which they were collected (Fig. 9a). However, three populations (ORO, BSR, ANR) were misassigned on different runs. BSR was consistently misassigned on all runs, while ORO was misassigned in 68% of the runs and ANR in 10% of the runs (50 total runs). Interestingly, ANR and BSR were both collected from along the Eel River in Northern California (see Discussion). At greater *K* values ($K = 3$-35), the partitioning of populations by STRUCTURE was much more complicated and population assignments were much less consistent among runs (Fig. 10). Out of all of the models tested with STRUCTURE, the model with $K = 29$ had the highest likelihood. In addition, I observed little evidence of isolation by distance, except for densely sampled populations in central California (analysis not shown, but see Fig. 10).

Our analysis with POPULATION GRAPH found that the coast and inland populations cluster as two distinct groups. There were 29 edges among inland populations, 21 edges among coast populations, but only 10 edges between coast and inland populations (Fig. 9b). This represents highly significant genetic differentiation between coast and inland groups ($N = 28$ populations, 10 loci, $P < 0.0001$).

Two marker loci (AAT217 and MgSTS278) are located on linkage group 8 near two previously identified Quantitative Trait Loci (QTLs), which are partly responsible for the phenotypic divergence between a pair of coastal and inland populations (Hall et al. 2006). Since selection at those QTLs may result in divergence of linked markers, I reran my analyses of population structure without those loci. Similar levels of clustering were observed using the eight remaining loci for both STRUCTURE (data not shown) and POPULATION GRAPH (23 edges among inland pops, 23 edges among coast pops, and 13 edges between coast and inland pops, $P < 0.0001$).

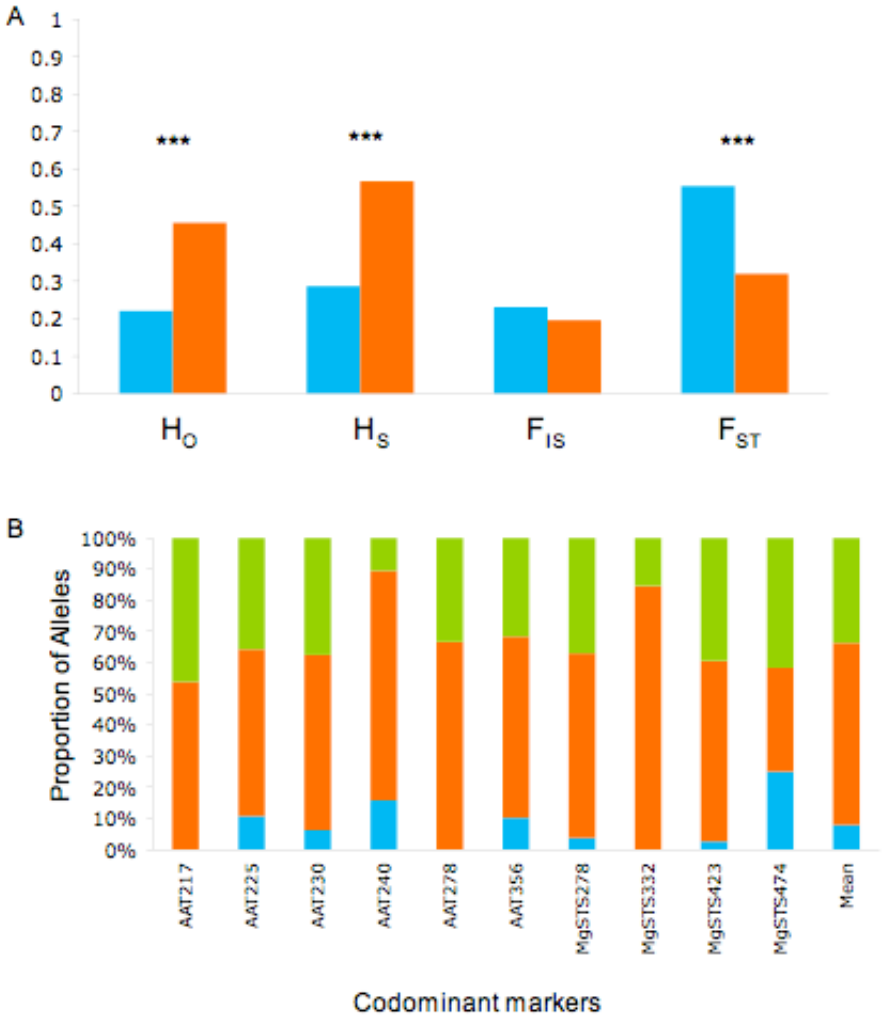

Figure 11: Comparison of population genetic summary statistics and habitat restricted alleles. (A) Coast (blue) and inland (orange) races significantly differed (*$P < 0.001$) in observed heterozygosity (H_O), gene diversity (H_S), and overall population structure (F_{ST}). The level of inbreeding (F_{IS}) did not differ between the races ($P = 0.486$). P-values calculated by permuting data 1000 times in FSTAT (Goudet 2001). (B) The proportion of alleles at each locus that are found exclusively in coastal populations (blue), exclusively in inland populations (orange), and shared between coast and inland populations (green). For both figures: N = 14 coast populations, 14 inland populations, 479 individuals, 10 codominant loci.**

Table 6: Populations genetic summary statistics for SSR and intron markers. SSR (microsatellite) markers are labeled "AAT" (Kelly and Willis 1998) while intron length polymorphism markers are labeled "MgSTS." Chromosome location of all markers has been established by ongoing genetic mapping studies. Data partitioned between coast and inland races as well as provided for all samples. Statistics include total number of alleles (n_a), number of private alleles that are restricted to a particular habitat (p_a), observed heterozygosity (H_O), expected heterozygosity (HS), overall gene diversity (H_T), allelic richness (R_S), and level of inbreeding (F_{IS}).

Locus	Linkage group	Type	n_a	n_h	H_O	H_S	H_T	R_S	F_{IS}
AAT217	8	Coast	6	0	0.390	0.392	0.711	4.01	0.013
		Inland	13	7	0.529	0.632	0.858	5.87	0.147
		All	13	-	0.444	0.494	0.817	5.28	0.092
AAT225	1	Coast	13	3	0.218	0.340	0.749	5.67	0.356
		Inland	25	15	0.455	0.498	0.723	5.27	0.091
		All	28	-	0.325	0.405	0.750	5.43	0.203
AAT230	6	Coast	19	3	0.196	0.369	0.877	7.64	0.439
		Inland	45	27	0.402	0.695	0.968	9.25	0.416
		All	48	-	0.289	0.514	0.944	8.66	0.424
AAT240	13	Coast	5	3	0.115	0.167	0.575	3.63	0.278
		Inland	16	14	0.447	0.570	0.866	6.36	0.246
		All	19	-	0.271	0.355	0.779	5.68	0.253
AAT278	10	Coast	2	0	0.043	0.044	0.229	1.91	0.045
		Inland	6	4	0.253	0.297	0.603	2.97	0.132
		All	6	-	0.143	0.164	0.553	2.77	0.118
AAT356	11	Coast	41	10	0.399	0.582	0.978	12.52	0.281
		Inland	88	57	0.689	0.834	0.984	10.48	0.144
		All	98	-	0.525	0.683	0.985	10.53	0.204
MgSTS278	8	Coast	11	1	0.278	0.345	0.819	6.14	0.223
		Inland	26	16	0.458	0.647	0.952	8.36	0.280
		All	27	-	0.355	0.478	0.914	7.50	0.258
MgSTS332	10	Coast	2	0	0.043	0.034	0.052	1.35	-0.264
		Inland	13	11	0.338	0.364	0.619	4.14	0.076
		All	13	-	0.184	0.192	0.413	3.06	0.047
MgSTS423	6	Coast	17	1	0.218	0.324	0.703	6.37	0.350
		Inland	38	22	0.587	0.693	0.956	8.87	0.172
		All	39	-	0.389	0.491	0.871	7.41	0.230
MgSTS474	3	Coast	8	3	0.306	0.289	0.742	4.38	-0.059
		Inland	9	4	0.419	0.481	0.816	5.32	0.132
		All	12	-	0.350	0.372	0.815	5.16	0.057
Overall		Coast	12.4	2.4	0.221	0.289	0.643	5.36	0.231
		Inland	27.9	17.7	0.458	0.571	0.835	6.69	0.196
		All	30.3	-	0.328	0.415	0.784	6.15	0.208

N=14 Coast populations containing 239 individuals
N=14 Inland populations containing 240 individuals

Table 7: Pair-wise F_{ST} among all populations. Blue is pair-wise F_{ST} among coast populations, orange is F_{ST} among inland populations, and yellow is between coast and inland populations. Note that pair-wise F_{ST} is greater among coast populations than inland populations, which is likely due to the lower heterozygosity within coastal populations.

	OSW	CKI	HEC	OPB	GBM	CMD	USB	NAV	SWB	SRN	MRR	DAV	BCB	ORO	SAM	LIN	SWC	RGR	BHI	BSR	ANR	SDA	RNC	LMC	USK	GUA	OAE	LOR
CKI	0.62																											
HEC	0.49	0.53																										
OPB	0.66	0.65	0.52																									
GBM	0.63	0.76	0.71	0.75																								
CMD	0.54	0.58	0.41	0.49	0.63																							
USB	0.59	0.59	0.52	0.63	0.69	0.57																						
NAV	0.49	0.61	0.38	0.56	0.62	0.45	0.58																					
SWB	0.54	0.68	0.37	0.60	0.74	0.54	0.59	0.41																				
SRN	0.52	0.53	0.41	0.56	0.66	0.47	0.52	0.49	0.51																			
MRR	0.54	0.55	0.35	0.54	0.68	0.51	0.47	0.42	0.48	0.40																		
DAV	0.56	0.58	0.41	0.59	0.67	0.44	0.51	0.50	0.57	0.50	0.41																	
BCB	0.57	0.62	0.40	0.60	0.73	0.53	0.48	0.52	0.51	0.44	0.37	0.47																
ORO	0.63	0.55	0.54	0.60	0.70	0.57	0.56	0.58	0.61	0.60	0.55	0.47	0.58															
SAM	0.58	0.54	0.56	0.61	0.66	0.54	0.55	0.59	0.63	0.50	0.51	0.47	0.55	0.48														
LIN	0.55	0.56	0.50	0.55	0.65	0.47	0.55	0.52	0.55	0.48	0.48	0.43	0.50	0.46	0.31													
SWC	0.55	0.56	0.49	0.59	0.67	0.49	0.52	0.53	0.58	0.50	0.45	0.39	0.49	0.43	0.27	0.27												
RGR	0.53	0.54	0.45	0.55	0.63	0.47	0.48	0.50	0.53	0.48	0.41	0.43	0.44	0.44	0.38	0.34	0.30											
BHI	0.57	0.58	0.52	0.58	0.67	0.52	0.52	0.55	0.57	0.52	0.49	0.50	0.52	0.53	0.45	0.47	0.47	0.37										
BSR	0.38	0.54	0.35	0.47	0.49	0.39	0.44	0.36	0.39	0.40	0.32	0.39	0.35	0.46	0.48	0.39	0.38	0.34	0.38									
ANR	0.49	0.49	0.42	0.47	0.57	0.41	0.41	0.42	0.45	0.42	0.41	0.46	0.43	0.44	0.40	0.40	0.35	0.36	0.38	0.34								
SDA	0.44	0.48	0.39	0.51	0.54	0.40	0.44	0.43	0.47	0.43	0.36	0.36	0.39	0.40	0.38	0.34	0.23	0.29	0.34	0.26	0.30							
RNC	0.39	0.41	0.32	0.45	0.48	0.39	0.38	0.36	0.40	0.37	0.25	0.29	0.33	0.34	0.32	0.30	0.21	0.23	0.29	0.21	0.28	0.13						
LMC	0.47	0.52	0.40	0.52	0.57	0.45	0.48	0.44	0.47	0.46	0.36	0.30	0.40	0.36	0.39	0.31	0.25	0.31	0.41	0.26	0.39	0.21	0.12					
USK	0.48	0.50	0.47	0.52	0.56	0.46	0.46	0.49	0.53	0.47	0.44	0.37	0.44	0.36	0.33	0.32	0.23	0.28	0.41	0.34	0.36	0.23	0.15	0.20				
GUA	0.50	0.58	0.49	0.59	0.61	0.50	0.53	0.51	0.55	0.52	0.44	0.39	0.48	0.43	0.39	0.33	0.26	0.34	0.43	0.31	0.40	0.26	0.17	0.20	0.17			
OAE	0.54	0.61	0.50	0.63	0.64	0.51	0.47	0.53	0.57	0.55	0.44	0.41	0.51	0.44	0.44	0.43	0.36	0.36	0.45	0.36	0.41	0.29	0.19	0.24	0.28	0.29		
LOR	0.45	0.47	0.40	0.49	0.50	0.38	0.45	0.42	0.48	0.45	0.41	0.38	0.46	0.41	0.40	0.37	0.29	0.33	0.37	0.31	0.32	0.23	0.22	0.30	0.25	0.31	0.34	
CAN	0.84	0.84	0.85	0.88	0.94	0.81	0.82	0.85	0.88	0.80	0.84	0.78	0.85	0.81	0.70	0.73	0.75	0.73	0.77	0.76	0.68	0.68	0.63	0.72	0.67	0.75	0.79	0.64

2.3.3 Reciprocal transplant experiment

Early season survival was high at three out of the four field sites while all experimental plants at coast site 2 died early in the season (Fig. 12, Table 9). As a result, I restricted my analyses to the three remaining field sites. Landslides destroyed three out of the ten blocks at coast site 1. Therefore, the sample size at coast site 1 is lower than at the two inland sites. At the three field sites with survivors, local plants consistently outperformed immigrant plants (Fig. 12, Table 9). In addition, there was very little overlap in flowering time between coast and inland plants (Fig. 12a).

Table 8: Results of analysis of molecular variation (AMOVA) preformed in Arlequin 3.11. The nested model included habitats (coast/inland), populations within habitats, and individuals within populations (*$P < 0.001$).**

Source of Variation	df	Sum of squares	Variance components	% of variation
Among coast and inland habitats	1	195.29	0.312	8.45***
Among popualtions within habitats	26	1299.71	1.456	39.48***
Within populaitons	898	1724.93	1.921	52.08***
Total	925	3219.93	3.689	

2.3.4 Analysis of local adaptation and hybrid performance

Selection was very strong against immigrants between habitats in both directions of migration. At the two inland sites, only one coastal plant out of 183 (0.5%) survived to flower and this plant only produced one flower. In contrast, 41% of inland plants at the two inland field sites survived to flower, and survivors produced an average of over 3 flowers. At coast site 1, over twice as many coast plants survived to flower than inland plants. In addition, coast plants that survived to flower at coast site 1 produced ~3.5 times as many flowers as inland plants at coast site 1 (Table 9). Overall, the population x site interaction (Fig. 12b,c) in the reciprocal transplant between coast site 1 and inland site 1 was highly significant (ASTER, $df = 11$, $z = -4.170$, $P < 0.0001$). Because all plants died prematurely at coast site 2, analysis of a site x genotype interaction was not

possible for experiment 2. Therefore, I restricted my analysis to a one-way comparison of the performance of coast and inland plants within inland site 2. Due to the fact that none of the coast (BCB) plants survived to flower at inland site 2, performance of inland plants (CAN) was significantly greater than the performance of coast plants at this site (Fig. 12d,e; ASTER, $df = 3$, $z = 2.459$, $P = 0.0139$).

At the inland site 1, the native inland (LMC) plants survived to flower at ~1.25 times the rate of F1 hybrids, and these inland plants produced ~1.25 times as many flowers as the F1 hybrids (Table 9, ASTER, $df = 3$, $z = 2.568$, $P = 0.0102$). At inland site 2, there was no difference in fitness between hybrids and local inland (CAN) plants (ASTER, $df = 3$, $z = -0.10$, $P = 0.92$). At coast site 1, hybrids outperformed native coast (SWB) plants (ASTER, $df = 3$, $z = 5.259$, $P < 0.0001$) as ~2.5 times more hybrids survived to flower and the survivors produced ~1.5 times more flowers (Table 9).

Table 9: Summary of results from reciprocal transplant field experiments. The percentage of plants surviving (Early Survival) and rosette diameter (mm) in late April are listed for inland, coast, and F1 hybrid plants at the three field sites. Survival to flowering is the amount of plants, which were planted in the field as seedlings and survived to flower. Number of flowers is the number of flowers produced of only plants that survived to flower. Means +/- standard errors are list with sample sizes in parentheses.

		Early Season Traits		Fitness Components	
Site	Type	Early Survival	Rosette diameter	Survival to flowering	Number of flowers
Mendocino	Inland (LMC)	68%(100)	36.00+/-2.06 (68)	57% (100)	3.53+/-0.90 (57)
Inland	Coast (SWB)	62% (99)	14.48+/-2.17 (61)	1% (99)	1.00 (1)
	F1 Hybrid	85% (98)	41.36+/-1.86 (83)	45% (98)	2.755+/-0.90 (44)
Mendocino	Inland (LMC)	49% (69)	31.56+/-4.54 (34)	17% (69)	2.83+/-5.85 (12)
Coast	Coast (SWB)	90% (69)	42.77+/-3.36 (62)	38% (68)	10.23+/-3.98 (26)
	F1 Hybrid	87% (68)	45.44+/-3.44 (59)	72% (68)	15.20+/-2.90 (49)
Big Creek	Inland (CAN)	52% (85)	18.07+/-2.21 (40)	22% (85)	3.37+/-2.08 (19)
Inland	Coast (BCB)	51% (84)	15.36+/-2.24 (39)	0% (84)	0.00 (0)
	F1 Hybrid	49% (79)	26.24+/-2.27 (38)	13% (79)	7.00+/-1.51 (10)

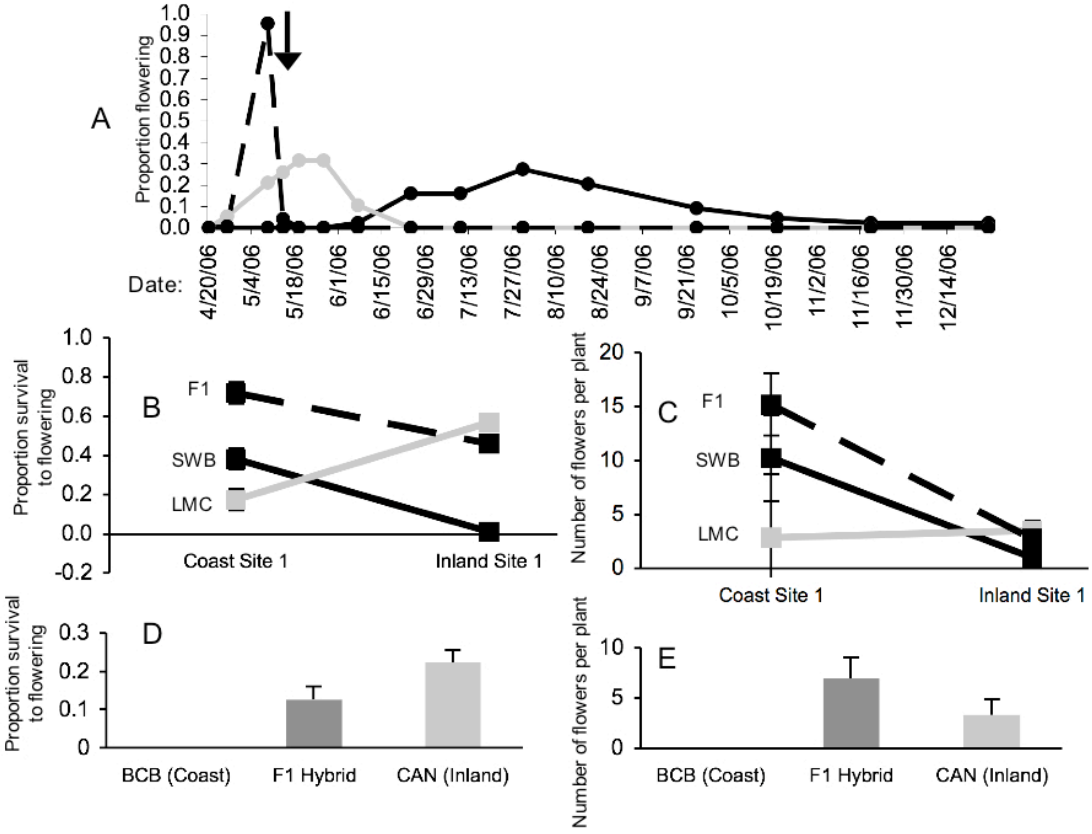

Figure 12: Results of reciprocal transplant experiment in Northern California. (A) Flowering time differences between coast and inland populations was assessed through a reciprocal transplant experiment between coast site 1 and inland site 1. There was no overlap in flowering time between inland (LMC) plants at inland site 1 (dashed line) and coast (SWB) plants at coast site 1 (black line). When placed into artificial sympatry, there was a slight overlap between inland immigrants (LMC) at coast site 1 (gray line) with coast plants at this site (black line). Only one coast (SWB) immigrant survived to flower at inland site 1 and it overlapped in flowering with 4% of the inland plants at this site (dashed line). (B) Survival to flowering of coast (black), inland (gray), F1 hybrids (dashed) between sites in experiment 1. (C) Number of flowers produced by plants that survived to flower between sites in experiment 1. (D) Survival to flowering of coast (BCB, black), inland (CAN, light gray), and F1 hybrids (dark gray) at inland site 2. (E) Number of flowers produced by plants that survived to flower at inland site 2. All error bars denote one standard error.

2.3.5 Salt spray tolerance

In the field, leaf damage (presumably due to salt spray) significantly differed among coast, inland, and F1 hybrids at coast site 1 in early spring (April 26th, 2006, $F_{2,148}$ =23.08, $P < 0.0001$, Fig. 13a). Leaf damage was an order of magnitude greater for

inland (LMC) plants than coast (SWB) plants. Both coast and F1 hybrids had significantly less leaf damage than inland (LMC) plants in the *post-hoc* analysis ($P < 0.05$, Fig. 13a). Further, 36% (13 out of 36) of the inland (LMC) plants alive in late April were subsequently killed by leaf damage. Leaves and flowers were severely wilted on all of the inland that survived to flower ($N = 12$) at coast site 1.

The greenhouse salt tolerance experiment confirmed that coastal populations are more tolerant to salt spray than inland populations. Inland plants accumulated salt spray damage at nearly three times the rate of coast plants ($F_{1,148} = 244.69$, $P < 0.0001$, Fig. 13c). Further, coastal plants survived salt spray treatment about twice as long as inland plants ($F_{1,149} = 155.65$, $P < 0.0001$, Fig. 13d). There was also significant variation in leaf damage ($F_{4,148}$=19.71, $P < 0.0001$) and time to mortality ($F_{4,149} = 12.46$, $P < 0.0001$) among populations within coast and inland habitats. F1 hybrids between coast (SWB) and inland (LMC) populations were significantly more tolerant than LMC, but

just as salt tolerant as SWB in a Tukey-Kramer *post-hoc* analysis ($P < 0.05$, Fig. 13c,d).

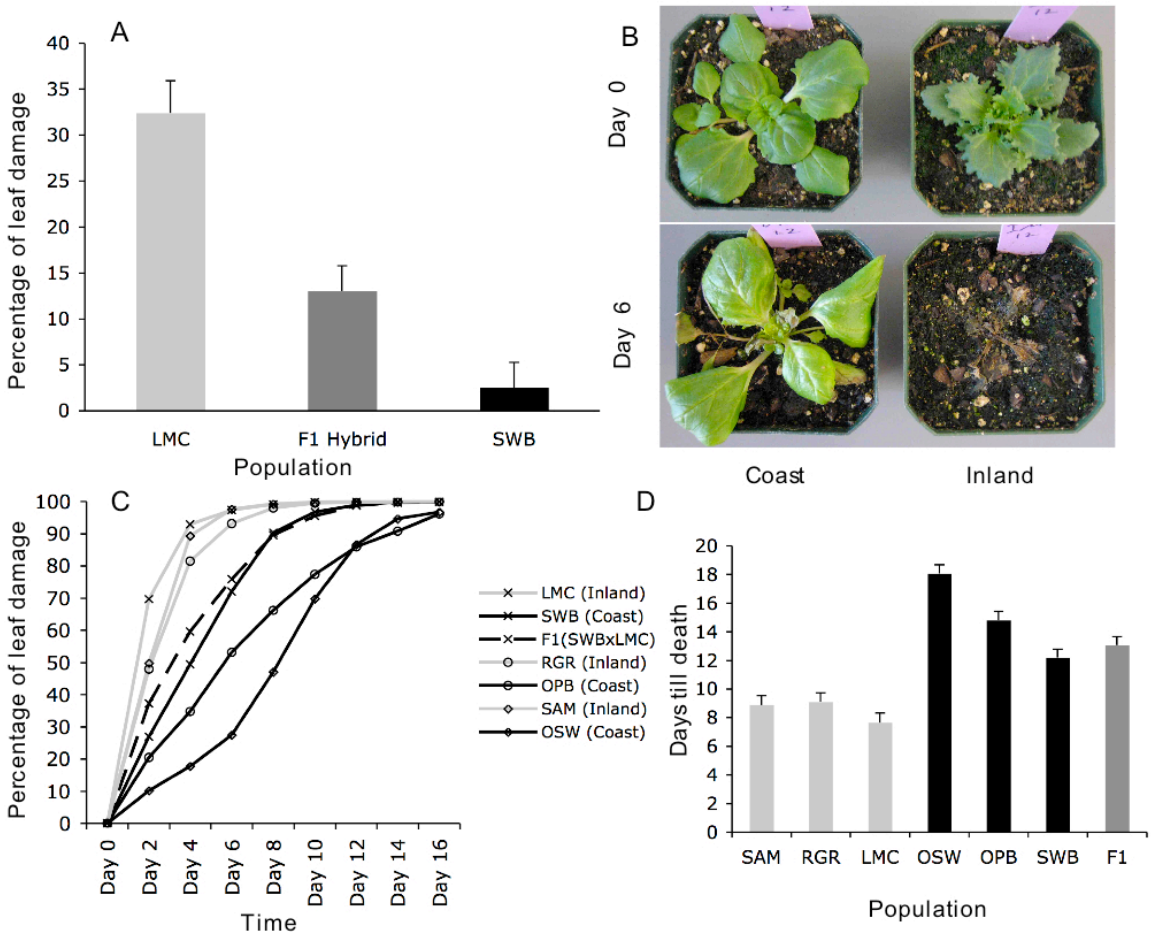

Figure 13: Salt spray tolerance differed among coast (black), inland (light gray), and F1 hybrids (dark gray, cross between LMC and SWB). (A) In the field, leaf damage was significantly greater for inland plants than coastal plants and F1 hybrids at coast site 1 ($F_{2,148} = 23.08$, $P < 0.0001$, Tukey-Kramer comparison of means with $\alpha = 0.05$). In the greenhouse: (B) Inland plants (right) had lower tolerance to 500 mM NaCl solution than coast plants (left). (C) Accumulation of leaf damage, from application of 500 mM NaCl solution, was significantly different between coast and inland populations ($F_{1,148} = 244.69$, $P < 0.0001$). (D) Days till mortality was also significantly different between coast and inland populations as well ($F_{1,149} = 155.65$, $P < 0.0001$). All error bars denote one standard error.

2.3.6 Intrinsic postzygotic reproductive isolation

Seeds germinated in all 20 of the interpopulation crosses, and 84 out of 86 the F1 progeny were fully viable. Only one cross out of 20 resulted in inviable F1 hybrids, and none of the crosses led to hybrid lethality. The cross leading to inviability was

conducted between the OSW (coast) and the SAM (inland) populations. The inviability appears to be a form of hybrid necrosis (Bomblies & Weigel 2007) as plants were dwarfed with most of the leaves turning brown. Overall, the affected F1 family contained two inviable hybrids and three fully viable hybrids. A cross between viable members of this F1 family and another independent F1 family also resulted in inviable plants (3 out of 20) in the F2 generation. The inviability was more severe in the F2 generation than the F1 generation, and F2 plants were severely dwarfed. I did not observe any additional inviable hybrids in the F2 generation of other population crosses.

2.3.7 The strength of reproductive isolating barrier

The level of reproductive isolation between coast and inland populations was near complete, with prezygotic barriers much stronger than postzygotic barriers in their contribution to overall reproductive isolation. For each barrier listed in Table 10, reproductive isolation ranged from ≤ 0 (unrestricted gene flow) to 1 (complete reproductive isolation). Since there was no overlap in flowering time between coast plants in coastal habitat and inland plants in inland habitat, there is complete ($RI_{F,A}$ = 1.000) temporal flowering isolation between habitats. However, it should be kept in mind that this barrier only applies to gene flow through pollen movement, not seed dispersal. Selection against immigrants was near complete ($RI_{I,I}$ = 0.999) for coastal immigrants moving into inland habitat, and was also strong for inland immigrants moving into coastal habitat ($RI_{I,C}$ = 0.874). The one immigrant coast (SWB) plant that survived to flower in inland habitat flowered during the last 4% of the flowering period of the native inland plants. However, since this immigrant's only flower was opened while native plants were still flowering I calculated that there was no reproductive isolation ($RI_{FS,I}$ = 0.000). At coast site 1, twelve inland plants survived to flower, but only 10.5% of the total immigrant (LMC) flowers were open at the same time as native

coast (SWB) plants ($RI_{FS,C}$ = 0.895). Extrinsic postzygotic isolation or ecological selection against hybrids provided a small barrier to gene flow into inland habitat ($RI_{EP,I}$ = 0.233), but was non-existent in coastal habitat ($RI_{EP,C}$ = -1.801), where hybrids outperformed local plants. The strength of intrinsic postzygotic isolation between coast and inland populations is very low (RI_{IP} = 0.023).

Table 10: The strength of reproductive isolating barriers between coast and inland populations. Data calculated from results of reciprocal transplant studies and controlled crossing experiments. Barriers range from negative values (unrestricted gene flow) to 1 (complete reproductive isolation). The first column is the strength of the barrier reducing gene flow into coastal populations, while the second column is the restriction on gene flow into inland populations.

	Strength of barrier	
Isolating barrier	Coast	Inland
Temporal flowering isolation among habitats (RI_{FA})	1.000	1.000
Selection against immigrants (RI_I)	0.874	0.999
Temporal flowering isolation in sympatry (RI_{FS})	0.895	0.00*
Intrinsic postzygotic isolation (RI_{IP})	0.023	0.023
Extrinsic postzygotic isolation (RI_{EP})	-1.801	0.233

*This was calculated for one suviving coast plant in inland habitat

2.4 Discussion

Our results indicate that coastal perennial and inland annual populations of *M. guttatus* comprise two distinct morphologically and molecular genetically diverged groups. Nearly complete prezygotic isolation through a combination of geography, selection against immigrants, and flowering time isolation likely maintains the genetic differentiation of these coast and inland groups. In the inland habitat, the onset of the summer drought prevents the successful immigration of late flowering coastal perennial plants, while early flowering annual life-history (Hall & Willis 2006) and low salt tolerance of inland plants inhibits their immigration into coastal habitat. Overall, these

results are consistent with the role of habitat-dependent natural selection in the formation of widespread reproductively isolated species.

2.4.1 Patterns of morphological and molecular genetic differentiation

Our analysis of morphological data clearly demonstrates that *M. guttatus* populations derived from coast and inland habitats are genetically distinct (Fig. 8; Table 4). This pattern is consistent with the findings of other botanists, who have long recognized a common suite of morphological differences between coast and inland plant populations of a variety of species (Turesson 1922a; Stebbins 1950; Clausen 1951; Clausen & Hiesey 1958; Grant 1981). Clausen (1951) argued that the consistent distinctness of coastal populations in species such as *Layia platyglossa*, *Potentilla glandulosa*, and *Achillea borealis* suggested that morphologically distinct coastal populations should be collectively classified as ecological races. Ecological races of plants are somewhat analogous to host races in insects (Dres & Mallet 2002; Berlocher & Feder 2002; Funk et al. 2002; Nosil 2007), and are defined as a large set of populations that are restricted to a particular type of habitat by abiotic and/or biotic factors (Clausen et al. 1951; Clausen & Hiesey 1958).

My analyses of the highly variable molecular genetic markers, using STRUCTURE and POPULATION GRAPH, are also consistent with the hypothesis that coast and inland populations of *M. guttatus* constitute distinct ecological races. My analysis implies that geographically distant coastal populations (> 1000 km apart from each other) are more closely related to each other than they are to adjacent inland populations, which are often only a few kilometers away. Despite the clear overall divergence between coast and inland races, only a relatively small proportion of the total molecular variation is partitioned between races in the AMOVA. Interestingly, far more of the variation in morphology (PC1), flowering time, and nine other traits (Table

4) is partitioned between coast and inland groups than genetic variation (Table 8). A high level of quantitative trait divergence coupled with modest levels of genetic divergence is consistent with habitat-mediated selection driving morphological evolution (Spitze 1993; McKay & Latta 2002). It should be noted that my common garden experiment was performed after only one generation in a common environment and thus, does not properly control for maternal effects. However, individual lines grown for multiple generations under common growth conditions maintain morphological distinctness between coast and inland habitats (data not shown).

Average pairwise F_{ST} for inland populations is high in both this study (0.32) and a previous study (0.32, Awadalla & Ritland 1997). Even greater F_{ST} was observed among coastal populations (0.55). Since F_{ST} depends inversely on within population diversity (Nei 1973, 1987; Charlesworth et al. 1997), the elevated among coast population F_{ST} is likely the result of consistently low within coast population heterozygosity. It should be noted that rare long-distance migration likely occurs among *M. guttatus* populations through dispersal by water (over 4.5 kilometer in a single season, Levine 2001), deer (over a kilometer in a season, Vickery et al. 1986), and birds (Lindsay 1964). Even so, restricted migration among all population of *M. guttatus* may increase the partitioning of molecular genetic variation among populations and individuals while diminishing the between group (coast versus inland) partition of genetic variation in the AMOVA analysis.

While most populations were correctly assigned to coastal or inland habitats using STRUCTURE, three of the populations (ANR, BSR, ORO) did not cluster with other populations from their respective habitats. One possible explanation is that these populations are derived from the admixture of coast and inland populations. The two misassigned inland populations (ANR & BSR) are located along the Eel River in Northern California. ANR appears to be admixed based on STRUCTURE results and

BSR may have been colonized by coastal plants from the nearby tidal estuary region of the Eel River. Further, BSR contained two different sized morphotypes, which may indicate that it is a mixed coast and inland population. Interestingly, the morphology of the ANR population is much like coastal plants in that these plants have many lateral branches and adventitious roots. More detailed sampling along the Eel River will be necessary to determine the evolutionary history of populations in this region. The misassignment of the ORO (coast) population may simply be the result of being located at the very southern end of my sampling area.

2.4.2 Habitat adaptation and reproductive isolation between coast and inland ecological races

The results of this study and a previous reciprocal transplant experiment (Hall & Willis 2006) clearly demonstrate that total reproductive isolation between coast and inland populations of *M. guttatus* is nearly complete as a result of local adaptation. The allopatric distribution of coast and inland populations may be a byproduct of ecological range limits of these two races and suggests that ecogeographic isolation (as defined by Schemske 2000; Sobel et al. 2010) may be the key barrier to gene flow. Even so, as explained above, rare dispersal of pollen or seed probably occurs between coast and inland populations since they are often in about as close proximity to each other as are different populations within habitats. Even if rare dispersal occurs, the high estimates of reproductive isolation due to immigrant inviability and flowering time differences will sharply limit the opportunity for gene flow.

Intrinsic postzygotic isolation between coast and inland populations appears to be insignificant. One caveat of my analysis of intrinsic postzygotic isolation is that I did not assess the strength of crossing barriers, hybrid sterility, or levels of transmission ratio distortion (but see Hall & Willis 2005). However, I were able to successfully

generate F2 hybrids in all intercrosses among F1 progeny. Therefore, the rate of hybrid sterility is likely to have limited effect on gene flow.

Extrinsic postzygotic isolation is thought to be a common byproduct of local adaptation of different species (Schluter 2001; Rundle & Whitlock 2001; Nosil et al. 2005). However, heterosis is known to offset the effect of intrinsic incompatibilities in early generation hybrids (Rhode & Cruzan 2005), and may offset extrinsic effects as well (Rundle & Whitlock 2001; Lowry et al. 2008b). Indeed, the high levels of coast/inland F1 hybrid performance in the field indicates that hybrids could actually facilitate gene flow into coastal habitats, while at most it would act as a weak barrier to gene flow into inland habitats. The elevated fitness of F1 hybrids in coastal habitat may be a product of heterosis combined with high F1 salt tolerance (Fig. 13). The effects of heterosis may be mitigated in inland habitat, where flowering time is key to fitness, since F1s flower later than inland parentals (Fig. 8b; Table 10).

While extrinsic postzygotic isolation is insignificant for coast/inland F1 hybrids, extrinsic postzygotic isolation may act on advance generation hybrids, where the effect of heterosis will be diminished (Burke & Arnold 2001; Rundle & Whitlock 2001). Even so, the patchy distribution of *M. guttatus* populations means that a large number of hybrids would be backcrosses to local individuals. Such backcrosses would be composed of genetic segregrants that are 50% homozygous for locally adapted alleles and heterozygous at the rest of their loci. Backcrosses to locally adapted populations typically perform as well as parent species under field conditions (Burke & Arnold 2001, Johnston et al. 2001; Rundle 2002; Lexer et al. 2003a,b,c). This also appears to be the case in a previous reciprocal transplant between coast and inland *M. guttatus* populations, where backcrosses to locally adapted populations performed just as well as locally adapted parentals (Hall & Wills 2006). Therefore, it appears unlikely that

extrinsic postzygotic isolation plays a major role in restricting gene flow between coast and inland populations.

Even though extrinsic postzygotic isolation may not be strong overall, particular genomic regions may not be able to introgress between habitats due to selection in advanced generation hybrids, while neutral loci introgress more readily (Wu 2001; Turner et al. 2005). Although it appears from my marker data that most genomic regions show at least some divergence, alternative alleles at loci involved in flowering time or salt tolerance may show even more restriction between habitats. I are currently in the process of creating near-isogenic lines with flowering time and salt tolerance QTLs to determine if habitat-dependent selection can restrict the spread of adaptive loci.

Although it is clear that prezygotic barriers result in essentially complete reproductive isolation between these ecological races, I did not calculate cumulative total isolation or proportional contribution of each barrier to total isolation, unlike several previous studies that have quantified reproductive isolating barriers (Ramsey et al. 2003; Coyne & Orr 2004; Nosil et al. 2005; Kay 2006). Such calculations, which are based on multiplicative functions, are not appropriate in most cases because sequential barriers are often not independent (Martin & Willis 2007). Further, my analysis of reproductive isolation was only based on a few field sites and reproductive isolation may be weaker in other geographic locations. Such context-dependent reproductive isolation could explain the apparently admixed populations (BSR, ANR, ORO) in the STRUCTURE analysis. I also did not measure all possible reproductive isolating barriers, which is important for a comprehensive understanding reproductive isolation (Lowry et al. 2008b). Finally, it is not clear in my case how much weight should be given to ecogeographic isolation versus temporal isolation and isolation due to immigrant inviability, since the frequency of rare dispersal events is notoriously difficult to study.

2.4.3 The origin, maintenance, and reproductive isolation of ecological races

Ecological races have long been thought of as an intermediate stage in process of plant speciation (Clausen 1951; Clausen & Hiesey 1958; Grant 1981; Barrett 2001). While my results demonstrate essentially complete prezygotic isolation and suggest that the coastal perennial and inland annual races of *M. guttatus* are in fact distinct biological species, the process by which most ecological races form, maintain their genetic distinctness, and accumulate further reproductive isolating barriers remains poorly understood.

Ecological races may be the product of a single evolutionary event or races may be derived from multiple geographically disjunct parallel evolution (speciation) events driven by repeated evolution of the same reproductive isolation mechanisms (Schluter & Nagel 1995; Rundle et al. 2000; Nosil et al. 2002; Rajakaruna et al. 2003). my molecular genetic results suggest that the coastal populations of *M. guttatus* may be the result of a single evolutionary origin since coastal populations consistently had a lower allelic diversity than inland populations. Further, populations throughout the coastal range appear to have a subset of the alleles of the inland race (Fig. 11; Table 6). Of course the hypothesis of parallel origins of ecological races is difficult to reject, since gene flow among parallel lineages can wipe out the molecular signal of such a history. Further, the low diversity of coastal populations of *M. guttatus* may also be the result a lower effective population size due the narrow band of suitable habitats along the Pacific coast, and their perennial, potentially clonal life-history.

Ecological races are thought to maintain their genetic integrity as a result of habitat mediated natural selection (Clausen 1951; Clausen & Hiesey 1958; Schemske 2000). Local adaptation can directly reduce gene flow through selection against immigrants between individual populations (Nosil et al. 2005; Rundle & Nosil 2005;

Nosil 2007) or through species-wide ecogeographic reproductive isolation as a result of the evolution of range limits of species (Mayr 1947; Clausen 1951; Schemske 2000; Ramsey et al. 2003; Husband & Sabara 2004; Kay 2006). However, studies of other ecological races will be necessary to draw any general conclusions about the relative importance of different types of reproductive isolating barriers.

Although the coastal and inland races of *M. guttatus* appear to show approximately complete reproductive isolation, the process by which ecological races become good species remains unclear. If ecological races actually are intermediates in the process of speciation, then there must be a mechanism by which additional reproductive isolating alleles spread between races to complete the speciation process. Levin (1993, 1995) argues that most intrinsic incompatibility alleles are at best mildly deleterious, such as those derived from underdominant chromosomal rearrangements, and will only be fixed in local populations through drift (Lande 1979, 1985). Therefore, Levin (1993, 1995) concludes that plant speciation must be initiated and completed in local populations. However, recent studies suggest that genic incompatibilities are frequently involved in intrinsic postzygotic isolation between plant species (Fishman & Willis 2001; Sweigart et al. 2006, 2007; Bomblies et al. 2007; Moyle 2007; Case & Willis 2008; reviewed in Bomblies & Weigel 2007 and Lowry et al. 2008b) and may be driven by natural selection or genomic conflict (Macnair & Christie 1983; Ting et al. 1998; Presgraves et al. 2003; Orr et al. 2007). If incompatibilities in plants are indeed driven by natural selection or genomic conflict, then incompatibility alleles may readily spread between widespread ecological races (Kane & Rieseberg 2007) and facilitate the conversion of ecological races into good species. Future research is clearly needed to resolve this issue.

Over a half century ago, Clausen (1951) envisioned that future studies and comparative analysis from the local population through ecological races to good species

would facilitate a general understanding of how ecology and geography interact to create new species. The rapid development of modern molecular techniques and expansion of genomic resources to many taxa make these prospects only brighter.

3. Genetic and physiological basis of adaptive salt tolerance divergence between coastal and inland *Mimulus guttatus*

3.1 Introduction

The natural landscape contains a heterogeneous array of environments that drive the adaptive differentiation of populations (Linhart & Grant, 1996; Kawecki & Ebert, 2004; Lexer & Fay, 2005; Schemske & Bierzychudek, 2007). Local adaptation to this habitat variation is thought to involve the composite of multiple phenotypic traits, each with a complex genetic basis, that have evolved in response to the mosaic of environmental factors that define habitats (Kawecki & Ebert, 2004; Keurentjes et al., 2008; Karrenberg & Widmer, 2008; Pauwels et al., 2008). Numerous reciprocal transplant experiments have demonstrated that locally adaptive population differentiation is a common phenomenon (Turesson, 1922a; Clausen, 1951; Grant, 1981; Linhart & Grant, 1996; Kawecki & Ebert, 2004; Lexer & Fay, 2005; Hereford 2009). However, most reciprocal transplant studies do not permit the determination of the traits responsible for local adaptation. Further, recent laboratory studies that have discovered loci and even genes putatively involved in particular adaptations (Colosimo *et al*., 2005; Hoekstra & Coyne, 2007; Stinchcombe & Hoekstra, 2008; Via & West, 2008) rarely test the effects of those loci on fitness under field conditions (Gardner & Latta, 2006; Verhoeven *et al*., 2004, 2008; Barrett *et al*., 2008; Stern & Orgogozo, 2008). The relationship between locally adaptive traits, their underlying genetic architecture, and selection in nature remains poorly understood.

Local adaptation is defined as a form of genotype by environment interaction, with genotypes from local populations outperforming foreign transplants (Linhart & Grant, 1996; Kawecki & Ebert, 2004; Lexer & Fay, 2005). How individual loci combine to cause local adaptation is largely unknown (Kawecki & Ebert, 2004; Keurentjes et al.,

2008; Karrenberg & Widmer, 2008). One possibility is that local adaptation is mediated by the net effects of loci that perform well in local habitat but are deleterious in foreign habitats (Fry *et al*., 1998; Kawecki & Ebert, 2004; Gardner & Latta, 2006). Such genetic tradeoffs could be caused by linkage to deleterious loci or antagonistic pleiotropy of adaptive loci, especially for traits that incur a physiological cost (Strauss et al., 1999; Kawecki & Ebert, 2004; Roff & Fairbairn, 2007). Alternatively, local adaptation may be caused by combination of loci that individually have fitness effects in one habitat but are effectively neutral in alternative habitats. The few field experiments that have quantified the fitness effects of loci across habitats do not support the hypothesis of local adaptation being caused by tradeoffs at individual loci (Weinig *et al*., 2003; Verhoeven *et al*., 2004, 2008; Gardner & Latta, 2006).

Coastal perennial and inland annual ecological races of *Mimulus guttatus* (yellow monkeyflower) occur throughout western North America and are locally adapted to their respective habitats (Hall & Willis, 2006; Lowry *et al*., 2008a). The inland habitat of annual *M. guttatus* is characterized by the rapid onset of a hot and dry summer drought (Lowry *et al*., 2008). Inland annual plants escape from this drought through early flowering (Hall & Willis, 2006; Lowry *et al*., 2008a; Wu *et al*., 2010). In contrast to inland habitat, persistent fog maintains low temperatures, high soil moisture, and reduces plant transpiration in coastal habitat during the summer drought (Corbin *et al*., 2005; Hall & Willis, 2006; Lowry *et al*., 2008a). In this way, coastal habitat favors the later flowering and perennial growth of the coastal race. The genetic basis of this flowering time divergence has now been established through Quantitative Trait Locus (QTL) mapping (Hall *et al*., 2006). However, while drought is not a major factor for coastal plant populations, they are inundated by persistent salt spray from the Pacific Ocean (Boyce, 1954; Barbour, 1978).

In a reciprocal transplant field experiment, inland annual *M. guttatus* plants transplanted into coastal habitat were observed to have much higher rates of leaf necrosis than coastal perennial plants, presumably due to salt from soil and/or oceanic spray (Lowry *et al*., 2008a). Subsequent laboratory experiments confirmed that salt water spray causes leaf necrosis and found that coastal populations of *M. guttatus* are genetically more tolerant to this salt stress than inland populations (Lowry *et al*., 2008a).

While the degree of salt spray tolerance of vegetation differs between coastal perennial and inland annual *M. guttatus*, salt spray also contributes to higher concentrations of salt in the soil (Lowry *et al*., 2008a). Further physiological assays are necessary to determine the contribution of such soil salinity to overall salt tolerance. Salt spray can enter the aboveground portion of a plant through the cuticle or stomata, which means that shoot tissue must tolerate high concentrations of toxic Na^+ ions (Boyce, 1954; Bukovac, 1973; Zobel & Nighswander, 1990; Griffiths & Orians, 2003). Similarly, plant tolerance to soil salinity is often mediated by the Na^+ tolerance of shoot tissue, but alternatively can involve tolerance to osmotic stress or the exclusion of Na^+ from the shoot (Munns & Tester, 2008). Osmotic stress acts to inhibit transpiration and the growth of the aboveground portion of a plant (Greenway & Munns, 1980; Fricke & Peter, 2002). One way that plants cope with this osmotic stress is to accumulate salt to come into osmotic balance with the surrounding environment (Munns & Tester, 2008). However, accumulation of too much Na^+ can lead to toxic concentrations in the shoot (Berthomieu *et al*., 2003; Munns *et al*., 2006; Rus *et al*., 2006). Therefore, soil salinity tolerance often involves a delicate balancing act of Na^+ accumulation and exclusion (Zhu, 2000; Munns & Tester, 2008).

Despite the importance of salt tolerance to the local adaptation of coastal populations, nothing is currently known about the genetic basis of salt tolerance in *M.*

guttatus. Since salt tolerance can involve multiple mechanisms, it is necessary to first determine which physiological traits contribute to salt tolerance before proceeding with genetic analysis. While there has been extensive work on salt tolerance across many other plant taxa, especially agricultural crops (Flowers, 2004; Bhatnagar-Mathur *et al*., 2008; Munns & Tester, 2008; Yamaguchi & Blumwald, 2005), very little is known about the genetic basis of adaptation to salt stress in coastal habitat (but see Rus *et al*., 2006), which is characterized by salt spray. Furthermore, it is currently unknown whether loci involved in salt tolerance adaptations to coastal habitat have effects on fitness in inland habitat.

In this paper, I examine the physiological basis and genetic architecture of local adaptation of *M. guttatus* to salt stress in the coastal habitat. First, I determine which mechanisms contribute to salt tolerance through multiple physiological assays. To discover loci involved in salt tolerance, I map salt spray tolerance and leaf Na^+ concentration QTLs using recombinant inbred lines (RILs) derived from a cross between a pair of coastal and inland populations (Hall & Willis 2006). I then determine whether these same loci play a role in local adaptation through a combined analysis of this new genotypic data with fitness data from a previously published reciprocal transplant experiment (Hall & Willis, 2006) to determine whether there are genetic tradeoffs across habitats for salt tolerance QTLs.

3.2 Methods

3.2.1 Mechanisms of soil salinity tolerance

To determine the mechanisms of physiological salt tolerance differences between a pair of coastal perennial and inland annual populations of *M. guttatus* I conducted a hydroponic experiment using various concentrations of NaCl and evaluated osmotic stress responses, Na^+ accumulation, as well as tissue tolerance. The coastal population

(DUN) is located in coastal sand dune habitat in the Oregon Dune National Recreation Area (43˚ 53' 35"N 124˚ 08' 16"W). The inland population (IM) is located in montane habitat on Iron Mountain in the Oregon Cascade Mountains (44˚ 24′ 03"N 122˚ 08' 57"W). Seeds of the inbred lines IM62 and DUN10 were planted in Fafard 4P soil and stratified at 4˚ C for a week. Seeds were then moved to the Duke University greenhouses for germination. Seven days after germination, seedlings were transplanted into 2.5-inch square pots that contained perlite. Transplanted seedlings (75 IM and 75 DUN) were moved to a growth chamber with 8 hour periods of light at 22° C and 16 hour periods of dark at 18° C. Plants were randomized into 30x18x10 cm plastic bins, with five IM and five DUN plants per bin (bin = block). Half-strength Hoaglands solution (pH = 6.0) was added to each block as a growth media, and solution was changed every third day to maintain a consistent concentration of nutrients in the solution. Salt treatment was initiated 14 days after transplantation. NaCl was added to the half-strength Hoaglands solution to produce treatment solutions. Sets of three blocks were randomly assigned treatments consisting of 0 mM, 25 mM, 50 mM, 100 mM, or 150 mM NaCl solution for a total of 15 blocks (3 blocks per each of the 5 treatments). Salinity concentrations were selected based on a recent review (Munns & Tester, 2008).

To examine ecotypic differences in the effects of osmotic stress on plants, I measured growth of one newly emerged leaf every two to three days after the initiation of the salt treatment as suggested by Munns & Tester (2008). These measurements were conducted for 14 days, but were subsequently terminated because many plants began to senesce in the higher concentration treatments (100 mM, 150 mM). To test for differences in the growth of young leaves between genotypes (DUN vs. IM) across treatments I conducted a two-way repeated measures MANOVA of leaf length data from all time points during the experiment.

To test for differential shoot accumulation of Na^+ ions, I harvested the entire aboveground biomass of randomly selected plants in the 0 mM and 100 mM NaCl bins 15 days after the initiation of the salt treatment. I harvested two plants of each type from each block for a total of 24 plants. I also collected 10 DUN and 10 IM plants, which were grown in 0 mM NaCl half strength Hoaglands solution for mapping of leaf sodium concentration QTLs (below). Collected tissue from the 44 samples were briefly submerged in 0.05% Triton, followed by a rinse in deionized water, placed into 15 mL tubes (VWR International), and dried in an oven for 24 hours at 90° C. Dried samples were shipped to Purdue University for ionomic analysis (Baxter *et al*., 2007). To determine if DUN and IM constitutively differ in concentrations of Na^+, and to test whether there is interaction between genotypes across treatments for Na^+ concentration, I conducted a two-way ANOVA with shoot ion concentration data from the 0 mM and 100 mM treatments. Because the concentration of K^+ across treatments is often associated with salt tolerance (e.g. Chen *et al*., 2007), I also conducted the same two-way analysis on the shoot concentration of K^+ ions.

To determine if ecotypes differ in tissue tolerance to NaCl, I photographed blocks every two to three days for 50 days after the initiation of the salt treatment. Subsequent analysis of photos was used to establish the date of death (100% leaf necrosis) for each plant. According to Munns and Tester (2008), measurement of the time to senescence of leaves is a good assay of shoot tissue tolerance to Na^+. I tested for differences between genotypes within each of the five treatments by survival analysis, where data was censored for plants that survived longer than 50 days. All analyses of salt tolerance mechanisms were preformed in JMP 7.0.1 (SAS, Cary, NC).

3.2.2 RIL Genetic map

To map salt tolerance QTLs and study fitness effects of those QTLs in the field, I genotyped previously constructed RILs. RILs were made through reciprocal crosses between an inbred inland montane (IM62) line and a field collected coastal dune (DUN) line and inbred for six to eight generations (Hall & Willis, 2006). To select markers for genetic mapping of RILs, I screened the inbred IM62 and DUN10 lines for polymorphism in hundreds of PCR-based markers. Markers used in this study are exon-primed intron-crossing (EPIC) markers derived from expressed sequence tags (ESTs). I evaluated polymorphism in terms of variation in the length of PCR products, which is typically caused by indel variation in the introns. The development of these markers is outlined elsewhere (Fishman *et al*., 2008) and primers can be found at the website *www.mimulusevolution.org*. Each primer pair included a forward primer fluorescently labeled with VIC, HEX or FAM (Invitrogen, Carlsbad, CA). Polymorphic markers were then tested in multiplex PCR reactions with 3-5 other markers. PCR products were subjected to capillary electrophoresis and fragment analysis on an ABI 3730xl DNA Analyzer. The size of the amplified fragments was scored using the programs GENEMAPPER (Applied Biosystems, Foster City, CA) and GENEMARKER (SoftGenetics, State College, PA).

We used an iterative process to assemble the linkage map for this study. Through regenotyping markers, genotyping additional markers with known locations in gaps between markers, and additional map assembly attempts I arrived at a final set of markers. Over the course of all iterations, I identified 239 markers that were polymorphic and amplified successfully in test multiplexes. These multiplex sets were used to genotype 186 RILs (113 with the DUN cytoplasmic background and 73 with the IM background), which had been used in the previous field experiment (Hall & Willis,

2006). Assembly of the linkage map was conducted with the program JOINMAP (Stam, 1993) using the Haldane mapping function with the default Maximum Likelihood settings. In addition, I used JOINMAP to identify markers with non-Mendelian segregation ratios.

3.2.3 QTL mapping of salt spray tolerance and leaf Na^+ concentration

To identify loci involved in salt spray tolerance, the 185 RILs (one RIL failed to germinate) were each tested for their respective tolerance to salt spray. Seeds of RILs and parental genotypes were stratified for two weeks at 4° C, before being transferred to a growth chamber. In total, five replicates of each RIL and 31 replicates of each parental type (DUN and IM) were potted individually in 2.5 inch square pots in Fafard 4P soil. Pots were then fully randomized, placed in flats, and grown in a growth chamber under the same conditions as the physiological experiments (above). This short day treatment prevented flowering of RILs, so that salt tolerance could be assessed across all plants at the same rosette stage of development. Plants were watered and flat positions were haphazardly rotated every other day. A regime of salt spray was initiated 25 days after germination. All plants were sprayed every other day with 5 mL of 500 mM NaCl, following Lowry *et al*. (2008a). The number of days of survival following the initiation of salt spray was recorded immediately before the application of each spray treatment, with death defined as no remaining green tissue. I statistically controlled for variation among flats in my analysis by fitting a single factor ANOVA, with flat as a fixed effect. Using the residuals of this model, I calculated the mean survival time for each RIL line. QTL analysis was then carried out using these centered line means.

To determine the loci involved in leaf Na^+ concentration, I grew 169 of the RILs in half-strength Hoagland's solution (pH = 6.0). Three replicates of each RIL were grown in a fully randomized design under the same growth chamber conditions as in the

physiological experiments. The second set of true leaves were collected from plants 30 days after germination and processed for ionomic analysis as described above. I bulked leaves from all three replicate plants of each RIL into a single tube for this analysis. Resultant Na^+ concentration data was used for QTL mapping. To test for the effect of cytoplasmic background on salt spray tolerance and leaf Na^+ concentration, I used one-way ANOVAs to compare RILs with DUN versus IM cytoplasmic backgrounds.

To map QTLs for survivorship in the growth chamber and leaf Na^+ concentration, I implemented the standard model, forward and backward composite interval mapping method in QTL CARTOGRAPHER 2.5 (Wang *et al*., 2007). The parameters for both analyses included 7 control markers, a 15 cM window size, and a 2 cM walk speed. The data were permuted 1000 times to estimate a significant experimentwise likelihood ratio threshold for each trait (Churchill & Doerge, 1994). I followed the initial mapping with single marker analysis (ANOVA) of loci located closest to the peak of significant QTLs. To determine the amount of variation between parental lines explained by each QTL, I calculated the additive effect of each QTL (2a) and divided it by the parental divergence of that trait. All analyses besides genome-wide QTL mapping were conducted in JMP 7.0.1.

3.2.4 Effects of salt spray tolerance and leaf Na^+ QTLs on fitness in the field

To determine if salt spray tolerance or leaf Na^+ concentration QTLs had an effect on fitness under field conditions, I incorporated the genotypic data into a reanalysis of fitness data of the RILs from a previous reciprocal transplant experiment (Hall & Willis, 2006). In that experiment, the same RILs used for QTL mapping in the growth chamber were backcrossed as the female parent to independent inbred lines from both parental populations (IM494 and DUN10) to eliminate the effects of inbreeding depression (Hall & Willis, 2006). The progeny of the RIL backcross lines are referred to

as BC-IM and BC-DUN. Three replicates of each BC-RIL type and 150 replicates of each parental type were planted at the seedling stage at the DUN field site (June 1, 2003) and at a field site on Browder Ridge (May 31, 2003) in the Oregon Cascades (3.2 kilometers from the IM population site; see Hall & Willis 2006 for details).

We analyzed the effect of QTLs on total lifetime fitness, lambda (λ), which incorporated both survival and seed production for each plant (Hall & Willis, 2006). However, salt stress may be more extreme in later developmental stages of a plant due to the accumulation of Na^+ ions over time (Munns & Tester, 2008). Further, salt spray declines with proximity to the ground (Martin 1959; Randall 1970; Barbour 1978), which is consistent with the observation that *M. guttatus* plants incur more necrosis in coastal habitat when they grow tall and flower (D. B. Lowry *personal observation*). Therefore, I also divided the field fitness data from 2003 into two components for each backcross RIL line: mean survival to flowering and mean seed production per lines with for surviving plants. These two fitness components were analyzed separately for QTL analysis.

We restricted my QTL analysis of the field data to an *a priori* determined set of loci based on the salt tolerance QTLs detected in the growth chamber. For each previously mapped QTL, I conducted a single marker analysis using only the marker within each QTL interval that was centered closest to the QTL peak in the original mapping experiment. All BC individuals were homozygous for coast (DUN) or inland (IM) alleles, depending on backcross direction, for 50% of alleles across the genome and heterozygous for the remaining 50% of alleles. Because of this difference in genetic composition, I analyzed backcrosses to DUN separately from backcrosses to IM. One-way ANOVAs were used to test for associations between genotype and λ as well as the two components of fitness at both field sites. Significant fitness effects in this analysis

were followed up by two-way ANOVAs to test for genotype by environment interactions across the DUN and IM field sites. All analyses were implemented in JMP 7.0.1.

3.3 Results

3.3.1 Mechanisms of soil salinity tolerance

Of the potential mechanisms of soil salinity tolerance, I found no evidence for the evolution of differences in osmotic stress tolerance between DUN and IM, as the growth rates of both ecotypes were affected similarly by the treatment with salt solution (Table 11, Fig. 14a). For DUN plants, growth was reduced by 24%, 30%, 47%, and 62% relative to the control in the 25 mM, 50 mM, 100 mM, and 150 mM treatments, respectively. Similarly, IM growth was reduced relative to the control by 23%, 23%, 36%, and 60% in the same treatments, but there was no interaction between genotypes and treatments.

We did find evidence consistent with differential accumulation of Na^+ between the ecotypes. Leaf Na^+ concentration was constitutively greater for DUN than IM plants and there was a significant interaction between genotypes and treatments (Table 12a, Fig. 14b). Leaf Na^+ concentration was 49% greater in DUN plants (Mean +/- SE = 1205 +/- 144 ppm) than IM plants (809 +/- 164 ppm) in the 0 mM treatment. Leaf Na^+ concentrations were much greater for both ecotypes in the 100 mM treatment. DUN plants (27074 +/- 3572 ppm) had a 44% greater concentration of Na^+ than IM plants (18789 +/- 1976 ppm) in the 100 mM treatment. While DUN and IM plants did not differ significantly in their leaf K^+ concentrations, there was a significant genotype by treatment interaction of K^+ (Table 12b, Fig. 14b). Concentration of K^+ decreased for both DUN and IM in the 100 mM treatment, but this decrease was more pronounced for

DUN (66% reduction in concentration) than for IM (28% reduction in concentration; Fig. 14b).

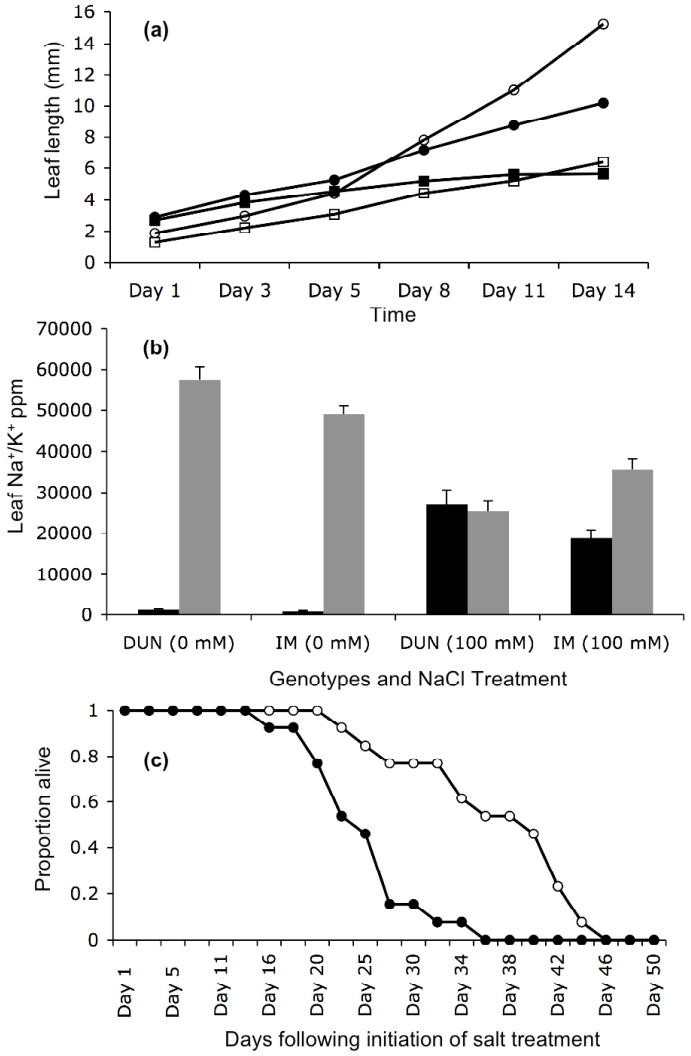

Figure 14: Physiological responses of DUN and IM plants grown in salt (NaCl) solution. (a) Comparison of young leaf growth (osmotic stress tolerance) differences between DUN (open circles) and IM (closed circles) in the 0 mM NaCl treatment, as well as between DUN (open squares) and IM (closed squares) in 150 mM NaCl. (b) Comparison of difference of the shoot concentration of Na^+ (black) and K^+ (gray) ions in DUN and IM plants in 0 mM and 100 mM NaCl treatments. Error bars denote one standard error. (c) Comparison of tissue tolerance differences (survival)

of DUN (open circles) and IM (closed circles) in the 150 mM NaCl treatment over the 50 day period following initiation of salt treatment.

Consistent with a substantial difference in tissue tolerance, the survival of DUN was significantly greater than IM in the 25 mM (Wilcoxon $\chi^2_{1,22} = 5.98$, $P = 0.0144$), 50 mM ($\chi^2_{1,23} = 21.98$, $P < 0.0001$), 100 mM ($\chi^2_{1,11} = 4.80$, $P = 0.0285$), and 150 mM treatments ($\chi^2_{1,24} = 11.54$, $P = 0.0007$, Fig. 14c). The mortality after 50 days for IM was 0%, 62%, 100%, 100%, and 100% in the 0 mM, 25 mM, 50 mM, 100 mM, and 150 mM treatments respectively. For DUN, the mortality rate was 0%, 0%, 0%, 67%, and 100% in the same treatments.

3.3.2 RIL Genetic map

Following multiple iterations of genotyping and map assembly attempts I were able to construct a linkage map that was consistent with other previous and ongoing mapping projects (Fishman et al., 2008; C. A. Wu, unpublished; Y. W. Lee, unpublished). Many markers were difficult to score when genotyped on all of the RILs due primarily to poor amplification. In total, I removed 50 markers from the data set before arriving at a final number of 189 markers. Heterozygotes were removed from the data set for map assembly (Fig. 15) and QTL mapping, which when combined with other sources of missing data resulted in a high level of missing individual data points per marker (Mean +/- SD = 17.85 +/- 11.83%).

Even with sufficient marker coverage, the assembly of linkage group 2 (Lg 2) was not initially possible because of extreme transmission ratio distortion in multiple regions of the linkage group. Assembly of Lg 2 with all of the marker data led to a map that was highly inconsistent with maps of Lg 2 from other linkage studies (Fishman et al., 2008; C. A. Wu, unpublished; Y. W. Lee, unpublished). Transmission ratio distortion on Lg 2 was especially strong in the DUN cytoplasmic background, with nearly complete distortion towards DUN alleles of some markers (Fig. 16). There was also strong

distortion toward IM alleles in the IM cytoplasmic background at other nearby markers along Lg2, but this was not as severe as in the DUN cytoplasmic background (Fig. 16). To assemble the map for Lg 2, I restricted my data set only to genotypes with the IM cytoplasmic background. The final assembly of Lg 2 had a consistent marker order with other mapping studies, where the IM population was used in crosses (Fishman et al., 2008; C. A. Wu, unpublished; Y. W. Lee, unpublished).

Significant transmission ratio distortion ($P < 0.05$) was also observed on a portion of all other linkage groups, except for Lg 10 (Fig. 15). None of these other distortion locations included obvious cytonuclear incompatibilities, as on Lg 2. Of the 189 markers included in the framework map, 91 (48%) were distorted at $P < 0.05$ and 33 (17%) were distorted at $P < 0.001$. Of the significantly distorted markers ($P < 0.05$), 59% had an excess of DUN alleles while 41% had an excess of IM alleles.

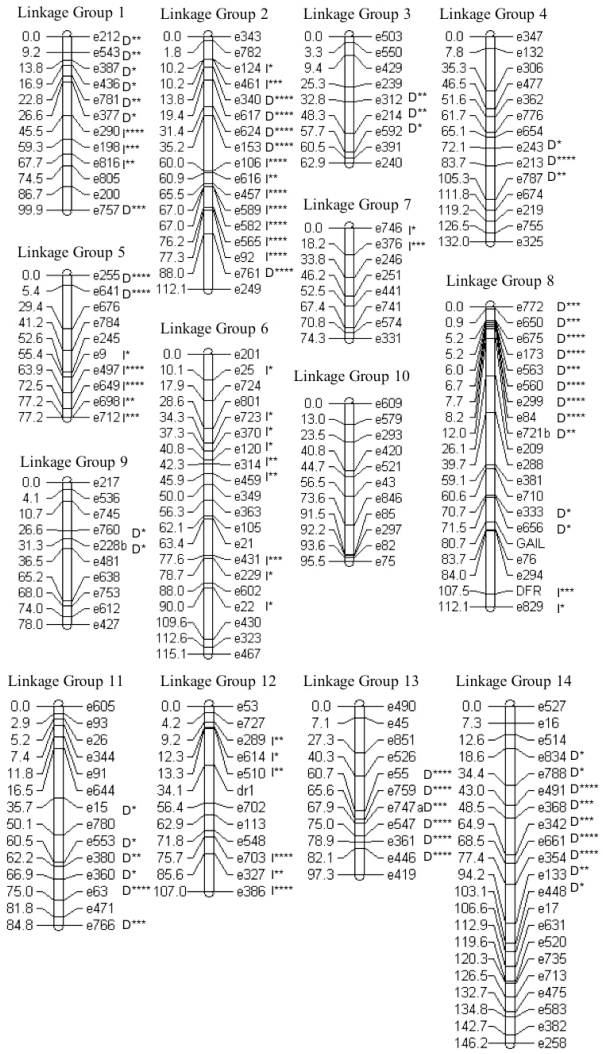

Figure 15: Linkage map of *M. guttatus* for RILs generated from a cross between a coastal perennial (DUN) and inland annual (IM) population. Regions with non-Mendelian inheritance (segregation distortion) are indicated at the right of each linkage group. The direction of segregation distortion (more DUN alleles = D/more IM alleles = I) and the level of significance are reported for each locus (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$). Segregation distortion calculated with both cytoplasmic types combined as one group.

Table 11: Comparison of tolerance to osmotic stress between DUN (coast) and IM (inland) populations. Osmotic stress tolerance was assessed through the growth rate of young leaves after initiation of salt stress.

Source of variation	Num df	Den df	*F*	*P*
Leaf growth (Osmotic stress)				
Population	1	107	1.46	0.2298
Treatment	4	107	8.76	<0.0001
Population X Treatment	4	107	2.05	0.0926

Table 12: Analysis of shoot ion concentration differences between DUN (coast) and IM (inland) populations. Two-way ANOVA of (a) Na^+ and (b) K^+ shoot ion concentration of plants in 0 mM (control) and 100 mM NaCl treatments.

Source of variation	df	*F*	*P*
(a) Concentration of Na^+ ions			
Population	1	13.06	0.0008
Treatment	1	333.28	<0.0001
Population X Treatment	1	10.78	0.0021
Error	40		
(b) Concentration of K^+ ions			
Population	1	0.13	0.7243
Treatment	1	96.12	<0.0001
Population X Treatment	1	15.94	0.0003
Error	40		

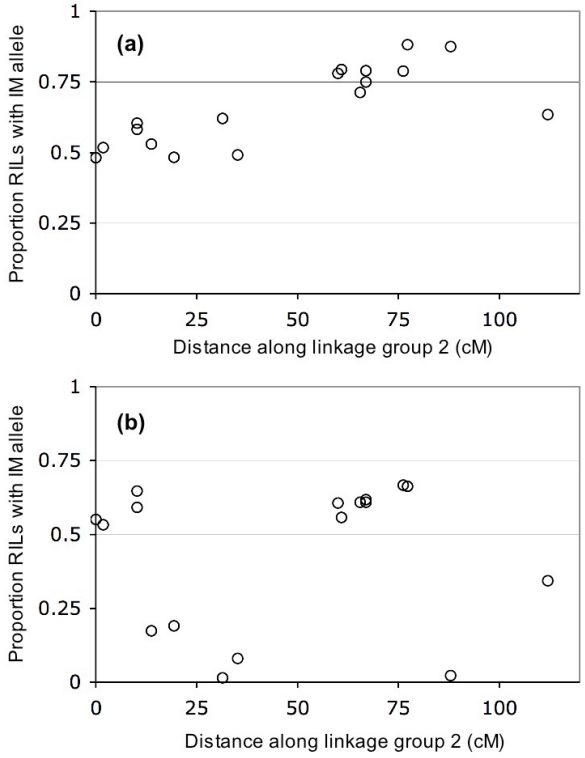

Figure 16: Non-Mendelian inheritance of markers on linkage group 2 when divided into (a) RILs with IM cytoplasmic background versus (b) RILs with DUN cytoplasmic background. All markers used in the assembly of the linkage map were included in this figure. The expectation for normal Mendelian inheritance is 50% IM alleles and 50% DUN alleles across RILs.

A total of 189 markers were used for the construction of the linkage map (Fig. 15). These markers formed 14 distinct linkage groups, which is consistent with previous mapping and cytogenetic studies of *M. guttatus* (Fishman et al., 2001; Hall & Willis, 2005; Fishman et al., 2008). The total map length was 1394.4 cM Haldane, which is marginally shorter than map lengths in other studies in the *M. guttatus* species complex (Fishman et al., 2001; Hall & Willis, 2005). Recombination rate was extremely suppressed on a large portion of Lg 8 in comparison to ongoing mapping studies (C. A. Wu, unpublished; Y. W. Lee, unpublished). In other crosses involving the IM population, the distance between markers e299 and e278 on Lg 8 ranged from 23.3 to 32.0 cM (Y. W. Lee, unpublished). In this study, the distance between e299 and e278 was 2.5 cM. Additional studies have determined that a large chromosomal inversion is the cause of suppressed recombination here (see Chapter 4), and may at least partially account for the short genome-wide map length.

3.3.3 QTL mapping of salt spray tolerance and leaf Na^+ concentration

Consistent with previous research (Lowry *et al*. 2008a), my new experiments showed that salt spray tolerance significantly differed between DUN (Mean +/- SE time of mortality = 20.32 +/- 1.87 days) and IM (13.24 +/- 0.64 days) parentals (t-test; df = 58, $t = 3.49$, $P < 0.001$, Fig. 17a). Cytoplasmic background had a significant effect on the salt spray tolerance ($F = 17.52$, $P < 0.0001$) but not on leaf Na^+ concentration ($P > 0.05$). Counterintuitively, RILs with IM cytoplasmic background had significantly higher salt spray tolerance than RILs with the DUN cytoplasmic background (Fig. 17b). Because cytoplasm had an effect on salt spray tolerance, I controlled for its effect in my QTL analysis.

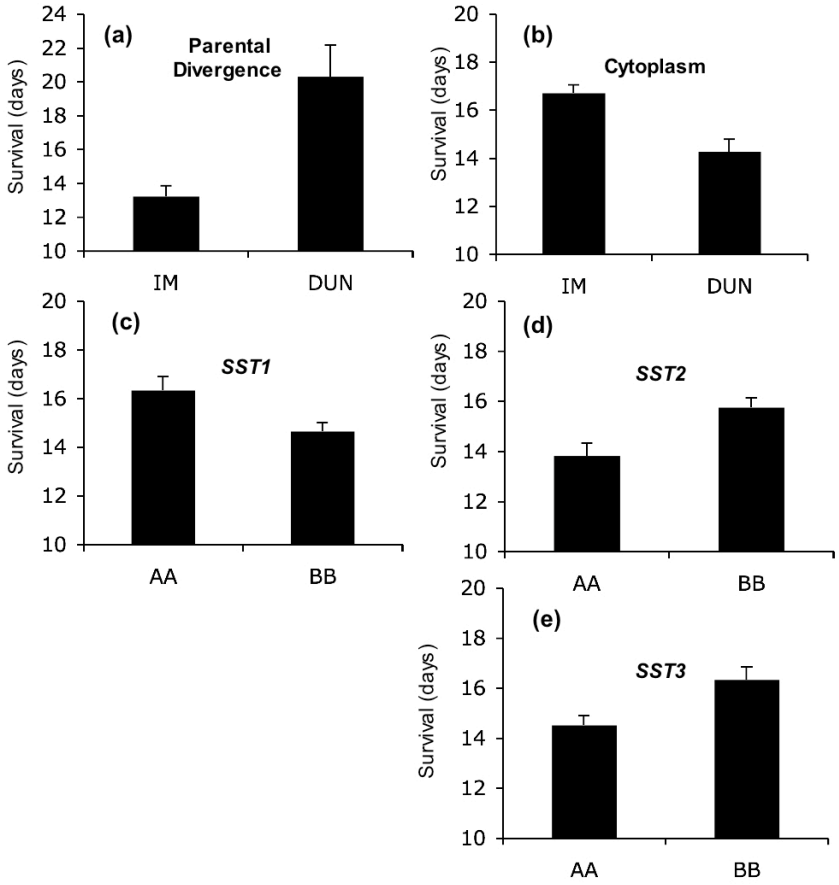

Figure 17: Effects of genotypes on salt spray tolerance (survival) in growth chamber experiment. (a) Difference in survival between IM and DUN parental plants. (b) Difference in survival of RILs with DUN or IM cytoplasmic background. Effect of (c) SST1, (d) SST2, and E) SST3 loci on survival, where AA are RILs homozygous for IM alleles and BB are RILs homozygous for DUN alleles. Note that the y-axis is not set to zero.

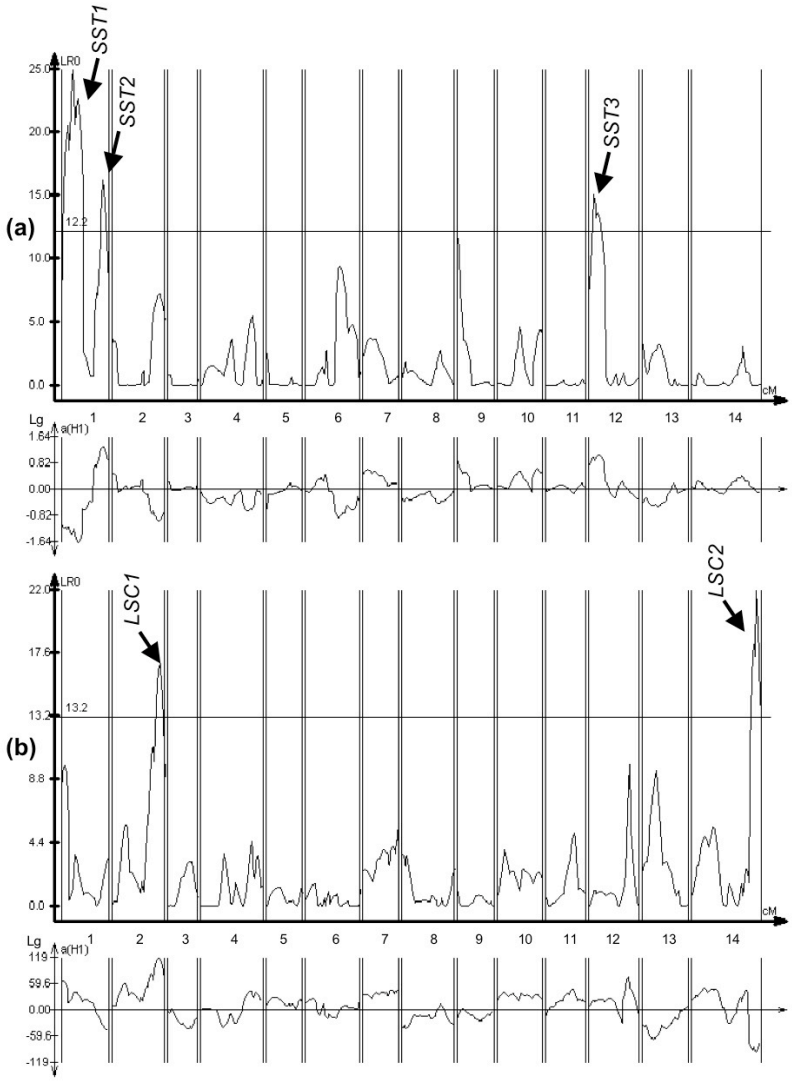

Figure 18: Three significant QTLs were mapped (a) for salt spray tolerance (survival) and (b) for leaf Na+ concentration in growth chamber conditions. Composite interval mapping significance threshold (P < 0.05) of LR = 12.2 for *SST* QTLs and LR = 13.2 for *LSC* QTLs was established by 1000 permutations in QTL cartographer 2.5. Linkage group number and additive effects are displayed below the QTL maps.

We identified three significant SALT SPRAY TOLERANCE (*SST*) (Fig. 18a, Table 13) and two significant LEAF SODIUM CONCENTRATION (*LSC)* QTLs (Fig. 18b). The significance threshold for the *SST* loci was LR = 12.22 and was LR = 13.22 for the *LSC* loci ($P < 0.05$, 1000 permutations). *SST1* was located on Lg 1 centered closest to e543, *SST2* on Lg 1 closest to e757, and *SST3* on Lg 12 closest to e510. For two out of the three *SST* QTLs (*SST2* and *SST3*), RILs homozygous for the DUN allele had significantly greater survival in my experimental assay than RILs homozygous for IM (Fig. 17, Table 13a). For the third QTL, *SST1*, RILs homozygous for the IM allele outperformed RILs homozygous for the DUN allele (Fig. 17, Table 13). *LSC1* was located on Lg 2 between e761 and e249 and RILs with the DUN allele had higher leaf Na^+ concentrations (Fig. 18b). *LSC2* had the opposite effect on leaf Na^+ concentration and was located on Lg 14 closest to e583 (Fig. 18b). It should be noted that there were multiple sharp non-significant peaks detected in the QTL analyses of both traits (Fig. 18).

3.3.4 Effects of salt spray tolerance and leaf Na^+ QTLs on fitness in the field

To determine if QTLs identified in the growth chamber had an effect on fitness in the field, I conducted single marker analysis with the markers e543 (*SST1*), e757 (*SST2*), e510 (*SST3*), e249 (*LSC1*), and e583 (*LSC2*). At the DUN field site, all three *SST* QTLs had a significant effect on lifetime fitness (λ) in the BC-IM lines (Table 13). None of the loci had an effect on λ in the BC-DUN lines ($P > 0.05$). Cytoplasm also had no effect on λ for either the BC-IM or BC-DUN lines. Separation of fitness components revealed that none of the QTLs had a significant effect on survival to flowering.

However, the three *SST* QTLs had a significant effect on seed set of surviving plants for the BC-IM lines at the DUN site (Table 13). For each of the three *SST* QTLs, heterozygous lines produced almost 3-fold more seeds per plant that survived to flower than lines homozyogous for the inland allele (Fig. 19). Interestingly, eight out of the ten top seed producing lines had a least one copy of the DUN allele at all three QTLs. The other two highest fitness lines had a copy of the DUN allele at two of those three loci. Neither of the *LSC* QTLs had a significant effect ($P > 0.05$) on fitness in either genetic background at either field site.

We found no evidence of a genotype by environment interaction for any of the three *SST* QTLs across the DUN and IM field sites ($P > 0.05$). While all three *SST* QTLs affected λ and seed set of surviving plants at the DUN site, none of these three QTLs had a significant effect on fitness at the IM (Browder Ridge) field site ($P > 0.05$; Fig. 19).

Table 13: The effect of three significant salt spray tolerance QTLs on RIL line means for (a) tolerance to salt spray (survival) under controlled growth chamber conditions. Fitness (λ) of BC-IM RILs at the (b) IM and (c) DUN field sites. Seed production of BC-IM RILs that survived to flower at the (d) IM and (e) DUN field sites. The number of lines (N), the divergence of alternative homozygous alleles (2a), the proportion of the parental divergence (2a/diff), as well as F and P values are provided for the growth chamber study. Comparisons in the field were between individuals homozygous for IM alleles or heterozygous for DUN and IM alleles and thus, only the additive effect "a" is given. Because RILs were backcrossed for field experiments, the proportion of the line mean variance explained (r^2) is provided instead of parental divergence.

Source of variation	*N*	2a	2a/diff (r^2)	*F*	*P*
(a) Growth chamber					
SST1 (e543)	179	-1.70	-0.2395	7.33	0.0075
SST2 (e757)	164	1.93	0.2726	8.57	0.0039
SST3 (e510)	179	1.82	0.2570	8.87	0.0033
(b) IM field site (λ)					
SST1 (e543)	178	-0.23	0.0013	0.23	0.6311
SST2 (e757)	162	-0.08	0.0001	0.03	0.8715
SST3 (e510)	175	-0.20	0.0011	0.20	0.6568
(c) DUN field site (λ)					
SST1 (e543)	177	0.06	0.0286	5.15	0.0245
SST2 (e757)	161	0.06	0.0280	4.58	0.0338
SST3 (e510)	174	0.06	0.0280	4.95	0.0274
(d) IM field site (seeds)					
SST1 (e543)	145	1.49	<0.0001	0.01	0.9138
SST2 (e757)	129	-3.93	0.0005	0.07	0.7988
SST3 (e510)	141	5.09	0.0010	0.15	0.7037
(e) DUN field site (seeds)					
SST1 (e543)	155	55.64	0.0334	5.29	0.0228
SST2 (e757)	141	61.97	0.0361	5.21	0.0239
SST3 (e510)	152	57.98	0.0370	5.76	0.0177

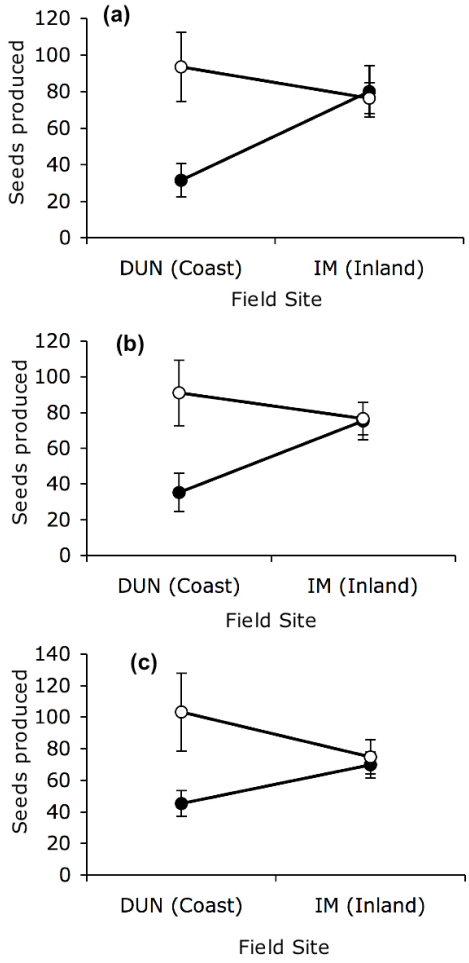

Figure 19: Effects of three salt spray tolerance (SST) QTLs on seed production of plants that survived to flower for RILs backcrossed to IM at the DUN and IM field sites. Effects of (a) SST1, (b) SST2, and (c) SST3 loci on mean seed production. Comparisons made between backcross RILs homozygous (AA) for IM allele (closed circles) or heterozygous (AB) for DUN and IM alleles (open circles). Error bars indicate one standard error.

3.4 Discussion

In this study, I sought to understand the differential response of coastal perennial and inland annual populations of *M. guttatus* to salt stress, its genetic architecture, and the fitness effects of any QTLs involved in salt tolerance in native field habitats. The physiological divergence of the DUN and IM populations appears to involve differential accumulation of Na^+ ions and tissue tolerance of the shoot, but not osmotic stress tolerance. These physiological results are consistent with adaptation of the DUN population to both soil salinity and salt spray. Genetic differences between DUN and IM in salt spray tolerance are due to at least three QTLs of moderate effect, while two other QTLs affect leaf Na^+ concentration. All three of the salt spray QTLs contribute to fitness in coastal habitat but have no detectable fitness effects in inland habitat. Leaf Na^+ concentrations QTLs had no fitness effects at either field site.

3.4.1 The physiology of salt tolerance in coastal populations

Although I had previously determined that coastal perennial and inland annual populations differ in salt spray tolerance (Lowry *et al.*, 2008a), I did not know whether soil salinity tolerance mechanisms contributed to this divergence. Physiological assays in this study suggest a major role for shoot tissue tolerance to Na^+ ions. Tissue tolerance in plants is thought to involve cellular processes such as the sequestration of toxic Na^+ ions in vacuoles (Zhu, 2000), and is consistent with adaptation to soil salinity (Munns & Tester, 2008) or oceanic salt spray (Boyce, 1954).

Both DUN and IM had over an order of magnitude more Na^+ in their leaves in the 100 mM treatment, which suggests that the roots of both ecotypes cannot exclude Na^+ ions under saline conditions. The higher concentration of Na^+ in DUN than IM plants suggests that DUN may be actively accumulating Na^+ ions to come into osmotic balance with the saline coastal soils (Barbour, 1978; Rus *et al.*, 2006; Munns & Tester,

2008). Even so, the leaf growth assays suggest that there is no difference in osmotic stress tolerance between DUN and IM. One possible reason for this finding is that both populations are adapted osmotic stress, but by different mechanisms. Inland annual populations may be adapted to osmotic stress from rapidly drying soils during the summer drought while coast perennial populations are adapted to osmotic stress caused by soil salinity (Hall & Willis, 2006; Lowry *et al*., 2008a). This hypothesis is supported by studies in other coast and inland ecotypes of plants such as the salt brush, *Atriplex halimus*, where tolerance to the osmotic stress of drought and soil salinity differ in their underlying physiological mechanisms (Hu *et al*., 2007; Teixeira & Pereira, 2007; Ben Hassine *et al*., 2008).

Retention of K^+ when subjected to saline conditions is thought to be crucial for salt tolerance of plants and has been found to be predictive of grain yield in crops such as barley and wheat (Wu et al., 1996; Zhu et al. 1998; Ren *et al*., 2005; Chen *et al*., 2007). Unexpectedly, K^+ ion loss was significantly greater in the more salt tolerant DUN plants. Thus, it may be that K^+ shoot concentration is not important for the adaptation of *M. guttatus* to salt stress in coastal habitats.

3.4.2 Genetic basis of salt spray tolerance

Very little is known about the genetic basis of the adaptation of coastal ecotypes to salt spray or soil salinity (but see Rus *et al*., 2006). While RILs homozygous for the DUN allele at *SST2* and *SST3* survived longer in the salt spray treatment, this was not the case for *SST1*. The longer survival of RILs homozygous for IM at the *SST1* locus is consistent with Lexer *et al*. (2003), who found salt tolerance QTLs to act in opposing directions in *Helianthus* hybrids. However, direction of effect of *SST1* could have been influenced by the nature of the salt spray assay. The NaCl solution used in my experiment was concentrated enough to eventually kill all of the plants, making it an

easily measured assay. However, the overall dose is likely greater than that experienced by plants that were exposed to salt spray in coastal habitat. At this high level of salt stress, salt tolerance alleles that are neutral or beneficial under natural field conditions could have negative consequences in the laboratory. This would be especially true for a locus involved in the accumulation of Na^+ ions in order to come into osmotic balance with the environment. Under low soil salinity levels, an allele that elevates Na^+ accumulation would be beneficial, but at higher salinity levels the shoot concentration of Na^+ would become toxic (Zhu, 2000; Munns & Tester, 2008). The QTL *LSC1*, and to a lesser extent *SST1*, appear to show such a tradeoff pattern in a comparison of the direction of effect of these loci on salt spray tolerance and leaf Na^+ concentrations. In other words, these QTLs co-localize with peaks that have opposing effects in the salt spray tolerance and leaf sodium accumulation assays, as seen in Fig. 18. Alternatively, the negative effect of *SST1* in the growth chamber may be caused by an inbreeding depression or hybrid inviability allele. The RILs in the growth chamber experiment were not outcrossed and thus, any recessive deleterious alleles would be homozygous across many lines.

Beyond the significant salt spray tolerance and leaf Na^+ concentration QTLs, I detected many sharp non-significant peaks (Fig. 18) that may also influence these traits. my power to significantly detect these other potential QTLs was likely diminished by three major causes. First, there was a large amount of missing genotypic data in my QTL analysis. Second, the precision of the assays used for trait measurement may have influenced detection of QTLs. Finally, transmission ratio distortion may have played a role in QTL detection. Nearly 50% of loci were significantly distorted in this study and in a previous study that involved a F2 mapping population generated from a cross between the same DUN and IM populations (Hall & Willis, 2005).

While the cause of transmission ratio distortion is unclear for many of the linkage groups in this cross, cytonuclear incompatibilities appear to play a role on Lg 2. Strong distortion occurs in at least one place (markers: e340, e617, e624, e153) and possibly two (markers: e761, e294) in RILs with the DUN cytoplasmic background. Since the *LSC1* QTL is located between e761 and e249, the distortion in this region may have had consequences for the estimation of the effect of this locus in the greenhouse and the field. Interestingly, distortion favoring IM alleles appears in the IM cytoplasmic background (Fig. 2). However, this distortion is less likely to be caused by cytonuclear incompatibilities in the IM background because similar distortion occurs in the DUN background. The distortion toward IM alleles, present in both cytoplasmic backgrounds, appears to be tempered by the cytonuclear effects in the DUN background.

3.4.3 Genetic basis of local adaptation

Local adaptation is often assumed to be caused by alleles that perform well in local habitats but have negative consequences in foreign environments (Hawthorne & Via, 2001; Kawecki & Ebert, 2004). In my study, however, the three *SST* QTLs that had an effect on fitness at the DUN field site did not have a significant effect on fitness at the IM site. Ongoing analysis has also revealed that there are no negative fitness consequences at the DUN site for QTLs that affect fitness at the IM field site (Hall et al. *in review*). Therefore, while there is a genotype by environment interaction for QTLs involved in local adaptation, I found no evidence of negative consequences of QTLs across habitats. This finding is consistent with the handful of other studies that have assessed the fitness effect of a locus between environments in reciprocal transplant experiments (Weinig *et al*., 2003; Verhoeven *et al*., 2004, 2008; Gardner & Latta, 2006).

The collective implication of the few reciprocal transplant QTL studies is that locally adaptive alleles may not have deleterious fitness consequences in other habitats.

If these alleles are truly neutral under other environmental conditions, then they could diffuse unidirectionally into other habitats, since selection acts on them only in one habitat (Gardner & Latta, 2006). Alternatively, these QTLs could have slight deleterious effects in other habitats that were not detected due to statistical power. Even so, the question arises as to whether the different levels selection across habitats, found in this and other studies (Weinig *et al*., 2003; Verhoeven *et al*., 2004, 2008; Gardner & Latta, 2006; Hereford 2009), implies that local adaptation mostly involves the action of non-overlapping sets of loci for each habitat. Answering this question will require detailed genetic analysis and field experimentation, but is crucial to the determination of the ultimate causes of local adaptation (Fry *et al*., 1998; Schemske, 2000; Stinchcombe & Hoekstra, 2008; Keurentjes et al., 2008).

Different but tightly linked genes may underlie QTLs that appear to affect both traits in the lab and fitness in the field, causing spurious associations (Kawecki & Ebert, 2004; Stinchcombe & Hoekstra, 2008). This could be the case for *SST1*, where the IM allele performed better under artificial salt spray conditions, but the DUN allele preformed better at the DUN field site. Cloning of genes that underlie QTLs will help to better understand QTL effects across experiments. Further, as genomic resources and advanced molecular techniques are applied to reciprocal transplant field experiments, the mechanisms of local adaptation should come into focus (Stinchcombe & Hoekstra, 2008; Wu et al. 2008).

4. Adaptive chromosomal inversion involved in life-history shift of *Mimulus guttatus* ecological races

4.1 Introduction

The highly polygenic nature of most complex traits means that populations with similar phenotypes may have different frequencies of alleles underlying those traits. Further, because phenotypic divergence can be achieved through parallel evolution, genetic redundancy, or small shifts in allele frequencies at multiple loci, evolutionary theory predicts that locally adapted populations, experiencing divergent natural selection, may not have fixed allelic differences (Goldstein & Holsinger 1992; Brookfield 1997; Kelly 2006; Novembre & Rienzo 2009; Lowry 2010). If diverging taxa experience ongoing gene flow, then migration will limit the extent of divergence in allele frequencies at loci underlying adaptive traits. Even if alleles are maintained at high frequency by strong selection in one population, opposing selection may be weaker in other populations, facilitating introgression and preventing the fixation of adaptive alleles (Gardner & Latta 2006; Verhoeven et al. 2008; Latta 2009; Lowry et al. 2009).

An additional factor expected to influence allele frequency divergence between locally adapted populations is genetic recombination, which is increasingly thought to play a key role in the maintenance of population and species differences (Noor et al. 2001; Rieseberg 2001; Butlin 2005; Hoffmann & Rieseberg 2008; but see Noor & Bennett 2009; Feder & Nosil 2009). For example, if locally adapted populations or closely related species differ by chromosomal rearrangements such as inversions, then the suppressed recombination between different chromosomal types will maintain multiple locally adaptive genetic changes that might otherwise have been eliminated by the homogenizing force of gene flow (Rieseberg 2001; Bultin 2005; Hoffman & Rieseberg 2008). The converse situation may also occur, as a recent population genetic study

shows that rearrangements can actually facilitate the fixation of novel inversions in the face of gene flow between locally adapted populations when two or more adaptive loci become trapped in a rearrangement (Kirkpatrick & Barton, 2006). Such rearrangements, with the cumulative effects of multiple locally adaptive loci, are predicted to exhibit greater allelic differentiation than individual loci under migration-selection balance (Hoffmann & Rieseberg 2008). Therefore, the fixation of locally adaptive chromosomal polymorphism between diverging taxa is hypothetically more probable than the fixation of adaptive polymorphism in collinear regions.

Adaptive divergence and reproductive isolation of coastal perennial and inland annual ecological races of the yellow monkeyflower, *Mimulus guttatus*, is driven by flowering time adaptations to seasonal drought in inland habitat and salt tolerance in coastal habitat (Hall & Willis 2006; Lowry et al. 2008a; Lowry et al. 2009; Hall et al. *in review*). Coastal perennial plants transplanted into inland annual habitat have severely reduced fitness because they fail to flower before the onset of the summer drought (Hall & Willis 2006; Lowry et al 2008a). In contrast, inland plants flower early and produce far more flowers before the drought becomes too harsh for survival. Later flowering is beneficial in coastal habitats because year-round soil moisture, maintained by fog and cool air temperatures, permits coastal perennial plants to allocate resources to growth instead of reproduction, which leads to greater fitness in the long-term (Hall & Willis 2006; Lowry et al. 2008a; Fig. 20). Previous QTL analysis of morphological and life-history found two large effect *DIVERGENCE* (*DIV*) QTLs and many smaller effect loci to be responsible for differentiation in many traits between a pair of coastal perennial and inland annual populations from central Oregon (Hall et al. 2006; Hall et al. *in review*).

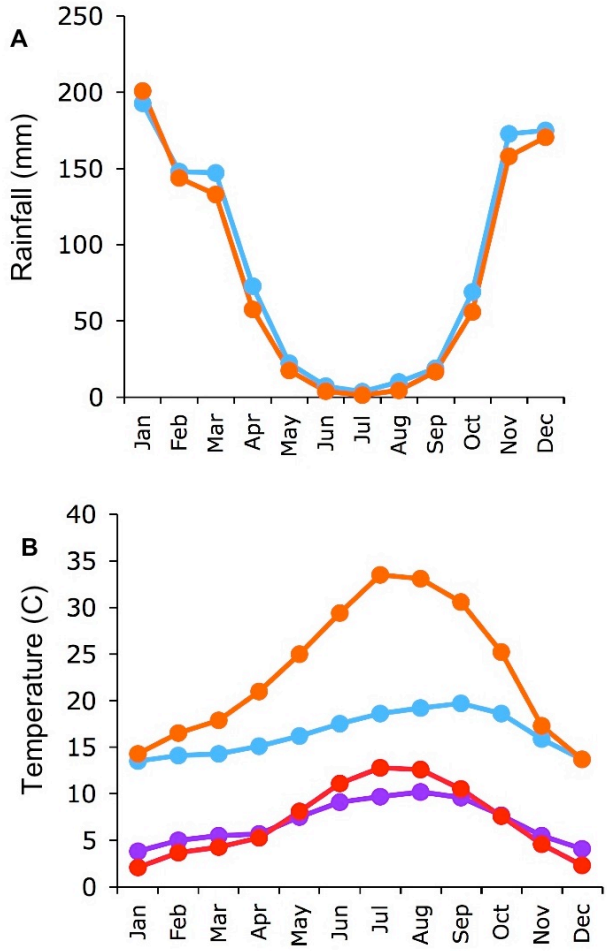

Figure 20: Annual rainfall and temperatures. Thirty year (1961-1990) average monthly data from the closest weather stations (Ukiah: Inland, Point Arena: Coast) to the field sites of the reciprocal transplant experiments. A) Rainfall (mm) in coast (blue) and inland (orange) habitats. B) Average high (coast: blue, inland: orange) and low (coast: purple, inland: red) temperatures.

In this study, I investigated the two largest-effect (Hall et al. 2006) *DIV* QTLs to determine their roles in shaping morphological and life-history divergence among perennial and annual *M. guttatus* populations across western North America. My initial goal was to determine if the alleles underlying the *DIV* loci are fixed between coastal and inland populations. In the process of examining the geographic distribution of *DIV* allelic effects I discovered that *DIV1* mapped to a chromosomal inversion while *DIV2* mapped to a collinear region. I then hypothesized that the inversion at *DIV1* might play

a role in the evolutionary transition between perennial and annual life-histories. I tested this hypothesis by creating an additional series of crosses within and among perennial and annual populations across the range of *M. guttatus*. In addition, I tested whether or not allelic differences at the *DIV* loci underlie local adaptation to coastal perennial versus inland annual habitat through a reciprocal transplant field experiment. My field experiment incorporated near-isogenic lines (NILs) where both *DIV* loci were individually reciprocally introgressed into coastal and inland genetic backgrounds. This design allowed us to test the effects of putatively adaptive loci and genetic background across natural habitat conditions.

4.2 Materials and Methods

4.2.1 Replicated QTL analysis

To determine if the same QTLs contribute to the divergence of morphology and flowering time over the range of the perennial and annual ecological races I carried out a replicated QTL experiment. Here, latitude-paired annual and perennial populations were crossed to create F2 mapping populations. Populations used in this experiment (Table 14) were collected in the summer of 2005 as described in Lowry et al. (2008a). For each population pair I grew 19-24 of each parental type, 17-25 F1 hybrids, and 126-172 F2s. Differences in samples sizes were due to a combination of number of seeds available, germination rate, and space availability. The replicated QTL studies were conducted first with plants from the LMC/SWB and SAM/OSW population pairs in August-October 2006 followed by the CAN/BCB and RGR/OPB population pairs in March-May 2007. Finally, tests were performed on the LMC/BOG population pair in September-November 2009. The plants were grown under 18 hour days at 21 C, 6 hour nights at 16 C, and 30% relative humidity in the Duke University greenhouses. Flowering time, second internode thickness and length, as well as mean corolla width and length of

the first two flowers were recorded for all coastal x inland crosses. The amount of aboveground nodes that produced roots was quantified in the 2007 experiment but not in the 2006 experiment. Only flowering time was measured in the LMC x BOG mapping population.

Table 14: Location of populations used in this study. Descriptive location and GPS coordinates are provided. All populations were used for analysis of the chromosomal orientation of linkage group eight by recombination suppression or marker order analysis.

Type	Pop ID	Location	Latitude (N)	Longitude (W)
M. guttatus	ALA	Himmel Creek, Chichagof Is., AK	58° 00' 26"	135° 44' 55"
(Coast perennial)	TSG	Ted's Spring, Graham Is., BC	53° 25' 07"	131° 54' 56"
	BOB	Botanical Beach, Vancouver Is., BC	48° 31' 42"	124° 27' 03"
	OSW	Oswald West SP, Tillamook Co., OR	45° 45′ 39″	123° 57′ 56″
	HEC	Heceta Beach, Lane Co., OR	44° 08′ 06″	124° 07′ 22″
	DUN	Oregon Dunes, Lane Co., OR	43° 53' 35"	124° 08' 16"
	OPB	Otter Point SP, Curry Co., OR	42° 27′ 50″	124° 25′ 22″
	SWB	Irish Beach, Mendocino Co., CA	39° 02′ 09″	123° 41′ 25″
	PR	Point Reyes, Marin Co., CA	38° 02′ 55"	122° 52′ 10"
	BCB	Big Creek Reserve, Monterey Co., CA	36° 03′ 46″	121° 35′ 31″
M. guttatus	SAM	Saddle Mountain SP, Clatstop Co., OR	45° 57′ 33″	123° 40′ 46″
(Inland annual)	SWC	Sweet Creek Road, Lane Co., OR	43° 57′ 34″	123° 54′ 08″
	IM	Iron Mountain, Linn Co., OR	44° 24′ 03"	122° 08' 57"
	RGR	Rouge River, Curry Co., OR	42° 29′ 21″	124° 12′ 30″
	LMC	Yorkville, Mendocino Co., CA	38° 51′ 50″	123° 05′ 02″
	MED	Moccasin Point Camp, Tuolume Co., CA	37° 48' 53"	120° 18' 42"
	CAN	Big Creek Reserve, Monterey Co., CA	36° 04′ 08″	121° 33′ 05″
M. guttatus	QNT	Lonestar Basin, Teton Co., WY	44° 25' 53"	110° 48' 48"
(Inland perennial)	BOG	Bog Hot Spring, Humboldt Co., NV	41° 55' 25"	118° 48' 21"
	ANR	Angelo Reserve, Mendocino Co., CA	39° 44′ 12″	123° 37′ 51″
	FAL	Fales Hot Spring, Mono Co., CA	38° 21' 18"	119° 24' 39"
M. nasutus	SF	Sherar's Falls, Wasco Co, OR	45° 15' 52"	121° 01' 21"

Table 15: Morphological trait measurements. Hybrid and parental traits for crosses between coastal perennial and inland annual populations grown in greenhouse. Means and standard errors of traits measured for parentals, F1, and F2 generation plants. Sample sizes are in parentheses. Number of nodes producing aboveground roots was measured only in two of the four crosses.

		Class			
Cross	Character	Coastal	F1 Hybrid	F2 hybrid	Inland
BCB/CAN	Flowering time (days)	37.67+/-0.61 (24)	29.60+/-0.54 (25)	34.34+/-0.45 (172)	27.50+/-0.68 (24)
	Stem thickness (mm)	6.79+/-0.13 (24)	4.74+/-0.25 (25)	3.11+/-0.22 (172)	1.28+/-0.11 (24)
	Internode length (mm)	31.97+/-2.24 (24)	113.71+/-4.89 (25)	52.72+/-2.24 (172)	55.47+/-6.96 (24)
	Corolla width (mm)	36.49+/-0.55 (24)	31.20+/-0.47 (25)	24.94+/-0.33 (172)	18.50+/-0.46 (23)
	Corolla tube length (mm)	26.54+/-0.29 (24)	22.19+/-0.26 (25)	19.86+/-0.16 (172)	15.87+/-0.28 (23)
	Aboveground roots	2.06+/-0.33 (24)	0.04+/-0.04 (25)	0.32+/-0.07 (170)	0.00+/-0.00 (23)
SWB/LMC	Flowering time (days)	37.35+/-0.87 (20)	29.6+/-0.74 (20)	31.49+/-0.33 (157)	30.10+/-0.62 (20)
	Stem thickness (mm)	5.23+/-0.15 (20)	3.47+/-0.14 (20)	4.25+/-0.08 (158)	2.95+/-0.17 (20)
	Internode length (mm)	20.59+/-1.80 (20)	74.63+/-3.92 (20)	47.51+/-2.31 (158)	2.24+/-0.10 (20)
	Corolla width (mm)	33.26+/-0.74 (20)	29.16+/-0.88 (20)	28.72+/-0.24 (158)	22.90+/-0.59 (20)
	Corolla tube length (mm)	24.34+/-0.39 (20)	19.94+/-0.30 (20)	19.68+/-0.13 (158)	16.52+/-0.56 (20)
OPB/RGR	Flowering time (days)	33.16+/-0.44 (19)	27.22+/-0.43 (23)	30.17+/-0.15 (126)	23.47+/-0.43 (17)
	Stem thickness (mm)	4.98+/-0.12 (19)	3.27+/-0.10 (23)	2.83+/-0.07 (126)	2.59+/-0.13 (17)
	Internode length (mm)	26.19+/-2.23 (19)	65.14+/-2.19 (23)	42.7+/-1.77 (126)	53.68+/-1.71 (17)
	Corolla width (mm)	33.12+/-0.48 (19)	29.19+/-0.51 (23)	24.97+/-0.32 (126)	24.16+/-0.49 (17)
	Corolla tube length (mm)	24.85+/-1.17 (19)	18.75+/-0.24 (23)	18.49+/-0.19 (126)	16.07+/-0.26 (17)
	Aboveground roots	5.37+/-0.35 (19)	0.65+/-0.24 (23)	0.72+/-0.13 (124)	0.00+/-0.00 (17)
OSW/SAM	Flowering time (days)	43.60+/-0.88 (20)	30.00+/-0.58 (18)	29.94+/-0.27 (157)	26.95+/-0.56 (20)
	Stem thickness (mm)	5.50+/-0.17 (20)	2.96+/-0.46 (18)	3.83+/-0.08 (157)	1.98+/-0.07 (20)
	Internode length (mm)	12.13+/-1.06 (20)	25.08+/-1.16 (18)	22.75+/-0.91 (157)	36.54+/-2.02 (20)
	Corolla width (mm)	31.17+/-0.97 (20)	31.88+/-0.45 (18)	30.64+/-0.30 (157)	26.46+/-0.90 (20)
	Corolla tube length (mm)	23.66+/-0.30 (20)	21.86+/-1.34 (18)	21.48+/-0.16 (157)	17.48+/-0.40 (20)

To be more confident that detected QTLs are fixed among coastal perennial and inland annual populations and robust to differences in genetic background a highly outbred breeding design was employed. For the 2006 experiment, each F2 mapping population was derived from 8-10 parental plants and involved eight different crosses to produce F1s. Due to difficulties with following multiple parental alleles in hybrids in the 2006 experiment, the 2007 F2 mapping populations was only derived from four parental plants, where F1 hybrids were intercrossed. This outbred design, with multiple parentals from each population, contrasts with many QTL studies where only two inbred lines are used in the initial cross. However, it should be noted that only one pair of parental lines was used for the LMC x BOG mapping population.

Tissue was collected from all F2 individuals after flowering and stored in 96-well plates in an -80C freezer. Genomic DNA was extracted from these plants with a

modified hexadecyl trimethyl-ammonium bromide chloroform extraction protocol (Kelly & Willis 1998).

To test for the effect of QTLs on morphological traits and flowering time markers were genotyped in the vicinity of two previously discovered large-effect QTLs (*DIV1* and *DIV2*) on linkage group 8 (Hall et al. 2006). Multiple markers were then screened in each of the two QTL regions. Only two markers, Micro6046 (*DIV1*) and MgSTS76 (*DIV2*), were divergent among parentals and polymorphic in all five F2 mapping populations. Micro6046 primers (F = TGATAATTTGTCCAATTGCGT, R=TCCAAATCAATAATCAAATCCC) were designed using Primer3 (Rozen & Skaletsky 2000; rodo.wi.mit.edu/primer3/) targeted to a microsatellite on a sequenced bacterial artificial chromosome (GenBank accession number 154350257). Primer sequences for all other markers used in this study were designed previously and can be found at www.mimulusevolution.org. All PCR products were subjected to capillary electrophoresis and fragment analysis on an ABI 3730xl DNA Analyzer. The size of the amplified fragments was scored using the program GENEMARKER (SoftGenetics, 2005, State College, PA).

I tested for an association between markers and a composite of five traits with separate two-way MANOVAs for each population pair. In this model the genotypes of Micro6046, MgSTS76, and their interaction were treated as fixed effects. To test for an association between the QTLs and individual traits, I conducted separate one-way ANOVAs. All analyses were carried out in JMP 7.0.1 (SAS, Cary, NC).

4.2.2 Chromosomal inversion geographic distribution

As a part of an ongoing project to map loci involved in divergence between coastal perennial and inland annual populations, I constructed a genetic map of linkage group eight (Lowry et al. 2009). To construct the map, I genotyped recombinant inbred

lines (RILs) that were previously created from a cross between the DUN (coastal perennial) and IM (inland annual) population inbred lines. The creation of these RILs is described in a previous study (Hall & Willis 2006). The map of linkage group eight reveled a tight clustering of markers that were linked to Micro6046 (*DIV1*) and suggested that recombination was suppressed in this region (Lowry et al. 2009). BLAST searches using an unpublished 7X assembly (Department of Energy, Joint Genome Institute, Walnut Creek, CA) of the *M. guttatus* genome were used to confirm that Micro6046 is linked to markers in the region of suppressed recombination.

To determine if the suppression of recombination linked to the *DIV1* QTL is due to a chromosomal rearrangement, I conducted multiple crosses within and among populations from the annual and perennial ecological races (Table 18). I genotyped a set of markers, from within and on both sides of the presumed inverted region (Fig. 25b) to determine the order of markers and whether or not recombination was suppressed.

4.2.3 Creation of near-isogenic lines

Eight plants from each of the SWB (coastal perennial) and LMC (inland annual) populations were selected haphazardly in the field. Each of these sixteen plants were self-fertilized for five generations in the Duke University greenhouses to create independent inbred lines. During this process many lines were lost to inbreeding depression. After five generations of self-fertilization, I selected three of the remaining inbred lines from each population for the creation of NILs. Three independent NILs were seeded through crosses between LMC to SWB lines. This led to the production of three independently derived F1 hybrids, which were then reciprocally backcrossed to the parental lines from which they were derived. Parental lines were also self-fertilized each generation. Thus, all lines became progressively more inbred each generation.

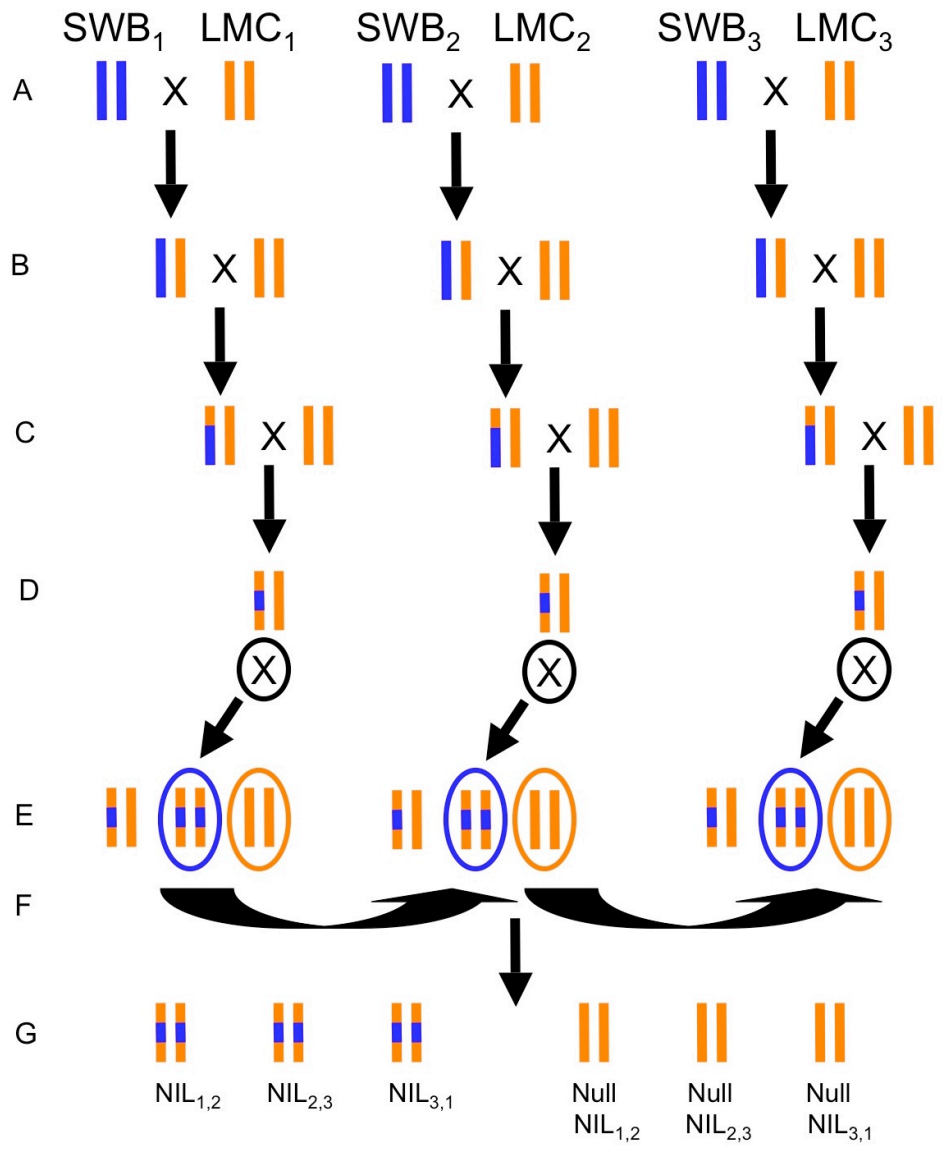

Figure 21: Breeding design for formation of NILs and Null-NILs. Figure shows breeding with the LMC (inland-orange) genetic background. Breeding of plants with SWB (coastal-blue) genetic background not shown. A) Three independent pairs of independently derived inbred LMC and SWB lines were crossed to create F1 progeny. B) F1s backcrossed to parental inbred lines. C) Marker-assisted selection used for four generation of backcrossing to move *DIV* alleles into alternate genetic backgrounds. D) Heterozygous NILs are self-fertilized. E) NILs that were homozygous with (blue oval) and without (orange oval) the introgressed *DIV* allele were selected for further breeding. F) Round-robin cross conducted among the three independent groups to create oubred NILs and Null-NILs. G) Outbred NILs and Null-NILs now ready to be planted into field reciprocal transplant experiment.

To facilitate the introgression of *DIV1* and *DIV2* into each genetic background two flanking makers (*DIV1*:e571, e772; *DIV2*:e381, e829) were genotyped around each QTL and one marker (*DIV1*: e173; *DIV2*: e76) was genotyped in the middle of each QTL. Each generation, 32 backcross hybrids of each type were genotyped for the appropriate markers. Hybrids heterozygous for the three markers were then backcrossed to each parental line. For four backcross generations only hybrids heterozygous for both QTLs were backcrossed. Forth generation backcrosses were then self-fertilized and progeny were used to for crosses to create the final generation. To eliminate the effects of inbreeding depression, the selfed backcross progeny were intercrossed with the other two parallel introgression lines. In this penultimate generation, plants homozygous for either coastal or inland alleles were selected through genotyping to create the final generation. I conducted a round robin cross with the three independent lines that had been selected for the introgressed region (NILs) or for the same allele as its genetic background (Null-NILs). I also conduct the same round robin cross among the parental inbred lines.

4.2.4 Field trial of fitness effects of *DIV* alleles

To test the fitness effects of the *DIV* loci across coast and inland habitats, I set up a reciprocal transplant experiment in Northern California. I planted seeds from all outbred NILs, Null-NILs, and parents in plug trays filled with Ocean Forest Potting Soil (Fox Farm, Arcata, CA) on February 8, 2009 in the Bodega Marine Reserve greenhouse. Seeds were germinated on a regime of misting three times daily for five minutes with no supplemental lighting.

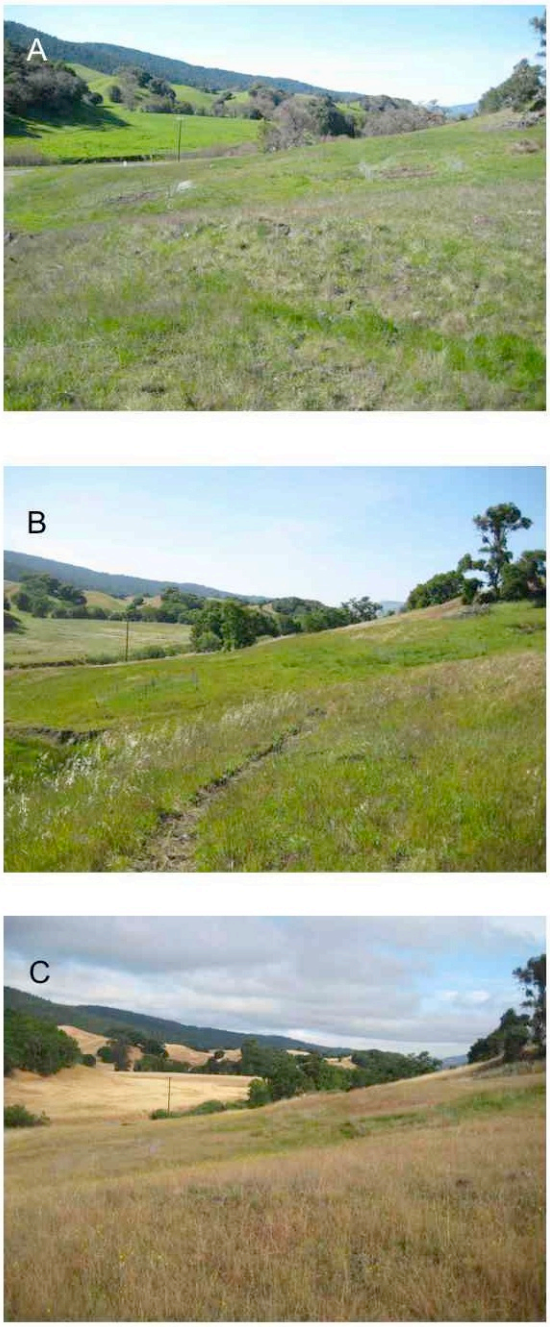

Figure 22: Photos documenting onset of drought at inland field site. View of the inland field site (Boonville, CA) from same perspective over the course of the spring on A) March 3rd, B) May 7th, and C) June 12th, 2009.

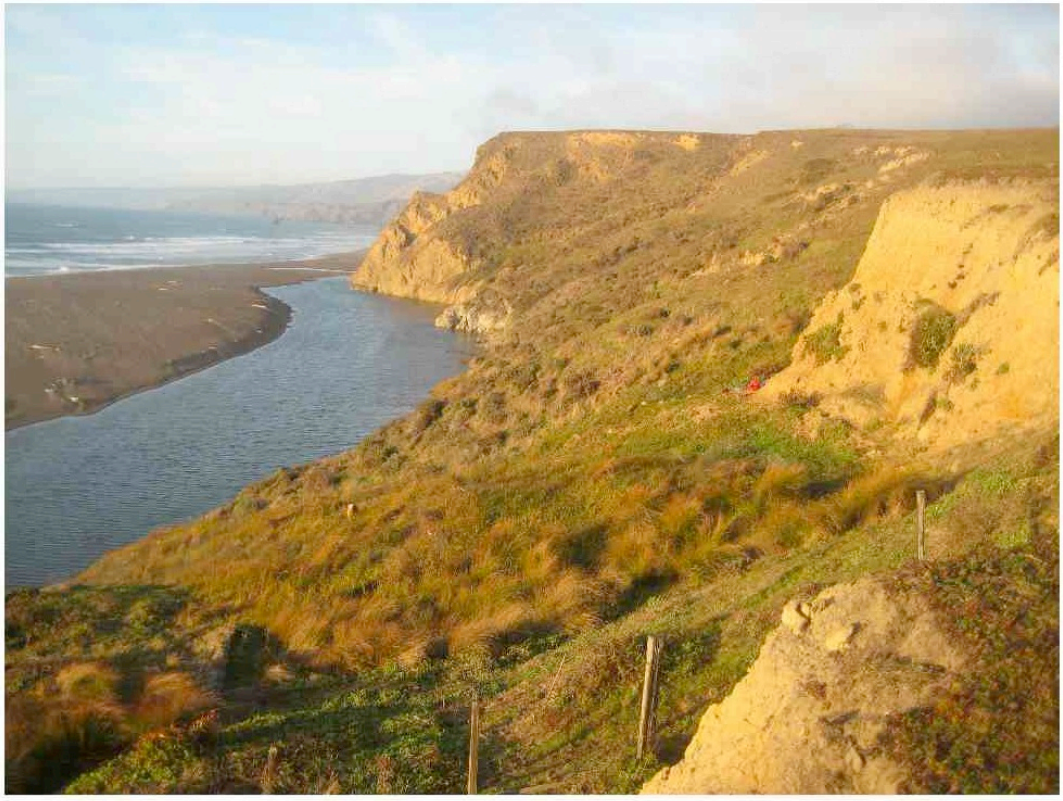

Figure 23: Photo of the coastal field site. Located near Manchester, CA in a seep on a shallow cliff at the edge of the coastal terrace.

We selected one inland annual (Boonville, CA N 38°59.221, W 123°21.059; Fig. 22) and one coastal perennial (Mancester, CA N 39°00.498, W 123°41.637; Fig. 23) field site. These field sites are described in detail in a previous study (Lowry et al. 2008a). I set up 20 blocks per site with 10 replicates of each parental, NIL, and Null-NIL type randomized within each block. Due to low germination success of some lines I were not able to achieve 10 replicates in all blocks. To prevent trampling by livestock I set up exclosures around all of the blocks. Transplanting of seedlings to the field sites was conducted on March 9-16, 2009.

We censused field sites over the course of the experiment at different intervals based on results from a previous field experiment (Lowry et al. 2008a). Survivorship, flowering time, and number of flowers produced were recorded during each census. I

collected data at the inland site until all plants had died as a result of the summer drought by June 23, 2009. At the coastal site, data collection was terminated on October 29, 2009 because flowering had ceased and plots were overgrown with other plant species. For the final coastal census, I carefully removed all remaining plants in the blocks, assessed survivorship, and counted fruits derived from previously open flowers.

4.2.5 Analysis of field data

ASTER (Geyer et al. 2007) was used to analyze the composite of two fitness components: Survival to flowering, which was modeled as Bernoulli (0 or 1) and the number of flowers per surviving individual, which was modeled as zero-truncated Poisson. Initially, the parents of the NILs were analyzed alone to test effects of site, genotype, and site x genotype interaction (local adaptation). Nested null models were used for comparison to test this alternative hypotheses through a likelihood ratio tests.

To determine the effect of DIV loci across habitats, a similar analysis was conducted on the same two components of fitness as the parents but this time using the data from the NILs and Null-NILs. The effects of site, genetic background, DIV allele, and all the interactions of these three factors were tested by fitting a series of nested models and comparing them with likelihood ratio tests. To test the significance of any given factor, null models were compared to alternative models that only differed by the addition of the factor of interest. Finally, maximum likelihood estimates of fitness were calculated with ASTER for all parental, NIL, and Null-NIL types across both field sites.

To test for effects of genetic background and the DIV loci on survival over the course of the season, I conducted survival analysis using ASTER. Here, survivorship for each field census, modeled as Bernoulli, was used for analysis. The dates of censuses as well as the total number of censuses differed between the coastal (N=11) and inland

(N=16) field sites. Therefore, I conducted separate survival analyses at each field site to test the effects of the two *DIV* loci.

To determine if there was an effect of the *DIV* loci on flowering time divergence I conducted two-way ANOVAs within each field site, where *DIV* allele and genetic background were factors. The least square means for the *DIV* allele factor were used as a quantification of the magnitude of the effect of *DIV* alleles on flowering time. This analysis of flowering time was conducted in JMP 7.0.1.

4.3 Results

4.3.1 Effects of *DIV* loci are replicable across multiple independent crosses

To determine if the two previously mapped *DIV* loci are fixed between coastal perennial and inland annual races, I conducted replicated QTL analysis using four latitudinal pairs of coastal and inland populations distributed over an 1100 km range along the Pacific Ocean of western North America (Table 15). Crosses were conducted between paired coastal and inland populations. Single marker analysis was then used to establish if there was an effect of *DIV* loci on a suite of measured morphological traits. If alleles at the *DIV* loci are fixed between coastal and inland races, then there should be an association between these loci and the phenotypes for all population crosses.

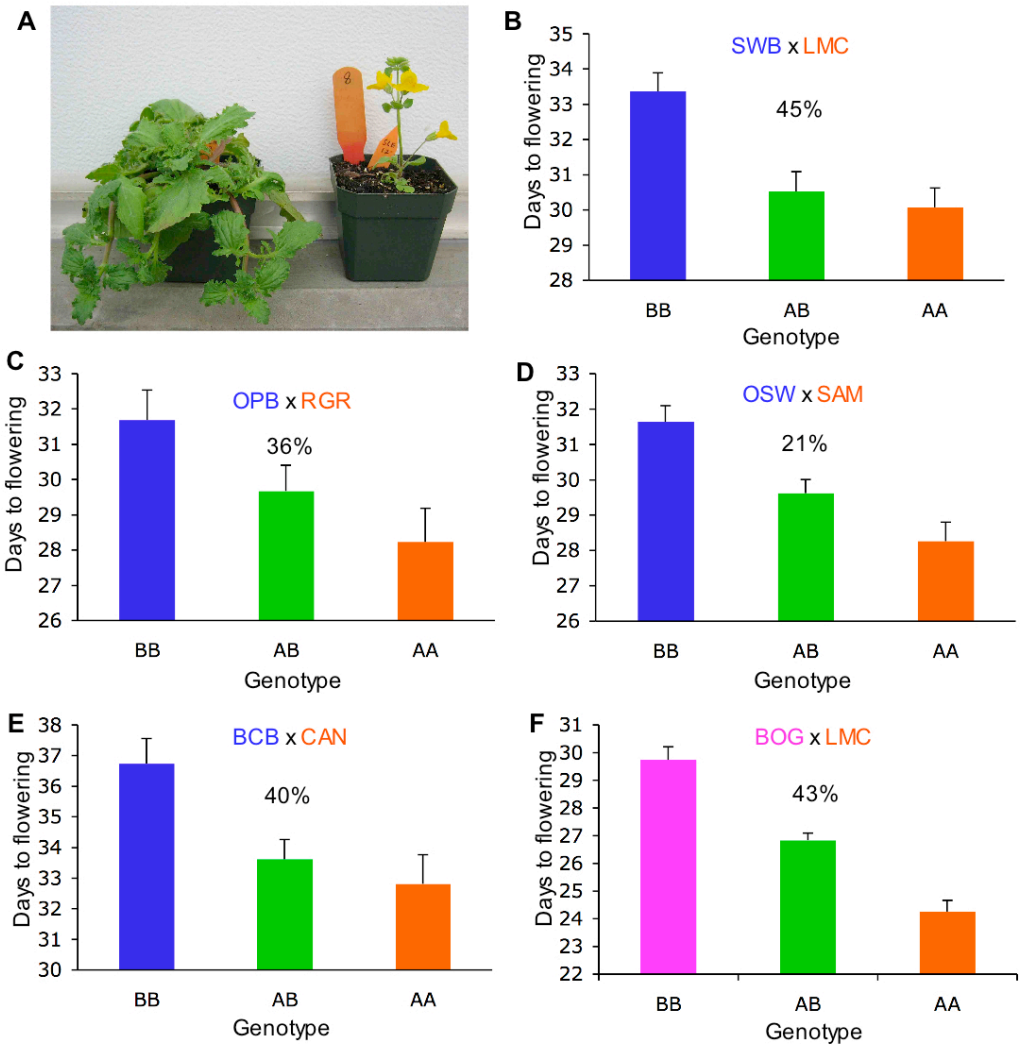

Figure 24: Replicated effect of *DIV1* locus. A) Parental morphological phenotypes were recovered within the F2 mapping populations as seen in a cross between SWB (Coast) and LMC (Inland) populations. B-E) Effect of the *DIV1* locus on flowering time in four independently derived F2 mapping populations between coastal perennial and inland annual populations. F) Effects of *DIV1* locus on flowering time in cross between inland annual and inland perennial populations. The mean flowering times (+/- 1 SE) of F2s that were homozygous for the annual allele (AA), heterozygous (AB) and homozygous for the perennial allele (BB) at Micro6046 are indicated. Also, the percent of parental divergence explained by *DIV1* is presented above each bar graph. Note: y-axes do not originate at zero.

Table 16: Effects of *DIV*1 and *DIV*2 on a composite of morphological traits. Results from replicated QTL analysis in four independent coastal perennial x inland annual populations crosses. MANOVAs were conducted for all four F2 mapping population to determine if QTLs affected multiple traits (second internode thickness, second internode length, corolla length, corolla width). *DIV1* had a significant effect in all population crosses, while *DIV2* had an effect in all but the northernmost cross (SAM x OSW). No evidence for an interaction was found among *DIV*1 and *DIV*2.

Cross: CAN x BCB				
Source	df	Wilk's λ	F	P
DIV1	8, 310	0.670	8.61	<0.0001
DIV2	8, 310	0.841	3.51	0.0007
Interaction	16, 474	0.919	0.83	0.6560

Cross: LMC x SWB				
Source	df	Wilk's λ	F	P
DIV1	8, 280	0.857	2.80	0.0053
DIV2	8, 280	0.881	2.29	0.0218
Interaction	16, 428	0.895	0.99	0.4640

Cross: RGR x OPB				
Source	df	Wilk's λ	F	P
DIV1	8, 218	0.746	4.31	<0.0001
DIV2	8, 218	0.821	2.82	0.0054
Interaction	16, 334	0.872	0.96	0.5011

Cross: SAM x OSW				
Source	df	Wilk's λ	F	P
DIV1	8, 276	0.830	3.37	0.0011
DIV2	8, 276	0.954	0.83	0.5782
Interaction	16, 422	0.930	0.64	0.8555

In the F2 generation, parental phenotypes were recovered in all four coastal perennial x inland annual mapping populations, indicating that few loci of large effect may underlie morphological divergence of these ecological races (Fig. 24a). Both *DIV1* and *DIV2* had a large effect on morphological and life-history divergence of coastal perennial and inland annual populations (Tables 17, 19, 20). *DIV1* significantly affected the composite of measured traits (MANOVA) in all four replicate mapping populations, while *DIV2* was associate with the composite of traits in all but the northern Oregon mapping population (Table 16).

Table 17: The effects of *DIV1* and *DIV2* on flowering time and morphological traits. For each QTL/trait combination: the additive effect (2a), dominance deviation (d), and the proportion of the parental population divergence explained (2a/diff). Data from four independent F2 mapping populations established from crosses between latitudinal paired coastal perennial and inland annual populations.

QTL	Cross	Flowering time			Stem thickness			Internode length			Corolla length			Corrola width			Aboveground roots		
		2a	d	2a/diff	2a	d	2a/diff	2a	d	2a/diff	2a	d	2a/diff	2a	d	2a/diff	2a	d	2a/diff
DIV1	CAN X BCB (N = 167)	3.93**	-1.16	0.40	0.70*	0.04	0.13	-5.60	6.22	-0.23	5.61****	0.36	0.32	2.74****	0.16	0.26	0.73****	-0.14	0.32
	LMC x SWB (N = 153)	3.29****	-1.20	0.45	0.55*	-0.09	0.18	-19.39***	5.98	-0.33	2.04***	-0.47	0.20	0.81*	-0.49	0.10	N/A	N/A	N/A
	RGR x OPB (N = 123)	3.47*	-0.279	0.36	0.65**	0.21	0.27	2.76	1.15	0.10	4.31****	0.59	0.49	2.65****	0.23	0.30	1.12**	-0.26	0.21
	SAM x OSW (N = 151)	3.43****	-0.364	0.21	0.65**	-0.07	0.18	2.96	0.49	0.12	2.62**	-0.43	0.56	1.43**	-0.23	0.23	N/A	N/A	N/A
DIV2	CAN X BCB (N = 172)	3.77**	-1.985	0.38	0.38	-0.22	0.07	-5.91	5.81	-0.24	2.65**	-1.74	0.1498	0.67*	-0.80	0.06	0.44*	-0.16	0.19
	LMC x SWB (N = 158)	3.46**	0.04	0.48	0.48**	0.39	0.16	-21.18**	-5.06	-0.36	2.53***	0.64	0.24	0.69	0.09	0.09	N/A	N/A	N/A
	RGR x OPB (N = 124)	0.66	-1.83	0.07	-0.02	0.17	-0.01	13.00**	8.96	0.47	0.86	0.87	0.10	1.00	-0.01	0.11	-0.17	-0.12	-0.03
	SAM x OSW (N = 157)	0.73	-0.135	0.04	-0.08	-0.03	-0.02	4.62	2.47	0.19	0.88	0.12	0.19	0.02	0.19	0.00	N/A	N/A	N/A

*P<0.05,**P<0.01,***P<0.001,****P<0.0001

In terms of individual traits, *DIV1* explained a large percentage (21%-45%) of the parental divergence in flowering time in all four of the mapping populations (Fig. 24, Table 17). *DIV2* had a similarly large effect (38-48% of the parental divergence) on flowering time in the two California mapping populations, but had no effect on flowering time in two Oregon mapping populations (Table 17). In one cross (LMC x SWB), *DIV1* and *DIV2* together explained 72 % of the coast and inland parental divergence in flowering time (Table 17). *DIV1* was more pleiotropic in its action than *DIV2*, in that *DIV1* significantly affected more of the traits across mapping populations than *DIV2* (Table 17).

4.3.2 Inversion associated with coastal perennial versus inland annual distribution

Previously, I found highly suppressed recombination of markers linked to the pleiotropic *DIV1* locus in a RIL mapping population derived from the IM and DUN populations of central Oregon (Lowry et al. 2009). Such suppression is indicative of a chromosomal inversion, but definitive evidence of an inversion requires additional crosses. If there is a fixed chromosomal inversion between the coastal perennial and

inland annual races, then there should be suppressed recombination in all crosses between the ecological races. To determine if this is the case, I generated new F2 populations by crossing individuals from five coastal perennial and five inland annual populations, including the same populations used for the replicated QTL analysis. Out of 429 F2s screened for recombination (48-96 per population pair; Table 18), I found zero recombination events between markers e299 and e278. Ongoing mapping experiments, involving crosses within the IM population, estimate the distance between markers e299 and e278 is 23.3 to 32.0 cM. Genotyping of additional markers in the region confirmed that no recombination had occurred in the presumed inverted region.

Table 18: Crosses to determine the chromosomal inversion orientation. The number of hybrids genotyped (N), type of hybrid cross, and recombination status are shown for each cross.

Cross	N	Hybrid type	Recombination status
SAM x OSW	95	F2	No recombination
HEC x SWC	48	F2	No recombination
RGR x OPB	95	F2	No recombination
LMC x SWB	95	F2	No recombination
LMC x BOG	282	F2	No recombination
CAN x BCB	96	F2	No recombination
IM62 x QNT	90	F2	No recombination
IM62 x DUN10	186	RIL	Highly supressed recombination[1]
IM767 x PR	148	RIL BC[2]	No recombination
IM1H x IM1L	383	F2	Marker orientation is A type[3]
IM2H x IM2L	382	F2	Marker orientation is A type[3]
IM3H x IM3L	378	F2	Marker orientation is A type[3]
IM62 x SF	480	F2	Marker orientation is A type[4]
IM62 x LMC	93	F2	Marker orientation is A type
IM62 x MED	43	BC	Marker orientation is A type
DUN10 x BOB	46	F2	Marker orientation is P type
DUN10 x TSG	46	F2	Marker orientation is P type
DUN10 x BOG	91	F2	Marker orientation is P type
DUN10 x FAL	101	F2	Marker orientation is P type
DUN10 x ANR	46	F2	Marker orientation is P type
DUN10 x SWB	48	F2	Marker orientation is P type
DUN10 x QNT	91	F2	Marker orientation is P type
SWB x BOG	42	F2	Marker orientation is P type
HEC x ALA	44	F2	Marker orientation is P type

[1]From Lowry et al. 2009

[2]Hybrid populaiton composed of RIL backcrossed to IM767 and then self-fertilized

[3]Crosses between lines within Iron Mountain (IM). Map on www.mimulusevolution.org

[4]Map on www.mimulusevolution.org

Table 19: Effects of the inversion (*DIV1*) locus on components of fitness in the reciprocal transplant experiment. The following are listed: Number of samples (N), days to first flower, number of flowers produced per plants surviving to flower, total number of flowers produced per seedling planted, percentage of plants still alive at the end of the first field season, and percentage of plants surviving to the end of the first season that had yet to flower.

Field Site	Genetic Background	N	Days to flower	Survial to flower	Flowers produced	Total Flowers Produced	End of season	Yet to flower
Boonville	Inland parent	204	52.04 (0.61)	89.71	14.78 (0.88)	13.25 (0.85)	0.00	NA
(Inland)	Inland NIL: Coast orientation	178	57.60 (0.69)	87.08	9.45 (0.65)	8.23 (0.61)	0.00	NA
	Inland Null-NIL: Inland orientaiton	191	53.59 (0.61)	94.76	11.70 (0.64)	11.08 (0.63)	0.00	NA
	Coastal parent	199	77.57 (1.35)	6.03	3.00 (0.72)	0.18 (0.07)	0.00	NA
	Coast NIL: Inland Orienation	195	73.49 (0.92)	51.28	5.26 (0.48)	2.70 (0.31)	0.00	NA
	Coast Null-NIL: Coast Orientation	201	82.54 (3.00)	6.47	3.77 (0.57)	0.24 (0.07)	0.00	NA
Manchester	Inland parent	195	80.56 (2.45)	9.23	4.44 (0.85)	0.41 (0.12)	0.00	NA
(Coast)	Inland NIL: Coast orientation	184	90.22 (2.88)	12.50	6.43 (1.53)	0.80 (0.24)	0.00	NA
	Inland Null-NIL: Inland orientaiton	190	86.00 (3.19)	8.95	4.53 (1.17)	0.41 (0.14)	0.00	NA
	Coastal parent	191	138.08 (2.91)	35.07	16.82 (6.02)	5.90 (2.18)	38.22	43.83
	Coast NIL: Inland Orienation	191	118.14 (2.50)	46.32	12.00 (2.33)	5.49 (1.15)	10.53	5.00
	Coast Null-NIL: Coast Orientation	195	139.46 (3.69)	34.87	12.12 (1.92)	4.23 (0.78)	36.92	45.21

Table 20: Result for *DIV2* locus from reciprocal transplant field experiment. The following are listed: Number of samples (N), days to first flower, number of flowers produced per plants surviving to flower, total number of flowers produced per seedling planted, percentage of plants still alive at the end of the first field season, and percentage of plants surviving to the end of the first season that had yet to flower.

Field Site	Genetic Background	N	Days to flower	Survial to flower	Flowers produced	Total Flowers Produced	End of season	Yet to flower
Boonville	Inland parent	204	52.04 (0.61)	89.71	14.78 (0.88)	13.25 (0.85)	0.00	NA
(Inland)	Inland NIL: Coast allele	204	52.38 (0.66)	90.68	11.85 (0.73)	10.75 (0.70)	0.00	NA
	Inland Null-NIL: Inland allele	191	53.59 (0.61)	94.76	11.70 (0.64)	11.08 (0.63)	0.00	NA
	Coastal parent	199	77.57 (1.35)	7.04	3.00 (0.72)	0.18 (0.07)	0.00	NA
	Coast NIL: Inland allele	199	74.36 (1.16)	27.04	4.89 (0.51)	1.32 (0.21)	0.00	NA
	Coast Null-NIL: Coast allele	196	77.26 (1.40)	19.10	3.50 (0.49)	0.67 (0.13)	0.00	NA
Manchester	Inland parent	195	80.56 (2.45)	9.23	4.44 (0.85)	0.41 (0.12)	0.00	NA
(Coast)	Inland NIL: Coast allele	201	85.78 (2.87)	11.44	3.61 (0.63)	0.41 (0.11)	0.00	NA
	Inland Null-NIL: Inland allele	190	86.00 (3.19)	8.95	4.53 (1.17)	0.41 (0.14)	0.00	NA
	Coastal parent	191	138.08 (2.91)	34.55	16.82 (6.02)	5.90 (2.18)	38.22	43.83
	Coast NIL: Inland alllele	198	122.28 (3.04)	39.90	8.28 (0.73)	3.30 (0.41)	22.22	20.45
	Coast Null-NIL: Coast allele	198	123.44 (2.61)	36.36	7.92 (0.73)	2.88 (0.38)	33.33	34.85

To prove that suppressed recombination is due to a chromosomal inversion, I made crosses among coastal perennial populations and among inland annual populations (Table 18). If there is a chromosomal inversion in the vicinity of the *DIV1* locus, then recombination should occur for these crosses within ecological races. Further, the marker order should be reversed for coastal perennial versus inland annual populations. Recombination did occur in the presumed inverted region in crosses among six coastal perennial populations from California (SWB), Oregon (HEC and DUN), Vancouver Island, BC (BOB), the Queen Charlotte Islands, BC (TSG), and Southeastern

Alaska (ALA). These recombination events also confirmed that marker order is reversed along a stretch of linkage group 8 in the coastal perennial populations relative to inland annual populations. For purposes of clarity, I denote the inland annual arrangement as A and the coastal perennial arrangement as P (Fig. 25). Comparisons to previous and ongoing mapping studies indicate that the inversion spans at a minimum 33.0 cM or ~1.6% of the genome, given the unrealistic assumption of equal recombination across the genome (Lowry et al. 2009; Markers and linkage maps are available on www.mimulusevolution.org).

We also observed recombination in the inverted region in the cross between IM and two other inland annual populations, LMC and MED (Table 18). The marker order for theses crosses was the same orientation (A) as replicated crosses within the IM population. An annual obligate self-fertilizing species *M. nasutus* (SF population), known to be derived from *M. guttatus* (Sweigart et al. 2003), was also found to have A type inversion orientation (Fig. 25b, Table 18).

4.3.3 Inland and coastal perennials share the same functional *DIV1* inversion orientation

Perennial life history is not limited to coastal populations of *M. guttatus*. While many inland populations of *M. guttatus* are annual, inland perennial populations are found in areas of year round soil moisture, such as on the edge of lakes as well as in rivers, hot springs, and alpine habitats (Vickery 1952; Clausen & Hiesey 1958). Coastal and inland perennial *M. guttatus* populations have many traits in common (Vickery 1952; van Kleunen 2007), but the relationship of these ecological races is yet to be evaluated.

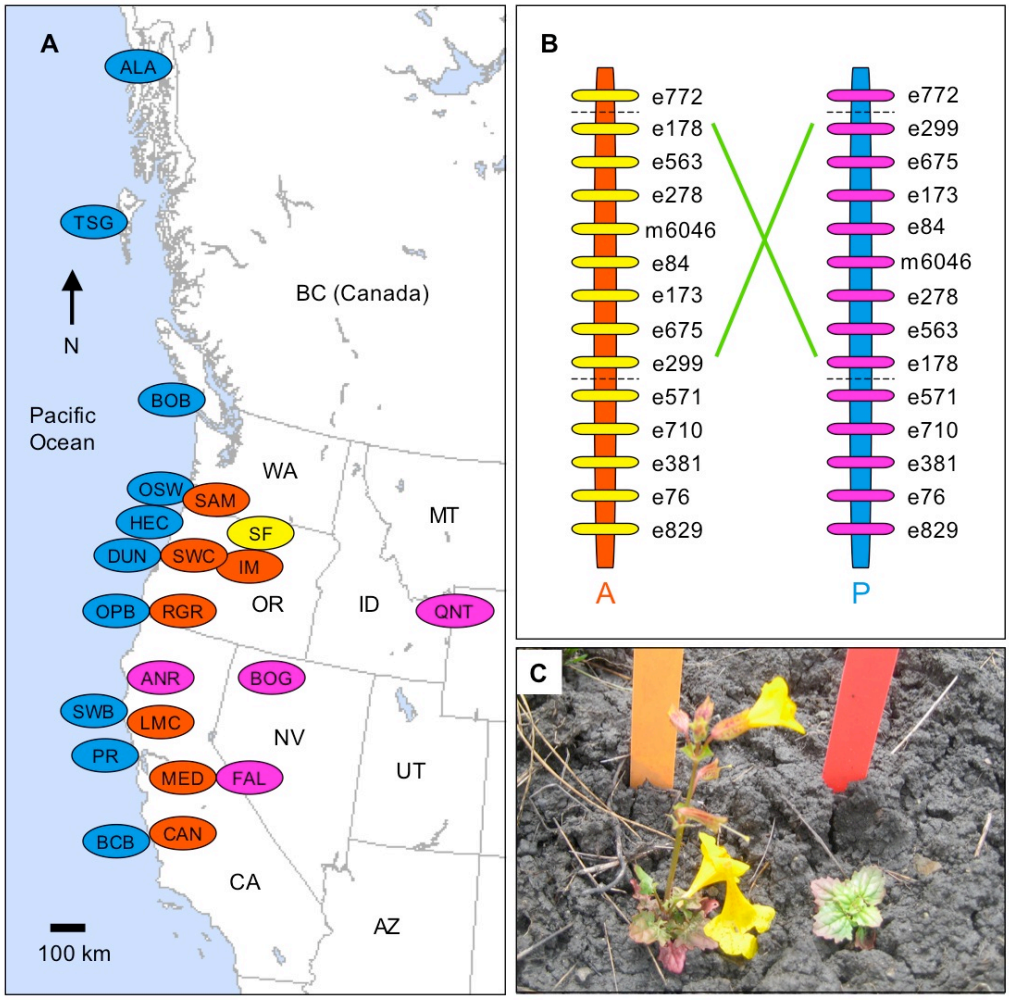

Figure 25: Geographic distribution of the *DIV1* chromosomal inversion. A) Map of Western North America with the locations of populations of coastal perennials (blue), inland annuals (orange), inland perennials (purple), and obligate self-fertilizing species *M. nasutus* (yellow). B) Marker order of the annual (A) and perennial (P) chromosomal arrangements along linkage group eight. C) Difference in flowering at inland field site for NIL and Null-NILs with the inland genetic background. Plant with orange tag has inland *DIV1* allele and plant with red tag has coastal allele.

To determine if inland perennial populations have the same chromosomal orientation as coastal perennial populations in the inverted region I conducted independent crosses between the DUN coastal perennial populations and four inland perennial populations. The inland perennial populations were collected from the Eel River (ANR) of Northern California, two hot springs populations from the Great Basin

(BOG and FAL; Fig. 25, Table 18), and a population from northwestern Wyoming (QNT). The ANR population is of particular interest. In a previous study (Lowry et al. 2008a), ANR was the only population out of 29 (14 coastal and 15 inland), which was consistently admixed between coastal and inland groups over multiple runs with the program STRUCTURE (Pritchard et al. 2000). Patterns of recombination suppression and marker order were consistent with all four of these inland perennial populations having the P chromosomal orientation (Fig. 25b, Table 18).

Since different orientations of the inversion are associated with life-history in inland populations, I hypothesized that *DIV1* would also have an effect on flowering time divergence between inland annual and perennial populations. To test this hypothesis I scored flowering time in a F2 population created through a cross between lines from the LMC and BOG populations. *DIV1* significantly explained 43% of the parental divergence in flowering time divergence in this inland annual x perennial cross (Fig. 24f; $F_{2,266} = 42.02$; $P < 0.0001$). *DIV2* also had a significant, albeit smaller (15% of parental divergence), effect on flowering time ($F_{2,276} = 4.61$, $P = 0.0107$).

4.3.4 Inversion affects local adaptation and perennial life-history in the field

The apparent fixation of the inversion at *DIV1* between perennial and annual populations suggests that habitat-mediated natural selection is driving its distribution. To test whether the *DIV* alleles are locally adapted to inland annual versus coastal perennial habitat, I conducted a reciprocal transplant experiment that incorporated a set of near-isogenic lines (NILs).

Table 21: Analysis of coastal and inland parental types in ASTER. Analysis with the composite of two dependent components of fitness (survival to flowering and number of flowers produced). Comparisons between null and alternative models to test the effects (A) genetic background (genbac), (B) site, and (C) the site x genetic background interaction. The formulae for the two compared models are given above with the analysis of deviance between the models given below. "Graph nodes" refers to the directional Aster graph used to model fitness (1-> survival to flowering -> number of flowers produced).

Model name	Model df	Model deviance	Test df	Test deviance	Test *P*-value
(A) Genetic background					
Null model: Response=Graph nodes+site					
Alternative model: Response=Graph nodes+site+genbac					
Null	3	-12026.8			
Alternative	4	-12097.4	1	70.6	<0.0001
(B) Site					
Null model: Response=Graph nodes+genbac					
Alternative model: Response=Graph nodes+site+genbac					
Null	3	-12037.6			
Alternative	4	-12097.4	1	59.8	<0.0001
(C) Genetic background x site					
Null model: Response=Graph nodes+site+genbac					
Alternative model: Response=Graph nodes+site+genbac+genbac*site					
Null	4	-12097.4			
Alternative	5	-12512.6	1	415.3	<0.0001

Table 22: ASTER modeling of effect of *DIV1* locus in the field. Analysis on the composite of two dependent components of fitness (survival to flowering and number of flowers produced). Comparisons between null and alternative models to test the effects (A) genetic background (genbac), (C) *DIV1* genotype, and (B,D,E) the two-way interaction of these three factors. The formulae for the two compared models are given above with the analysis of deviance between the models given below. "Graph nodes" refers to the directional ASTER graph used to model fitness (1-> survival to flowering -> number of flowers produced).

Model name	Model df	Model deviance	Test df	Test deviance	Test *P*-value
(A) Genetic background					
Null model: Response=Graph nodes+site					
Alternative model: Response=Graph nodes+site+genbac					
Null	3	-13975.0			
Alternative	4	-14035.8	1	60.8	<0.0001
(B) Genetic background x site					
Null model: Response=Graph nodes+site+genbac					
Alternative model: Response=Graph nodes+site+genbac+genbac*site					
Null	4	-14035.8			
Alternative	5	-14658.9	1	623.0	<0.0001
(C) *DIV1* allele					
Null model: Response=Graph nodes+site+genbac+genbac*site					
Alternative model: Response=Graph nodes+site+genbac+genbac*site+*DIV1*					
Null	5	-14658.9			
Alternative	6	-14722.5	1	63.7	<0.0001
(D) *DIV1* allele x site					
Null model: Response=Graph nodes+site+genbac+genbac*site+*DIV1*					
Alternative model: Response=Graph nodes+site+genbac+genbac*site+*DIV1*+*DIV1**site					
Null	6	-14722.5			
Alternative	7	-14755.4	1	32.9	<0.0001
(E) *DIV1* allele x genetic background					
Null model: Response=Graph nodes+site+genbac+genbac*site+*DIV1*+*DIV1**site					
Alternative model: Response=Graph nodes+site+genbac+genbac*site+*DIV1*+*DIV1**site+*DIV1**genbac					
Null	7	-14755.4			
Alternative	8	-14775.0	1	19.6	<0.0001

Table 23: ASTER modeling of effect of *DIV2* locus in the field. Comparisons between null and alternative models to test the effects (A) genetic background (genbac), (C) *DIV2* genotype, and (B,D,E) the interaction of these three factors. The formulae for the two compared models are given above with the analysis of deviance between the models given below. "Graph nodes" refers to the directional Aster graph used to model fitness (1-> survival to flowering -> number of flowers produced).

Model name	Model df	Model deviance	Test df	Test deviance	Test *P*-value
(A) Genetic background					
Null model: Response=Graph nodes+site					
Alternative model: Response=Graph nodes+site+genbac					
Null	3	-13246.9			
Alternative	4	-13493.6	1	246.7	<0.0001
(B) Genetic background x site					
Null model: Response=Graph nodes+site+genbac					
Alternative model: Response=Graph nodes+site+genbac+genbac*site					
Null	4	-13493.6			
Alternative	5	-14231.2	1	737.7	<0.0001
(C) *DIV2* allele					
Null model: Response=Graph nodes+site+genbac+genbac*site					
Alternative model: Response=Graph nodes+site+genbac+genbac*site+*DIV2*					
Null	5	-14231.2			
Alternative	6	-14236.3	1	5.0	0.0247
(D) *DIV2* allele x site					
Null model: Response=Graph nodes+site+genbac+genbac*site+*DIV2*					
Alternative model: Response=Graph nodes+site+genbac+genbac*site+*DIV2*+*DIV2**site					
Null	6	-14236.3			
Alternative	7	-14237.4	1	1.1	0.2947
(E) *DIV2* allele x genetic background					
Null model: Response=Graph nodes+site+genbac+genbac*site+*DIV2*					
Alternative model: Response=Graph nodes+site+genbac+genbac*site+*DIV2*+*DIV2**genbac					
Null	6	-14236.3			
Alternative	7	-14236.8	1	0.5	0.4596

Briefly, NILs were created from three replicate crosses between independent inbred lines from the SWB (coastal perennial) and LMC (inland annual) populations. F1 progeny of each initial cross were backcrossed reciprocally as the pollen donor for four generations to their respective pair of coastal and inland lines. Marker assisted selection was used each generation to move the *DIV* alleles into the alternate genetic backgrounds. After the forth backcross generation of back-crossing the NILs were self-fertilized. Selfed progeny were then genotyped and introgression and non-introgression homozygotes were selected. Lines with the chromosomal region introgressed into the alternative ecotypic background are henceforth referred to as NILs, whereas lines with

the introgressed region removed in the last generation of breeding are Null-NILs. To prevent any effects of inbreeding depression, the final generation of breeding involved round robin intercrosses within independently derived NIL and Null-NILs of the same genetic background and introgression type (N = 3 NIL and 3 Null-NILs per two genetic backgrounds = 12 crosses; see supplementary methods and Fig. 21 for more complete description). Round robin crosses were also conducted among parental lines to create outbred lines for the reciprocal transplant experiment.

To test the effect of the two introgressed *DIV* QTLs in the field, I set up a reciprocal transplant experiment with the NILs, Null-NILs, and the parentals. I selected a coastal (Manchester, CA; Fig. 22) and an inland (Boonville, CA; Fig. 23) field sites near to the location of the parent populations used to make the NILs. The experiment was initiated with greenhouse-reared seedlings in mid-March 2009, approximately two months before the onset of the summer dry season in mid-May. I then monitored the experiment until October 29th, 2009, 15 days after the first storm of the wet season, at the time when new seedlings germinate.

To determine whether *DIV* loci contributed to fitness effects across field sites I analyzed the data with the program ASTER (Geyer et al. 2007; Shaw et al. 2008), which is a package of the statistical program R (R Core Development Team 2010). ASTER modeling is a method that conducts a single analysis of the combination of multiple components of fitness, even if they have different probability distributions. ASTER accounts for dependencies among fitness components by appropriately generating an overall likelihood for each individual over the course of its life.

Table 24: Analysis with ASTER on survival over the field season. Comparisons between null and alternative models to test the effects *DIV1* at the (A) inland and (B) coastal field sites as well as *DIV2* at the (C) inland and (D) coastal field sites. The formulae for the two compared models are given above with the analysis of deviance between the models given below. "Graph nodes" refers to the directional ASTER graph used to model survival (1 -> survival at census one -> survival at census two ->…->survival at census x, where x = 16 for the inland field site and 11 for the coastal field site).

Model name	Model df	Model deviance	Test df	Test deviance	Test *P*-value
(A) *DIV1* survival inland site					
Null model: Response=Graph nodes+genbac					
Alternative model: Response=Graph nodes+genbac+*DIV1*					
Null	16	3270.6			
Alternative	17	3264.3	1	6.3	0.0124
(B) *DIV1* survival coast site					
Null model: Response=Graph nodes+genbac					
Alternative model: Response=Graph nodes+genbac+*DIV1*					
Null	12	3265.8			
Alternative	13	3257.7	1	8.1	0.0044
(C) *DIV2* survival inland site					
Null model: Response=Graph nodes+genbac					
Alternative model: Response=Graph nodes+genbac+*DIV2*					
Null	16	3395.1			
Alternative	17	3392.7	1	2.4	0.1241
(D) *DIV2* survival coast site					
Null model: Response=Graph nodes+genbac					
Alternative model: Response=Graph nodes+genbac+*DIV1*					
Null	12	3360.3			
Alternative	13	3359.8	1	0.5	0.4910

As in previous studies (Hall & Willis 2006, Lowry et al. 2008a), coastal perennial and inland annual parents were highly locally adapted to their respective habitats (genotype x environment interaction, $P < 0.0001$; Table 21) based on composite of two fitness traits: Survival to flowering and number of flowers produced per plant. Analysis of the backcross near-isogenic lines also revealed a highly significant effect of genetic background and genetic background x site interaction across both the *DIV1* and *DIV2* NILs ($P < 0.0001$; Tables 19, 20, 22, 23).

In terms of individual loci, there were highly significant allele x site and allele x genetic background interactions for *DIV1* on fitness ($P < 0.0001$; Table 22). However, the three-way interaction of *DIV1* allele x site x genetic background was not significant

($P > 0.05$). Further, none of the interactions of the *DIV2* locus with site and/or genetic background were significant (Table 23).

Figure 26: Photo of flowering time differences caused by the *DIV1* locus in the coastal genetic background. From left to right: Coastal parent, coastal Null-NIL (homozygous for coastal perennial *DIV1* allele), and coastal NIL (homozygous for inland annual *DIV1* allele).

At the inland field site, *DIV1* explained similar amounts of the divergence in parental flower production in the inland (21.99%) and coastal (19.36%) genetic backgrounds (Fig. 27b). The effects of *DIV1* on flower production at the inland field site can be attributed largely to flowering time. Across the NILs, plants with the coastal allele of *DIV1* initiated flowering 6.54 days later than plants with the inland allele ($F_{1,445} = 23.10$; $P < 0.0001$). Later flowering plants produced fewer flowers before the summer drought made further survival impossible (Fig. 27; Table 19). This effect of flowering time and fitness was most dramatic in the coastal genetic background where survival to flowering was eight times greater for plants with the inland versus coastal *DIV1* allele (Figs. 26, 27; Table 19). *DIV2* did not have a significant effect on flowering time at the inland site ($F_{1,454} = 0.69$; $P = 0.4066$).

At the coastal field site, *DIV1* had a significant 12.77 day effect on flowering time across the NILs ($F_{1,192} = 8.34$; $P = 0.0043$), where plants with the inland allele at

DIV1 flowered earlier (Fig. 27, Table 19). However, in contrast to the inland site, earlier flowering only translated into slightly greater fitness for plants with the inland *DIV1* allele in the coastal genetic background (Fig. 27). Further, there was no statistically significant difference in fitness between coastal and inland *DIV1* alleles in the inland genetic background. *DIV2* also had no effect on flowering time at the coastal field site ($F_{1,185} = 0.0133$; $P = 0.9085$).

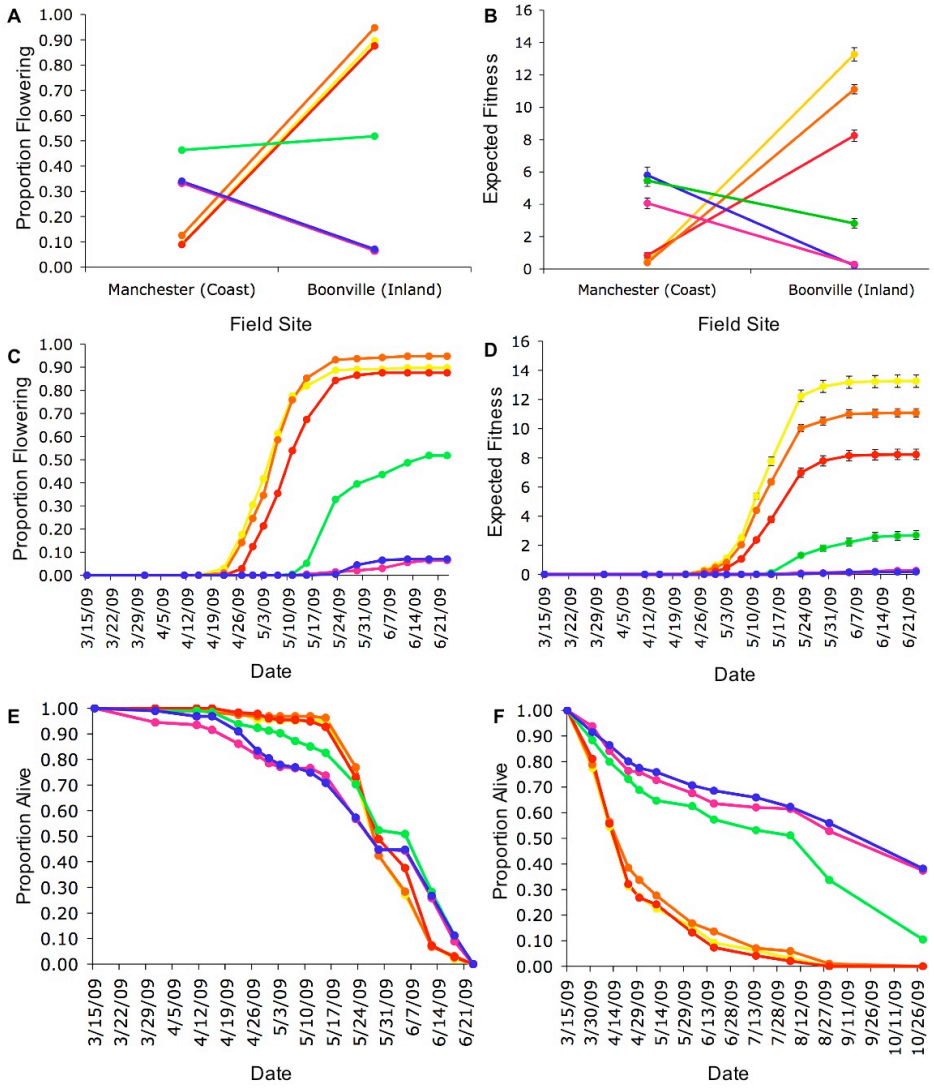

Figure 27: Results from reciprocal transplant field experiment for *DIV1* locus. A) Proportion of plants surviving to flower and B) Expected fitness per plants across field sites. Values plotted are maximum likelihood estimates +/- 1 SE following the alternative model in Table 3E. C) Cumulative proportion of plants surviving to flower and D) expected fitness +/- 1 SE per individual at the inland field site. Survival over time at the E) inland and F) coastal field sites. Yellow=Inland Parent, blue=coastal parent, orange=inland NIL, red=inland Null-NIL, green=coast NIL, pink=coast Null-NIL.

DIV1 had significant effects on patterns of survival over the course of the season at both the coastal ($P = 0.0044$) and inland ($P = 0.0124$) field sites, while *DIV2* had no

effect ($P > 0.05$) on survival at either site (Table 24; Fig. 27). At the coastal site, plants with the coastal *DIV1* allele had a 3.5 times greater survivorship (69% of the parental divergence) to the end of the first season (e.g. first rain of the 2009/2010 wet season) than plants with the inland allele (Fig. 27f). Nearly half of the plants that survived to the end of the first season, and were homozygous for the *DIV1* coastal allele, did not flower during the 2009 field season (Coastal parent = 45% and Coastal Null-NIL = 44% versus Coastal NIL = 5%). In other words, plants with the coastal *DIV1* allele allocated all of their resources to growth instead of reproduction at nearly 10 times the rate of those with the inland allele.

4.4 Discussion

Overall, my results suggest that chromosomal inversion polymorphism is distributed between the early flowering annual and late flowering perennial races of *M. guttatus* by habitat-meditated natural selection. Replicated QTL analysis demonstrated that the chromosomal inversion (*DIV1*) has a consistent effect on flowering time divergences between coastal perennial and inland annual populations over an 1100 kilometer range of western North America. Further crosses revealed that one orientation of the inversion is found in perennial populations while the other orientation is found in annual populations. Finally, I confirmed that the inversion contributes to local adaptation to perennial and annual habitat through a reciprocal transplant experiment. This result contrast with another flowering time QTL (*DIV2*), from a collinear region of the genome, that appears to have less consistent effects.

4.4.1 Geographic distribution of divergence alleles

Understanding the geographic distribution of adaptive alleles is a major goal of evolutionary biology (Kelly 2006; Joost et al. 2007; Coop et al. 2009; Novembre & Rienzo 2009; Lowry 2010) because it informs us how landscape-scale environmental patterns

shape the standing genetic variation within a species (Colosimo et al. 2005; Barrett & Schluter 2008; Stinchcombe & Hoekstra 2008; Steiner et al. 2009). However, even if I know the distribution of different sequence haplotypes thought to be adaptive, quantitative trait assessment is necessary to establish that actual effects on traits are as widespread as the underlying alleles. In this study, I used a replicated QTL approach to establish that *DIV1* consistently affects a suite of traits that diverge between annual and perennial populations. The hypothesis that alternative alleles are fixed between populations with different life-histories is strengthened by the finding that widespread chromosomal inversion polymorphism at the *DIV1* locus maps geographically onto the distribution of annual and perennial populations.

The results of the *DIV1* locus contrast to that of the collinear *DIV2* locus. Alleles of loci underlying the *DIV2* locus may be widespread since it was discovered in a cross among central Oregon population and affects flowering time by a similar number of days in populations as far south as central California. However, I did not detect the effect of the *DIV2* locus across all population crosses, the traits that *DIV2* affected were far less consistent than *DIV1*. I also detected no effect of *DIV2* in the field, even though it had a strong effect on flowering time in a cross between the same pair of coastal (SWB) and inland (LMC) populations in the greenhouse. Further, this result contrast with another field experiment involving RILs, where *DIV2* was found to affect a suite of traits including fitness (Hall et al. *in review*)

The inconsistent effects of *DIV2* could be the result of a segregating polymorphism or a locus with effects that are not robust to genetic background. Alternatively, I may not have had enough power to detect the effect *DIV2* in all of my mapping populations. This may especially be the case for the low sample size of OPB x RGR cross, where the effects of alleles on flowering time trended in the same direction as the California crosses. Indeed, the small effect of the *DIV2* locus in the BOG x LMC

cross indicates that I would not have detected its effects with a sample size comparable to the OPB x RGR cross. Another reason I may not have detected the effect of *DIV2* is recombination between the marker MgSTS76 and gene or genes that contribute to the phenotype. The detection of the effect of *DIV1* may have been so replicable because it is located within or near to the chromosomal inversion, where recombination between the marker and causative genes is suppressed (Noor & Bennet 2009).

Multiple causative genes may underlie either of the *DIV* QTLs. However, the suppressed recombination caused by the inversion in the region of *DIV1* would be much more likely to hold together allelic combinations of linked loci than for the collinear *DIV2* locus (Kirkpatrick & Barton 2006; Hoffman &Rieseberg 2008).

4.4.2 The role of the *DIV1* inversion

The pattern of distribution of the *Mimulus* inversion is similar to chromosomal rearrangement polymorphism in other organisms. Inversions have often been shown to be distributed geographically along environmental clines (Dobzhansky 1951; Etges & Levitan 2004; Feder et al. 2005; Umina et al. 2005; Manoukis et al. 2008; McAllister et al. 2008) and to contribute to phenological shifts (Feder et al. 2005) as well as desiccation and thermal tolerance (reviewed in Hoffmann & Rieseberg 2008). For *M. guttatus*, the geographic pattern of the *DIV1* inversion distribution appears to be dictated by temperatures and the availability of water in summer months.

The *DIV1* locus appears to contribute to a classic pattern of resource allocation trade-off in plants. The A allele of *DIV1* leads to a greater investment of resources into an early flowering "live fast die young" life-history strategy (Silvertown & Charlesworth 2001; Roux et al. 2006; Datson et al. 2008). In contrast, the P allele of *DIV1* contributes to investment in vegetative growth and as a consequence to later flowering and perennial life-history strategy. Interestingly, an independently derived segregating inversion

polymorphism had similar life-history effects within a population of *M. guttatus* (IM) in the Cascade Mountains (Scoville et al. 2009).

4.4.3 Deconstructing local adaptation

Local adaptation is usually defined as a trade-off of fitness across habitats, where local genotypes outperform foreign genotypes across habitats. Many theoretical models have argued that specialization to a particular habitat through local adaptation is mediated by antagonistic pleiotropy at individual loci (Hedrick 1986; Gillespie & Turelli 1989; Kawecki & Ebert 2004; Turelli & Barton 2004). However, the handful of studies that have attempted to detect such trade-offs across habitats for individual loci have only found fitness effects in one habitat, while the same locus has little or no effect in alternative habitats (Verhoeven et al. 2004, 2008; Gardner & Latta 2006; Hall et al. *in review*). A lack of trade-off was also found in a previous study of *M. guttatus*, where three salt tolerance loci contribute to fitness in coastal habitat but have no effect in inland habitat (Lowry et al. 2009). Thus, it is possible that local adaptation may be the result of the summation of effects of a non-overlapping set of loci that each only contribute to fitness in one habitat. However, if this were true then alleles that are adaptive to one habitat should move unidirectionally into other habitats, where they are effectively neutral.

Our geographic data suggests that alternative orientations of the *DIV1* inversion are restricted to perennial versus annual habitats. Even so, I found no evidence for antagonistic pleiotropy across habitats for the *DIV1* locus for fitness in the 2009 season. The local inland *DIV1* allele was highly favored in its native habitat, yet the coastal allele did not contribute to higher fitness in coastal habitat. So is there any trade-off at the *DIV1* locus that might explain the restriction of alleles between annual and perennial habitats?

The apparent restriction of the A orientations of the inversion to annual habitat may be due to the effects of the inversion on multi-season perennial survival. At the coastal field site the *DIV1* inversion locus accounts for 69% of the parental divergence for survival to the second wet season. But what advantage might be gained by pereniality? Consider foremost the observation that plants that survived to the second season become well-established large plants. These established plants may have an advantage in coastal habitat because it is primarily composed of other perennial plant species that may shade out and compete below ground except in areas of natural disturbance.

Natural landslides and animal trampling in the coastal habitat continually create disturbed habitat for new seedlings recruitment (Hampton & Griggs 2004; Lowry *personal observation*). My study mimicked natural disturbance since I cleared plots of most of the vegetation before planting. By August, these coastal experimental plots were completely covered again by a dense thicket of perennial competitor species, which could limit the success of seedling recruitment in subsequent seasons and lead to an overall advantage of established plants.

Our current study was not designed to directly test long-term advantages of perennial growth in coastal habitat. Even so, the differential survival caused by the *DIV1* locus does present a compelling hypothesis for future experiments. This situation is analogous to recent results in stickleback fish, where allelic trade-offs at the armor plate locus (*Eda*) occur at different life-history stages (Barrett et al. 2008, 2009).

4.4.4 The origin and maintenance of a chromosomal inversion

Phylogenetic studies have generally found that annual species are derived from perennial species (Andreasen & Baldwin 2001; Church 2003; Datson et al. 2008). Therefore, I hypothesize that the A chromosomal orientation, found in inland annuals

and the obligate self-fertilizing species *M. nasutus*, is the derived form. Further, I hypothesize that since its origin the A orientation has accumulated multiple genetic changes that have facilitated the invasion of habitat with harsh hot seasonal drought. Crosses within more distantly related species will be necessary to resolve which orientation is the ancestral.

The spread of the chromosomal inversion may have been the result of selection on phenotypic effects of alleles at multiple genes within the inversion. Alternatively, the inversion polymorphism could be maintained by hitchhiking with an adjacent locus, which contributes pleiotropically to the divergence traits but falls within the region of suppressed recombination just outside of the inversion (Noor et al. 2007; Noor & Bennett 2009). Future studies to map the inversion breakpoints will be necessary to rule out genes outside of the inversion as the cause of the *DIV1* phenotypic effects. Regardless, standing genetic variation of the chromosomal inversion appears to be maintained in this system by habitat-mediated natural selection.

5. Conclusion: Landscape evolutionary genomics

Tremendous advances in genetic and genomic techniques have resulted in the capacity to identify genes involved in adaptive evolution across numerous biological systems. One of the next major steps in evolutionary biology will be to determine how landscape-level geographic and environmental features are involved in the distribution of this functional adaptive genetic variation. Here, I outline how an emerging synthesis of multiple disciplines has and will continue to facilitate a deeper understanding of the ways in which heterogeneity of the natural landscapes mold the genomes of organisms.

In 2003, the year before I embarked on my dissertation research, three landmark papers envisioned an emerging integration of ecology, evolution, and population genetics. Luikart et al. (2003) defined the field of *Population Genomics* as the "simultaneous study of numerous loci or genome regions to better understand the roles of evolutionary processes that influence variation across genomes and populations." Feder and Mitchell-Olds (2003) recognized the synthetic discipline *Ecological and Evolutionary Functional Genomics* or EEFG. The main goal of EEFG was to use all the genetic and genomic tools available to determine the exact functional genetic changes involved in the evolution of adaptations. A third field, *Landscape Genetics*, was born out of the fusion of population genetic techniques and landscape ecology's layered geographic information system (GIS) maps (Manel et al. 2003). Landscape Genetics has thus far primarily focused on how various landscape features affect gene flow of neutral genetic variation, usually with the goal of identifying threatened or endangered populations for conservation purposes.

In this piece, I will briefly outline the current states of the fields of Population Genomics, EEFG, and Landscape Genetics. I then discuss how a further synthesis of

these fields has and will continue to facilitate a better understanding of the nature of adaptive genetic variation.

5.1 The genome scan gold rush

Genomic scans are a major hallmark of Population Genomics. The last few years have seen an expansion of scans focused on genomic heterogeneity across habitats in a plethora of biological systems (Nosil et al. 2009). Here, a number of individuals from populations located in distinct habitats or across an ecological cline are genotyped for multiple markers. The logic behind such genomic scans is that neutral regions of the genome will freely move between populations via gene flow while loci under selection will show higher genomic divergence across habitats. Genomic scans can range in size from couple of hundred markers to true population genomics through resequencing of the whole genomes with the aid of tiling arrays or next generation technologies (e.g. Turner et al. 2005).

Genetic differentiation resulting from habitat-mediated selection can result in divergence of neutral markers linked to locus under selection for many centimorgans. For example, a recent study claimed to find very long-distance genetic differentiation in the vicinity of Quantitative Trait Loci (QTLs) for divergently selected traits in pea aphids (Via & West 2008). However, there are many instances of genetic differentiation extending only a few kilobases around a selected gene and even being limited to a single exon (Storz & Kelly 2008). Ultimately, the ratio of the selection coefficient to recombination rate determines the width of elevated divergence along a chromosome. Yet expectations differ depending on whether the region was the subject of a recent selective sweep (Slatkin & Wiehe 1998) or long-term habitat-mediated balancing selection (Charlesworth et al. 1997). Unfortunately, if regions of elevated molecular divergence are small, any genomic scan with less than hundreds of thousands of markers

will miss most important loci involved in adaptation. On the other hand, if the region of divergence is large, fewer markers will be required. Even so, determination of the ultimate cause of why any particular region is distorted and the extent to which a given locus contributes to adaptation will still require forward genetic approaches.

Beyond the difficulty in determining the causal mutations involved in adaptation exclusively through genomic scans, there are some fundamental problems with genomic scans that are often ignored. Population structure is a major challenge. When population structure is high, as is often the case for sessile organisms with discrete populations, it may be very difficult to detect outlier loci above the cloud of the high F_{ST} null distribution. Further, demographic histories are very difficult to determine. Past population bottlenecks and hierarchical population structure can contribute to high genome-wide variances in summary statistics (Excoffier et al. 2009). As a result, genomes can be extremely heterogeneous, which can lead to a high rate of false positives. Thus, it is possible that insufficient modeling of demographic history and not rampant selection may be the cause of the 5-10% rate of outlier loci found in a recent review of genomic scans studies (Nosil et al. 2009).

5.2 The gene first approach

The best landscape-scale EEFG studies have first identified the genes involved in adaptive divergence and then established the spatial distribution of functional allelic variation through multi-population resequencing. The greatest of these successes have arguably come from studies of stickleback fish (Shapiro et al. 2004; Colosimo et al. 2005; Barrett et al. 2008) and *Peromyscus* mice (Steiner et al. 2007, 2009; Storz & Kelly 2008; Linnen et al. 2009). In both systems, genes that are involved in adaptations to very divergent habitats have been cloned by forward genetic techniques in conjunction with knowledge of candidate genes. After gene identification, population genetic

analysis was conducted to determine the geographic distribution of alleles involved in ecotype-defining traits. This approach allowed the researchers to distinguish between phenotypes that result repeatedly from standing genetic variation and parallel phenotypes arising from new mutation.

Critically, field experimentation after gene identification can be used to confirm the adaptive significance of particular phenotypes. In the case of sticklebacks, field experiments with natural mutants of the armor control gene *eda* allowed researchers to test whether particular alleles are favored in fresh water habitat (Barrett et al. 2008).

The gene-first approach is definitely more rigorous than genomic scans in terms of ability to identify novel gene functions and understand the forces involved in the geographic distribution of adaptive genetic variation. However, the cloning of genes remains an expensive and labor-intensive bottleneck in the process. Further, the difficulty of fine-mapping and cloning adaptive genes means that they have for the most part been biased toward large-effect loci underlying discrete phenotypic traits.

Incorporation of QTL analysis into reciprocal transplant experiments may also be effective in determining the factors governing the spatial distribution of adaptive alleles, such as whether trade-offs at individual loci (i.e. antagonistic pleiotropy) underlie habitat-mediated adaptation. Recently, a study used field QTL analysis to determine the fitness effects of loci across habitats for plant ecotypes known to be locally adapted to coastal and inland habitats (Lowry et al. 2009). Here, three salt tolerance QTLs, previously identified in the laboratory, were found to have fitness effects in coastal but not inland habitat. This result may suggest that different sets of loci are responsible for adaptation to each habitat. Further, if adaptive alleles are indeed conditionally neutral, then they could diffuse unidirectionally by gene flow between habitats. More field studies are necessary to determine the extent to which

trade-offs determine the spatial distribution of adaptive alleles among natural populations.

5.3 Adaptive alleles as a GIS layer

Since Manel et al. 2003, much thought has been put into how to combine multivariate-layered GIS maps with population genetic data. Many methods have been developed to assess population genetic structure (reviewed in Holderegger & Wagner 2006, 2008; Stofer et al. 2007; Balkenhol et al. 2009), and have been used to determine how landscape features contribute to the structuring of what is presumed to be neutral genetic variation. While exploring the distribution of neutral genetic variation can definitely inform us about the patterns and processes that limit gene flow, landscape genetics has yet to develop a framework to understand how landscape features contribute to the distribution of adaptive genetic variation.

Taking a landscape perspective could have huge implications for evolutionary biology. Studies of the genetics of adaptation commonly focus on a single environmental factor as it is distributed across a cline or compare phenotypes across binomial habitats (e.g. coast versus inland). Natural landscapes are much more heterogeneous. Further, the distribution of adaptive alleles can be influenced by multiple environmental factors.

Landscape genetics is a maturing field that incorporates many types of data collected through remote sensing, weather stations, and geologic maps. These multivariate data are layered on top of each other and subsequent analyses are conducted. Genetic data can also be incorporated as a layer that can be used to understand the distribution of neutral genetic variation and gene flow (Kozak et al. 2008). Comparisons between the geographic distributions of neutral alleles and alleles thought to be involved in local adaptation could also be used to test for selection.

Joost et al. (2007) recently developed a methodology that utilizes GIS to compare geographic and genetic data to detect alleles associated with particular environmental factors. While this is a significant step forward, comprehensive analysis of the spatial distribution of alleles with regard to the distribution of environmental heterogeneity and barriers to gene flow has yet to be developed. The great hope is that multivariate geographic information could be incorporated with population genetic models to create more robust analyses of landscape-level natural selection. Further, field experimentation to "ground truth" hypotheses as well as sampling design are very important with any landscape study and should be carefully considered before populations are selected for analysis.

5.4 Future directions

As evolutionary biologist begin to get a better handle on what loci are involved in adaptations to different habitats, a new set of questions is likely to emerge. For example it is currently unknown the extent to which fitness trade-offs at individual loci occur across the landscape, how geographic barriers influence the spread of adaptive versus neutral alleles, and whether ecotypic divergence is due to the fixation of adaptive alleles or small shifts in allele frequencies at many loci. Current genome scans and gene-first approaches may not be representative of the complexity of landscape-scale adaptations as they are biased toward finding large-effect alleles that are fixed among taxanomic groups.

Recent studies on human population genetics provide a glimpse into what lays ahead for landscape evolutionary genomics (Coop et al. 2009; Novembre & Rienzo 2009). Coop et al. (2009) examined global allele frequencies across numerous populations at hundreds of thousands of SNPs to search for loci under selection. Overall, very few genes in the human genome had extreme allele frequency differences

among populations. This may indicate that selection has only acted on a few loci. Alternatively, local selection may have been more widespread, but adaptive phenotypic change was achieved through small allelic changes at multiple loci. With ongoing improvements and decreased costs of genome sequencing technologies, much broader analyses will soon be possible in many other systems. It will be important that this data be viewed in a landscape ecological context to better understand factors contributing to the geographic distribution of adaptive alleles.

5.5 Final thoughts

Indeed, fully understanding adaptation on landscape scale is a monumental task even for one system. Habitat-mediated adaptation almost invariably involves multiple phenotypic changes each of which have a complex genetic basis. The complexity of this pursuit becomes multiplicative when landscape level environmental variation is added to the equation. Understanding adaptation at the level of the natural landscape may be especially difficult for evolution of polygenic traits, where adaptation has occurred through small allelic shifts across loci. Even so, there are now a few good examples of successfully connecting the distribution of functional genetic variation to coarse landscape features (Colosimo et al. 2005; Steiner et al. 2007; Storz & Kelly 2008). As more systems enter the genomic era we will gain greater insight into how the mosaic of the natural landscape molds the genomes of the organisms distributed across its vastness.

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Biography

David Bryant Lowry was born during a heat wave on May 28th, 1979 in Santa Rosa, CA. David received a B.S. in Genetics and Plant Biology from the University of California, Berkeley in 2001. During graduate school, David was an author for the following peer-reviewed scientific articles: "Landscape evolutionary genomics," "Natural variation for drought response in the *Mimulus guttatus* species complex," "Genetic and physiological basis of adaptive salt tolerance divergence between coastal and inland *Mimulus guttatus*," "The strength and genetic basis of reproductive isolating barriers in flowering plants," "Ecological reproductive isolation of coast and inland races of *Mimulus guttatus*," and "*Mimulus* is an emerging model system for the integration of ecological and genomic studies."