

ECOGEOGRAPHICAL AND INTRINSIC POSTZYGOTIC ISOLATION BETWEEN
MIMULUS GLAUCESCENS AND *MIMULUS GUTTATUS*

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Nicole M. Habecker

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ABSTRACT

ECOGEOGRAPHICAL AND INTRINSIC POSTZYGOTIC ISOLATION BETWEEN *MIMULUS GLAUCESCENS* AND *MIMULUS GUTTATUS*

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The mechanisms by which species are formed is a central question in evolutionary biology. The Biological Species Concept has provided a framework for quantifying, via reproductive isolation, the extent of divergence between populations. The relative strengths and importance of individual reproductive isolation barriers is unclear. This study estimated the strength of ecogeographic (prezygotic) isolation and intrinsic (postzygotic) isolation barriers (genetic incompatibilities between hybrids) between the closely related annual monkeyflowers, *Mimulus glaucescens* and *M. guttatus*. Georeferenced herbarium records and environmental data were used to model each species ecological niche and to measure their habitat overlap. Reciprocal crosses between species were performed to measure hybrid seed set, germination success of the resulting seeds, biomass of F₁ adult plants, total number of days to first flower (developmental rate), total flower production, ovule numbers, and pollen viability, as intrinsic postzygotic isolation. The species exhibited both ecogeographical isolation and non-reciprocal intrinsic isolation (observed in seed set, developmental rate, and ovule production). These results suggest that isolation was a result of

geologic history causing divergent ecological selection, where ecogeographical isolation acted before and limited a minimum of 45% gene flow compared to intrinsic postzygotic isolation factors, which acted later and limited a minimum of 21% of the gene flow on average between *M. glaucescens* and *M. guttatus*. The morphological differences between these species may reflect their genetic differences due to local adaptation.

CHAPTER I

INTRODUCTION

The mechanism of speciation is a central question in evolutionary biology (Darwin 1859, Coyne and Orr 2004, Schemske 2000). The Biological Species Concept (Mayr 1942) offers a framework for quantifying the amount of divergence between populations, which may represent the extent to which speciation has proceeded, by measuring reproductive isolation (Coyne and Orr 2004, Sobel *et al.* 2010). Speciation is usually viewed as a process of divergence that occurs over time (Coyne and Orr 1998, Coyne and Orr 2004). Species often continue to accumulate additional barriers to reproductive isolation even after gene flow is initially disrupted, thus closely related species may be less reproductively isolated than more distant relatives (Coyne and Orr 2004).

A wide variety of traits and biological processes can impose barriers to reproduction between populations. For example, adaptation to different habitats (Lowry *et al.* 2008a), or shifts in the timing of reproduction (Martin and Willis 2007), can make it unlikely that populations will have opportunities to mate. Similarly, alternative alleles (mutations) that accumulate in different populations may lead to reduced fertility (Martin and Willis 2010) or even sterility (Fishman and Willis 2001) in offspring resulting from matings between populations.

Multiple barriers typically work in concert to limit gene flow, and these act sequentially, such that barriers imposed earlier in potential interactions between populations (e.g., prezygotic barriers) limit the opportunities for subsequent gene flow to occur (Coyne and Orr 2004,

Ramsey *et al.* 2003, Sobel and Chen *in review*). Unraveling the ways in which these different barriers evolve is a topic of active research (Coyne and Orr 2004, Kay and Schemske 2008, Sobel *et al.* 2010).

One unresolved area in reproductive isolation research is the relative strength of barriers responsible for speciation. Some studies have concluded that prezygotic mechanisms are more efficient and stronger, because they limit gene flow before gametes are committed (Ramsey *et al.* 2003, Coyne and Orr 2004, Wu *et al.* 2008, Sobel *et al.* 2010). Postzygotic barriers, however, such as sterility in hybrid offspring, can indicate permanent genetic divergence between species (Dobzhansky 1937, Muller 1942, Orr 1995, Coyne and Orr 1998) and can be more important barriers driving speciation in some taxa (Cozzolino *et al.* 2004, Jewel *et al.* 2012). Regardless, reproductive isolation conferred by a single trait is seldom sufficient to limit all gene flow, thus multiple barriers may work together to limit gene flow (Coyne and Orr 2004, Rieseberg and Willis 2007). Several recent studies have compared the strength of reproductive isolation within and among multiple barriers (Schemske 2000, Ramsey *et al.* 2003, Coyne and Orr 2004, Kay 2006, Martin and Willis 2007, Lowry *et al.* 2008b). The relative paucity of studies such as these has been identified as an important obstacle toward resolving speciation mechanisms (Widmer *et al.* 2009, Sobel *et al.* 2010).

Sobel *et al.* (2010) suggested that, in particular, estimating the strength of ecogeographic isolation—potential differences in spatial distribution of species based on habitat preferences, as opposed to effective geographic separation (distance only)—has been neglected in past studies of reproductive isolation. Few studies have attempted to estimate

this prezygotic barrier, but those that have, found it to limit gene flow (Ramsey *et al.* 2003, Kay 2006, Nakazato *et al.* 2010). For example, Sobel (2010) found ecogeographic barriers to reduce potential gene flow by no less than 64% on average between 12 pairs of closely related monkeyflowers (*Mimulus* spp.).

On the other hand, Jewell *et al.* (2012) found intrinsic postzygotic barriers, such as fruit mass, to account for 76.3% of reduction in gene flow on average between closely related species of Chilean bellflowers (*Nolana* spp.), whether in sympatric (where gene flow is geographically feasible) or allopatric (where gene flow is geographically discouraged) arrangements. This study also reported a correlation between postzygotic barriers and geographic and/or genetic distance. Because intrinsic postzygotic isolation is considered important for completion of reproductive isolation (Muller 1942, Coyne and Orr 2004, Rieseberg and Willis 2007), comparing the strength of postzygotic isolation relative to the amount of ecogeographic isolation identified between closely related species may indicate their combined importance in speciation events.

In my thesis, I estimate the strength of ecogeographic isolation and intrinsic postzygotic isolation between the annual wildflowers *Mimulus glaucescens* and *M. guttatus*. *Mimulus glaucescens* is a northern California endemic. Previous studies have supported the idea that *Mimulus glaucescens* is distinct from *M. guttatus* (Ritland and Straus 1993), although the two are closely related (Beardsley *et al.* 2004). Well-defined populations of each species have been observed in close proximity in the field, and some have reported observing hybrids in the field (Vickery 1964). The persistence of these closely related species despite that they are capable of hybridization (Vickery 1964), and despite broad overlap in their ranges (Hickman 1993), suggests that some reproductive isolation must

contribute toward maintenance of their species boundaries (Jiggins *et al.* 2001, Coyne and Orr 2004). I used georeferenced herbarium records and spatial environmental data to estimate ecogeographical isolation between these species using an ecological niche model. I also reciprocally crossed populations of both species using hand-pollinations and used the progeny to estimate intrinsic postzygotic isolation due to multiple barriers, such as reduced hybrid seed set, F₁ germination rate, developmental rate, total flower production, biomass, pollen viability and ovule production. I used the results of these studies to address the question: How important are intrinsic postzygotic isolation factors relative to ecogeographic isolation?

CHAPTER II

REVIEW OF THE LITERATURE

Darwin (1859) inspired many to explore the mechanisms for the origins of the impressive diversity of species on earth—a subject of active research to this day (Coyne and Orr 2004, Sobel *et al.* 2010). Multiple species concepts have attempted to wholly define a species, such as those based on morphology or phylogenetics (Coyne and Orr 2004). Ernst Mayr (1942) argued that, fundamentally, speciation begins when two populations diverge sufficiently that gene flow is unable or unlikely to occur. His concept, known as the “Biological Species Concept”, is favored among many biologists (Coyne and Orr 2004, Freeman and Herron 2007, Sobel *et al.* 2010), because it provides an operational framework for quantifying the extent of population divergence (Coyne and Orr 2004). Barriers causing species to be reproductively isolated from each other are categorized as those that prevent fertilization (prezygotic) and those that act to reduce fitness of hybrids (postzygotic) (Coyne and Orr 1998). Reproductive isolation factors were listed by Dobzhansky (1951) as indicators of both plant and animal divergence. Continued speciation research involves comparisons between closely related species, as some level of gene flow is needed to determine the order of appearance, rate of evolution, and the relative importance of barriers leading to speciation (Coyne and Orr 2004, Wu *et al.* 2008).

Prezygotic Isolation Barriers

Species that are prezygotically isolated can be geographically separated or exhibit ecological differences in their spatial isolation, and thus are unlikely to encounter each other for mating (Dobzhansky 1951, Mayr 1956, Coyne and Orr 2004). Examples can be observed in the wide geographical distribution and divergent habitats of Hawaiian flies (*Drosophila* spp.), which oviposit on plants, aquatic substrates, or even spiders (Kaneshiro and Boake 1987, Freeman and Herron 2007); and the diversity of ecological niches occupied by members of the Hawaiian silversword alliance (*Argyroxiphium*, etc.) (Baldwin 2006). In some cases, prezygotic isolation leads to differences in the timing of reproduction. For instance, differences in time of migration and spawning habits of reproductively isolated populations of Chinook salmon (*Oncorhynchus* spp.) were observed shortly after their introduction from California into New Zealand (Quinn *et al.* 2000). Temporal isolation was also reported in the asynchronous flowering phenologies of inland and coastal populations of the seep-spring monkeyflower, *Mimulus guttatus* (Lowry *et al.* 2008a). Behavioral isolation can be manifested in animals, through mating preferences or courtship behaviors that prevent mating, and has been noted in the female preference for conspecific over heterospecific mating calls in some frogs and toads (Gerhardt 1994). In plants, behavioral isolation can be mediated through the actions of pollinator preferences for divergent species (Kay 2006).

If ecogeographical, temporal, and behavioral isolation fail to prevent mating, other prezygotic barriers can prevent zygote formation. Mechanical isolation can restrict gene flow due to incompatible genitalia in animals, such as improper grasping of female damselflies (*Odonata* spp.) by heterospecific males, which prevents breeding (Paulson 1974,

Coyne and Orr 2004). Mechanical isolation between plants can occur when flower morphology constrains the placement of pollen on pollinators (e.g., Kephart and Theiss 2004; Kay 2006). In a recent study, differences in herkogamy, or anther-stigma separation, were hypothesized to be distributing pollen differently onto pollinators, and possibly driving the diversification of *Zingerberales* spp. (Ley and Classen-Brockhoff 2012). Several forms of gametic isolation exist (Coyne and Orr 2004), such as the inefficiency of interspecific sperm and egg attachment in sea urchins (Metz *et al.* 1994), or faster pollen tube growth in conspecific versus heterospecific plant matings (Levin 1978).

Postzygotic Isolation Barriers

Any trait that reduces hybrid fitness can lead to postzygotic reproductive isolation. Intrinsic postzygotic isolation is caused by genetic incompatibilities that affect hybrids in any environment, and can be explored in a controlled laboratory setting (Dobzhansky 1951, Orr 1995, Coyne and Orr 2004). Genetic differentiation in isolation may result in epistatic genetic interactions that reduce fitness in hybrids, which are known as Dobzhansky-Muller incompatibilities (Dobzhansky 1937, Muller 1942, Moore 1976, Orr 1995). Studies of postzygotic isolation usually compare the numbers, fecundity, and fitness in successfully produced hybrids with conspecific offspring. Most progress in identifying incompatible genetic traits has been in *Drosophila* (Coyne and Orr 1998), although other animal studies have observed differences in morphological and life history traits, such as hybrid diet and body shape in *Gasterosteus* spp. (Hatfield and Schluter 1999). Assessments in plants have compared parental and hybrid seed set, germination rates, biomass, ovule and pollen grain numbers, and ovule and pollen grain viability, among others (Vickery 1959,

Vickery 1964, Coyne and Orr 1998, Fishman and Willis 2001, Ramsey *et al.* 2003). Plant speciation can also occur via polyploidy, or the duplication of chromosome numbers. This is another form of intrinsic postzygotic isolation that can result – within a single generation - in hybrid offspring that are incompatible with their parent populations (Stebbins 1950, Grant 1963).

Evidence of intrinsic postzygotic isolation in animals is often reported as reduced fitness of the heterogametic (usually male) offspring, a phenomenon sometimes referred to as “Haldane’s rule” (Haldane 1922, Turelli and Orr 2000). This has been primarily observed in *Drosophila* and other animals (Turelli and Orr 2000, Coyne and Orr 2004). Dioecious plants, which produce separate male and female flowers, such as *Silene* spp., have also been shown to conform to Haldane’s rule (Brothers and Delph 2010). Because sex chromosomes evolved recently in most plants (Brothers and Delph 2010), male and female chromosomes tend to be genetically similar. As a consequence, decreased hybrid fitness may be equally likely in both male and female fitness components of plants (Rieseberg and Willis 2007).

Extrinsic postzygotic isolation occurs when postzygotic fitness reductions are observed only in certain environments. This can be an effective isolating barrier even if intrinsic postzygotic isolation does not prevent the successful formation of hybrids. Extrinsic postzygotic isolation factors are characterized in a similar fashion to intrinsic factors (whether they effect hybrid viability or sterility), but plants are often grown in both parental environments to determine whether ecological factors reduce the fitness of hybrids relative to parents (Stebbins 1950, Rundle and Whitlock 2001, Coyne and Orr 2004). Hatfield and Schluter (1999) found extrinsic postzygotic isolation in reciprocal transplants of F₁ hybrids of

stickleback fishes (*Gasterosteus* spp.), which grew much slower than their parents when transplanted out to the original parent field sites. Rundle and Whitlock (2001) suggested that improved transplant experiments would utilize F₂ and backcross hybrids to tease out habitat requirements for each species. Hall and Willis (2006) used backcross hybrids of *Mimulus guttatus* to demonstrate that divergence in flowering time led to local adaptation between montane and coastal populations.

Formation of Reproductive Isolation Barriers

Reproductive isolation evolves by selection and/or drift (Lesica and Allendorf 1995, Orr 1995, Rundle and Whitlock 2001, Schluter 2001), and its evolution is influenced by the mode of speciation. Under allopatric speciation, for example, both prezygotic and postzygotic isolation can evolve at equal rates (Coyne and Orr 1998). If speciation occurs sympatrically, however, prezygotic barriers are likely to evolve quickly through reinforcement, or selection against maladaptive/postzygotically incompatible hybrids (Coyne and Orr 1998, Coyne and Orr 2004, Widmer *et al.* 2009). Evidence of this in sympatry can sometimes be observed in phenotypic character displacement of diverging populations (Schluter 2000, Rice and Pfenning 2010, Grossenbacher and Whittal 2011).

Ecological isolation has recently received more attention in reproductive isolation studies (Hatfield and Schluter 1999, Ramsey *et al.* 2003, Kay 2006, Sobel *et al.* 2010). Ecological isolation is likely important to speciation in plants, which often display local adaptation or diversification along ecological gradients (Ramsey *et al.* 2003, Kay 2006). Plant spatial distributions follow a range of geologic (edaphic to lithological) attributes (Vickery 1966, Kruckeberg and Rabinowitz 1985), and likewise, multiple scales of climate

variation (Rajakaruna 2004), among other environmental characteristics. Evidence for reproductive isolation between closely related species has been observed between sharply contrasting soils (Macnair and Christie 1983, Rajakaruna 2004, Brady *et al.* 2005) and along elevation gradients (Ramsey *et al.* 2003).

Progress in Reproductive Isolation Research

Most studies of reproductive isolation have assessed only one or a few potential barriers to isolation (Widmer *et al.* 2009). However, for a comprehensive study, all prezygotic and postzygotic barriers need to be quantified to compare their relative importance to constructing species boundaries (Schemske 2000, Coyne & Orr 2004). Although individual isolating mechanisms rarely confer complete reproductive isolation, each contributes to reducing some gene flow, which ultimately maintains species boundaries (Lowry *et al.* 2008b). Studying reproductive barriers at all life stages is important for understanding which isolating mechanisms may have evolved first or has developed at a faster rate (Coyne and Orr 1998). Intrinsic and extrinsic postzygotic isolation studies are still current avenues for understanding the significance of ecology to reproductive isolation (Ramsey *et al.* 2003, Coyne and Orr 2004, Widmer *et al.* 2009), especially in recently derived species, which are likely to be ecologically radiated (Behm *et al.* 2010).

Few studies have explored how ecological contributions to isolation can be expressed on a geographical scale (Sobel *et al.* 2010). Ramsey *et al.* (2003) found contrasting adaptation to elevation to be an abiotic ecogeographical factor contributing to more than 60% of reproductive isolation between *Mimulus cardinalis* and *Mimulus lewisii*. Kay (2006)

calculated that differences in habitat use and pollinator relationships contributed more to the isolation of two closely related *Costus* species than their spatial distances. Sobel (2010) estimated that ecogeographic barriers between 12 sister *Mimulus* species reduced no less than 64% of potential gene flow between sister pairs, suggesting that adaptation to local ecological conditions may be an important step in population divergence in this group. Further studies of reproductive isolation should include a comparison of ecogeographical isolation with pure spatial isolation when delving into ecological niche scenarios (Sobel *et al.* 2010).

A Brief Overview of Ecological Modeling

Ecological modeling is gaining complexity as more environmental issues are addressed (Lek 2007). Species distribution models (SDMs), or Ecological Niche Models (ENMs), predict suitable habitat for a given species based on known presence and absence locations (latitude and longitude) across a defined area described by multiple environmental covariates (Elith *et al.* 2006). The development of large databases of species occurrence records, and compilation of environmental measurements into spatial datasets, has enabled users to interface geographical information systems (GIS) software with ecological modeling software to create distribution maps for conservation management, evolutionary patterns, or other applications (Guisan and Zimmerman 2000, Guisan and Thuiller 2005, Elith *et al.* 2006).

Models work by giving either discrete binary code for predicting species presence and absence, or they provide a continuous probability distribution across a given area (Liu *et al.* 2011). From a given area the model can pull 4 types of results: true and false positives/

presences and true and false negatives/ absences based on environmental constraints (Liu *et al.* 2005). These are identified by specifying a threshold that determines a range in which the discrete or continuous assessment values are false/absent (below the threshold) or true/present (above the specified threshold) based on the empirical average of the environmental constraints (Liu *et al.* 2005). There are numerous subjective and objective approaches to defining such thresholds exhibited across a variety of models, but the ROC (receiver operator characteristic) plot is often used in ecological modeling. The ROC is obtained by the combination of sensitivity and specificity measures, which are probabilities that the model has correctly predicted the observation of a species presence (sensitivity) or absence (specificity) at a random site in the matrix (Liu *et al.* 2005).

Model Choice

Choosing a model is very important for ensuring that its predictive performance will be efficient in explaining the desired goals of the research (Gaston and García-Viñas 2011). Many models have been compared for species distributions, and multiple comparison attempts have found generalized linear models (GLM), generalized additive models (GAMs), genetic algorithm for rule set production (GARP), and/or maximum entropy (MaxEnt) models all perform well using presence/absence data (GLM, GAM), or presence only data (GARP, MaxEnt) (Elith *et al.* 2006). MaxEnt ranked 5th out of 16 models used to explain species-level distributions of 226 different species within 6 different regions of the world, where numbers of occurrence records ranged from 2 to above 5000 (Elith *et al.* 2006). This accessible and user-friendly model has become a common modeling approach in ecology for both government and private research (Elith *et al.* 2011).

Ecological niche models (ENMs) have recently been used to identify ecological characteristics associated with the distribution of closely related species (Graham *et al.* 2004, Rissler and Apodaca 2007, Warren *et al.* 2008, Nakazato *et al.* 2010, Sobel 2010). For example, a maximum entropy model, MaxEnt (Phillips *et al.* 2006), was used to describe the ecological niches of parapatric populations of the black salamander (*Aneides flavipunctatus*) in California (Rissler and Apodaca 2007). When combined with phylogenetic approaches, the model results revealed cryptic lineages of *A. flavipunctatus* that have diverged ecologically (Rissler and Apodaca 2007).

Sobel (2010) employed a MaxEnt model to examine the distribution of multiple closely related pairs of *Mimulus* species. Habitat models of each pair were created, and their area of niche overlap was determined to estimate the index of ecogeographic (prezygotic) isolation between them. The contribution of ecogeographic barriers to isolation averaged no less than an index of 0.64, suggesting that adaptation to local ecological conditions may be an important step in population divergence in this group (Sobel 2010).

MaxEnt is a generative model that uses the theory of maximum entropy described by Jaynes (1957), where it obtains a uniform probability distribution of species over a defined area based on environmental constraints—expected values of each feature that should match its empirical average—between each pixel of the matrix (Phillips *et al.* 2008). In other words, entropy is a measure of dispersiveness in geographic space (Elith *et al.* 2011). MaxEnt uses presence-only data (such as obtained from most herbarium databases), so it obtains pseudo-absence measurements by background sampling (Elith *et al.* 2011). It uses area under the curve (AUC) of the ROC, as a measure of model accuracy (Liu *et al.* 2005,

Liu *et al* 2011). An acceptable model results in an AUC above 0.70 (Araújo *et al.* 2005, Scheleman and van Zonneveld 2010), where an AUC of 0.50 is no better than random (Phillips *et al.* 2006). The default settings were tested over various scenarios of sample selection bias, environmental conditions, and species ranges, and were considered acceptable for explaining species distributions (Phillips *et al.* 2008, Elith *et al.* 2011). With presence-only data, a majority of the data must be used to train the model, and a portion must be set aside for testing. The user must select “random seed” in order for default cross-validation to randomly select a new subset of test data with each replication (Sobel 2010), or select “random seed” and define a percentage of data to be set aside with other test types (Flory 2010).

Modeling Concerns

With all models, there are a various types of uncertainty of which to be wary, (Barry and Elith 2006, Lek 2007, Cressie *et al.* 2009), but the most basic precautions are listed here. Sample selection bias in database records will influence models, as locations may have been difficult for field biologists to access, skewing toward locations in close proximity to roads or frequently visited locations (Phillips *et al.* 2008, Elith *et al.* 2011). Misidentification by confusing closely related species can also occur (Phillips *et al.* 2008). Another important issue to consider about obtaining occurrence records from a database is that there are no absence records. Pseudo-absences can be generated in MaxEnt (Phillips *et al.* 2006), but the bias introduced with this problem is that models may consider some areas of suitable habitat as unsuitable (Barry and Elith 2006).

One must also assess the environmental inputs for collinearity (similar features such as annual and monthly precipitation), especially if the model is to be extrapolated to other scenarios (Flory 2010, Gaston and García-Viñas 2011). Some types of data are suitable to analyze only at particular scales; for example, annual precipitation acts on a meso- to global scale, whereas soil substrates can act on a topological to micro-scale (Phillips *et al.* 2008). Moreover, the grain size of the model may not be able to accurately predict the combined effects of environmental variables when fine-scale measurements are extrapolated to large pixel sizes and vice versa (Phillips *et al.* 2006). The exclusion of environmental variables may also cause erroneous predictions (Barry and Elith 2006). More variety of in-depth environmental measurements, validation of models by geo-referenced field surveys, and collaboration between statistical model experts and ecologists can ameliorate these concerns.

CHAPTER III

METHODS

Study System

Mimulus guttatus and *M. glaucescens* are annual, mixed-mating herbs, with bilabiate, yellow corollas (Ritland and Ritland 1989, Hickman 1993). The range of *M. glaucescens* is restricted to Butte and Tehama counties, whereas *M. guttatus* is widespread throughout western North America (Grant 1924, Vickery 1959, Hickman 1993). *Mimulus glaucescens* and *M. guttatus* appear to favor similar ephemeral, moist habitats (Hickman 1993), although *M. guttatus* has been reported to occur in elevations higher than that of *M. glaucescens* (Banchero 1987, Oswald 1994). Banchero (1987) suggested that *M. glaucescens* may be more frequently restricted to ephemeral habitats than *M. guttatus*. The barriers to gene flow that led to speciation between *M. glaucescens* and *M. guttatus* have not been identified.

Vickery (1964) reported variation in postzygotic isolation among members of the *Mimulus guttatus* complex (section *Simiolus*), indicating asymmetric gene flow between these species and recent adaptive radiations within the complex. He noted barriers that reduced seed set, affected hybrid seedling development, and/or resulted in hybrid breakdown in the F₂ or later generations, showing different types of reduced fitness such as failure to flower (Vickery 1964). Furthermore, Vickery (1964) observed partial F₁ barriers contributing

to seed set, as well as evidence for heterosis (increased fitness due to heterozygosity) in crosses between populations of *M. guttatus* and a population of *M. glaucescens* (Vickery 1978).

Ecogeographic Isolation

Map construction. To estimate the importance of divergence in habitat to reproductive isolation, a range map for each species was constructed using ArcMAP 10 (ESRI 2011). The map reflected the location of *M. glaucescens* and *M. guttatus* collections within Butte and Tehama counties, based on georeferenced records obtained from the Consortium of California Herbaria (2010). Included in the map were the following environmental features: geology (primary substrates), soils (series profiles), Jepson subecoregions (a combination of elevation, geology, and vegetational communities as defined in Hickman 1993), and elevation (meters) (Table 1). Aspect and slope (degrees) were calculated from the elevation raster in ArcGIS (ESRI 2011). All layers were projected to the same datum (US NAD 1983 zone 10 N), and extracted to the same spatial extent, using ArcGIS functions (ESRI 2011).

Ecogeographical modeling. Ecological niche models (ENMs) reference field data to empirically predict important environmental variables that correlate with species distributions (Guisan and Thuiller 2005, Elith *et al.* 2011). The machine-learning program MaxEnt is robust to sample selection bias, environmental conditions, and species ranges, and is commonly used to model species habitat (Phillips *et al.* 2006, Elith *et al.* 2006, Phillips *et al.* 2008); thus, its use has become common in government and private research (Elith *et al.*

2011). This program identifies the most uniform probability distribution of species occurrences across complex habitat landscapes, based on the principle of maximum entropy (see Jaynes 1957), by modeling species point data (latitude and longitude in .csv file form) against multiple environmental layers (ASCII files) built from ArcGIS (Phillips *et al.* 2006).

Because MaxEnt uses presence-only data (such as obtained from most herbarium databases), it creates pseudo-absence measurements by background sampling (Phillips *et al.* 2006). MaxEnt modeling is thus similar to the commonly used GARP (genetic algorithm for rule set production) model, which also uses presence-only data (Phillips *et al.* 2006), and performs well in comparison to other models distinguishing suitable from unsuitable species habitat (see Elith *et al.* 2006). Particularly as species occurrence records increase, it has better predictive power than logistic regression models as species occurrence records increase (see Gaston and García-Viñas 2011).

Data preparation for MaxEnt modeling in ArcGIS. The same projection, extent, and grain size were imposed on all environmental layers to be used in the MaxEnt model, using ArcGIS (Phillips *et al.* 2006, Phillips and Dudík 2008, Phillips *et al.* 2008). The National Elevation Dataset (NED) rasters (see Table 1) were combined into one raster GRID file and then extracted by the limits of the Butte-Tehama county shapefile (Flory 2010). Other environmental features were converted to same parameters as the NED file: 10 m² cell size, 32-bit float, UTM projection, and 1 band. Slope and elevation were calculated in degrees from the NED raster in ArcGIS. Spatial extent was confirmed to be identical across all rasters, and exported as ASCII files for MaxEnt modeling (Phillips *et al.* 2006).

Table 1. Sources for data used in the environmental range map of *Mimulus glaucescens* and surrounding populations of *M. guttatus* in Butte and Tehama counties, California, USA.

<i>Spatial data</i>	<i>Resolution</i>	<i>Date</i>	<i>Source</i>	<i>Accessed from</i>
NorCal 7.5 minute quad	1 - meter	August 2009	Cal-Atlas Geospatial Clearinghouse: SacValleyGeology 02	http://atlas.ca.gov
California counties	1: 24,000	February 2009		
Jepson subecoregions	1: 250,000	April 1997	Cal-Atlas Geospatial Clearinghouse: JepsonSubEcoregsions	http://atlas.ca.gov
National Elevation Datasets (by county)	10 – meter	January 2010	Geospatial Data Gateway (USDA NRCS); also see USGS for NED and geology	http://datagateway.nrcs.usda.gov/ (for all); http://seamless.usgs.gov/ (for NED); http://usgs.gov/ (for geology)
California geology	1: 750,000	December 2007		
Soils (by county)	1: 24,000	June 2009		
<i>Mimulus</i> spp. records	N/A	November 2011	Consortium of California Herbaria	http://ucjeps.berkeley.edu/consortium/

MaxEnt modeling. MaxEnt (version 3.3.3; Phillips *et al.* 2006) was used to predict suitable habitat for *M. glaucescens* and *M. guttatus* based on similarity to modeled characteristics of the environment at historical collection locations in Butte and Tehama counties. Models for each species were run within the combined extent of Butte and Tehama counties, with soils excluded due to county differences in description, and a model for each species was run within the extent of Butte county (all layers prepared as above to the extent of Butte county only) with the soil parameters included (4 models total).

To validate the models, a majority of the data was used to train the model, and the remainder was set aside for testing. This process was performed for 25 iterations, using a new subset of test data with each replication (Sobel 2010). The area under the curve (AUC) of the receiver operator characteristic (ROC) within MaxEnt was used to evaluate model accuracy (Phillips *et al.* 2006), as commonly practiced in ecological modeling (Liu *et al.* 2005, Liu *et al.* 2011). The ROC is obtained from the probabilities that the model has correctly predicted the observation of a species presence (sensitivity) or absence (specificity) at a random site in the matrix (Liu *et al.* 2005). Models with an AUC above 0.70 are considered acceptable (Araújo *et al.* 2005, Scheleman and van Zonneveld 2010), where an AUC of 0.50 is no better than random (Phillips *et al.* 2006). Model results, which characterized the potential habitat of each species, were imported to ArcGIS to estimate ecogeographical isolation, following Sobel (2010).

Estimating ecogeographical isolation. To characterize ecogeographical isolation, a threshold on the probability values reflecting likely habitat suitability must be imposed to convert probability into a binary presence/absence response (Liu *et al.* 2005, Phillips *et al.*

2006). Each pixel in the resulting species distribution map reflects either species presence or absence (Sobel 2010), and the choice of threshold value used can therefore strongly influence modeling results. Ideally, the threshold value should be motivated by the biology of the species involved (Liu *et al.* 2005), although some have used arbitrary values (Manel *et al.* 1999). In the absence of other information, I used the approach of Kozak and Weins (2006) Sobel (2010), and Ochoa-Ochoa *et al.* (2012), and applied the average 10th percentile logistic threshold. This threshold codes the lower 10 percent of probability values as absence and codes probabilities at the threshold value and above as presence.

ArcGIS was used to convert the MaxEnt model output from ASCII files to binary coded rasters, in which species absence (predicted after applying the 10th percentile logistic threshold) were reclassified to a pixel value of 0 and species presence was coded as either 1 for *M. glaucescens* or 2 for *M. guttatus*. These rasters were then combined to form a third raster with pixel values of 0 (absence of both species), 1 (presence of *M. glaucescens*), 2 (presence of *M. guttatus*), or 3 (presence of both species). The total area (numbers of pixels multiplied by total m²) of possible presences, was used to estimate ecogeographical isolation following Sobel and Chen (*in review*):

$$RI_{\text{Ecogeography of } x} = 1 - [S/(S + U_x)],$$

Where,

S = area shared by both species x and y (*M. glaucescens* and *M. guttatus*)

U_x = unshared area occupied only by species x (*M. glaucescens* or *M. guttatus*)

Intrinsic Postzygotic Isolation

Experimental design. Intrinsic postzygotic isolation was estimated under laboratory conditions, which indicates genetic barriers limiting the success of hybrid offspring between the species. For these experiments, I used two populations each of *M. glaucescens* and *M. guttatus* collected from Butte Creek Canyon, an area where populations of both species are found (Table 2). The populations I used all occurred within 0.4 – 3 km, which is within the flight range of their primary pollinators (Waddington 1979, Beekman and Ratnieks 2000, Gathmann and Tscharntke 2002, Guédot *et al.* 2009, Osgathorpe *et al.* 2012). One seedling from each of 100 field-collected families from each population was sown in 5.08-cm pots with potting soil composed of equal parts peat, sand, pumice, and redwood compost, kept at room temperature, with a 12-hour cycle of supplemental fluorescent light (6500 Kelvin, 1000bulbs.com) cycle. The plants were watered as needed and fertilized once every 2 weeks with 16-16-16 (N-P-K) fertilizer (MaxSea, Garberville, CA). After 40 days, the plants were placed in a 4°C walk-in cooler for 32 days, and were then returned to their previous conditions to encourage flowering. Following their return to room temperature, light exposure was increased by 30 minutes every 2-3 days from a 12-hr to a 16-hr photoperiod.

Crossing experiment. Plants from all populations and both species were crossed in all possible combinations, resulting in three categories of cross types for each species: conspecific within-population, conspecific between-population, and hybrid crosses. Flowering plants were assigned at random to serve as pollen donor or recipient. To avoid self-pollination, flowers assigned as pollen recipients were emasculated in bud no more than 24 hours prior to pollination. A single anther from a plant assigned as pollen donor was

Table 2. Locations within Butte County, California, of study populations for intrinsic postzygotic isolation.

Species	Population	Lat/Long	Elevation
<i>M. glaucescens</i>	1	N 39° 45.119' W 121° 38.208'	165 m
<i>M. guttatus</i>	2	N 39° 44.984' W 121° 38.435'	165 m
<i>M. glaucescens</i>	3	N 39° 46.068' W 121° 40.494'	112 m
<i>M. guttatus</i>	4	N 39° 43.646' W 121° 42.304'	178 m

gently pressed into the stigma lobes of its assigned recipient to pollinate the flower.

Seed production. Seed pods were collected and stored in coin envelopes when the calyx was dried (as a sign of maturity) and before the carpels split open for seed dispersal. Seeds from each fruit produced were spread on white printer paper, photographed, and counted using the computer software Image J[®] (Rasband 2011). Seed set among cross types was compared with a general linear model (GLM) in Minitab[®] 16 (Minitab Inc. 2010).

Seed viability: germination rates. Twenty successful fruits of each conspecific and F₁ hybrid type produced in the crossing experiment were randomly chosen, and 20 seeds from each were sown into 10.2-cm pots in the lab. After two weeks, the number of seedlings was censused in each pot. The germination rate was calculated as the proportion of seeds that germinated.

Post-germination performance. One seedling from each of the subset of fruits chosen for the germination experiment was randomly chosen to evaluate intrinsic performance in the lab. Plants were grown as described above. To compare fertility and

performance among crosses, ovule number and pollen viability were scored following the methods of Carr and Dudash (1997) for the first flower to open on each plant. Pistils of collected flowers were stored in 3:1 ethanol: glacial acetic acid until analysis. Ovules from a single carpel per flower were counted under a light microscope after staining with lactophenol aniline blue. All anthers of collected flowers were placed in lactophenol in microcentrifuge tubes until viability could be scored. The mean proportion of pollen grains staining in three 25 µl aliquots was scored for each sample (Carr and Dudash 1997). In addition, the number of days until the first flower opened was recorded and, after plants had grown for 59 days, the total number of flowers was recorded for each plant. On day 60, all above-ground biomass was dried to constant mass at 55° C and weighed to the nearest milligram (0.001g).

Statistical analysis of postzygotic isolation data. Means of postzygotic performance traits were compared among cross types within each species using one-way ANOVA. All non-proportional data were log-transformed prior to analysis to satisfy model assumptions. Similarly, proportion of seeds germinated and proportion of viable pollen were arcsine-square-root transformed prior to analysis. No other variables required transformation to meet model assumptions. Post-hoc comparisons among means were made with a Tukey test. All analyses were conducted using Minitab 16[©] (Minitab Inc. 2010) or SAS 9.3[©] (SAS Institute 2010).

Calculating postzygotic reproductive isolation. To assess the potential for postzygotic fertility to limit gene flow between species, the performance of offspring from within-population crosses was first compared to that of between-population crosses within

each species. If a significant difference in performance were observed, this was interpreted as evidence for local adaptation or heterosis, depending on the direction of the difference. Because these involved crosses between populations of the same species, however, they were not considered to reflect species-level barriers to reproduction (or disassortative mating). Instead, these results were used as a basis for interpreting comparisons between the performances of hybrid offspring to that of within-population crosses. A trait was considered to contribute significantly to reproductive isolation only if the difference in mean performance of hybrid vs. within-population crosses was more extreme than within-population vs. between-population crosses.

Reproductive isolation was estimated independently for each species, as species barriers can be asymmetric, where gene flow is hindered in one species direction for a particular barrier (Coyne and Orr 2004). The approach of Sobel and Chen (*in review*) was used to calculate reproductive isolation:

$$RI_{Factor} = 1 - 2 * [H/(H + C)]$$

where,

H = the mean fitness measurement of hybrids
and,

C = the mean fitness measurement of conspecifics

Reproductive isolation based on the mean number of days to first flower opening among the offspring of these crosses was calculated as described above for ecogeographic isolation, by comparing the number of days both plants were flowering to the number during which each flowered in isolation (Sobel and Chen *in review*).

CHAPTER IV

RESULTS

Ecogeographic Isolation

Map of collection records. Based on collection records, *M. glaucescens* is predominately within the Cascade Range Foothills, whereas *M. guttatus* populations are distributed more broadly (Fig. 1).

Ecogeographic analysis with MaxEnt. The potential habitat of *M. glaucescens* was modeled as the probability distribution of its presence across Butte and Tehama counties, resulting in a model with a mean (SD) test area under the curve (AUC) of 0.799 (0.031) (Figure 2). The Jepson subecoregions variable explained most of the variation in *M. glaucescens* distribution (Table 3). The model of *M. guttatus* resulted in a mean (SD) AUC of 0.703 (0.042) (Figure 3). Geology and slope appear to explain most of the variation in distribution of *M. guttatus* in Butte and Tehama counties (Table 4).

When the MaxEnt model was restricted to Butte County with the addition of soil series information, the potential habitat of *M. glaucescens* was explained best by soils (Table 5), and resulted in an AUC of 0.862 (0.072) (Figure 4). For *M. guttatus*, Butte County soils explained 75.1 percent (%) of the potential habitat distribution of the species (Table 6), resulting in a model with an AUC of 0.725 (0.160) (Figure 5).

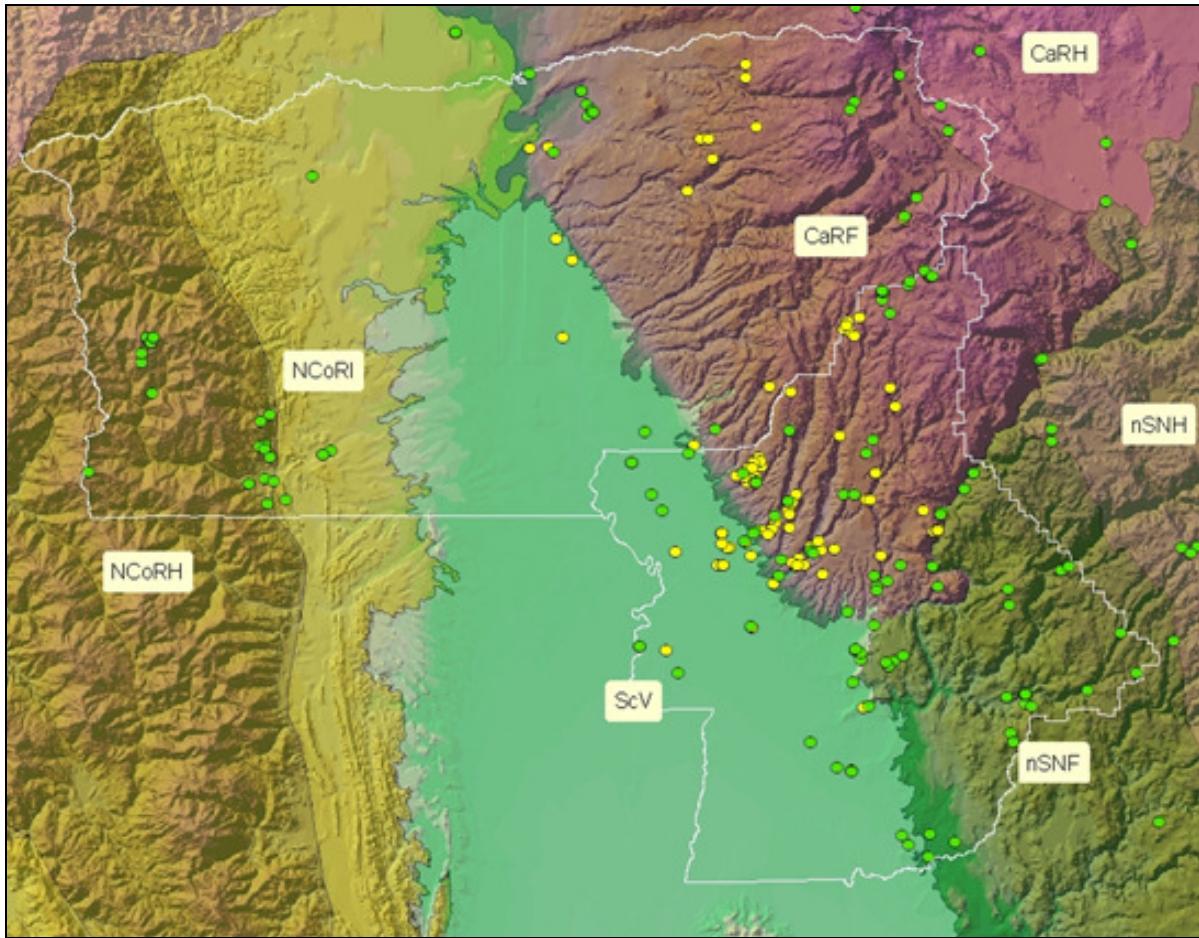


Figure 1. Collection record locations of *M. glaucescens* (yellow) within Butte (southern) and Tehama (northern) counties (white boundary lines) and surrounding populations of *M. guttatus* (green) from the Consortium of California Herbaria (2010). Region labels correspond to the Jepson subecoregions identified by Hickman (1993), which reflect vegetation associations: Interior North Coast Range (NCoRI), and Northern Coastal Range High (NCoRH), Sacramento Valley (ScV), Cascade Range Foothills (CaRF), High Cascade Range (CaRH), northern Sierra Nevada Foothills (nSNF), and northern High Sierra Nevada (nSNH).

Niche overlap as a measure of ecogeographic isolation. The combined MaxEnt model of habitat for both species predicted approximately 187 km² of habitat specific to the niche of *M. glaucescens*, 289 km² of habitat specific to *M. guttatus*, and 233 km² of habitat overlap between them (Figure 6). This suggests that habitat preferences limit about 45% of

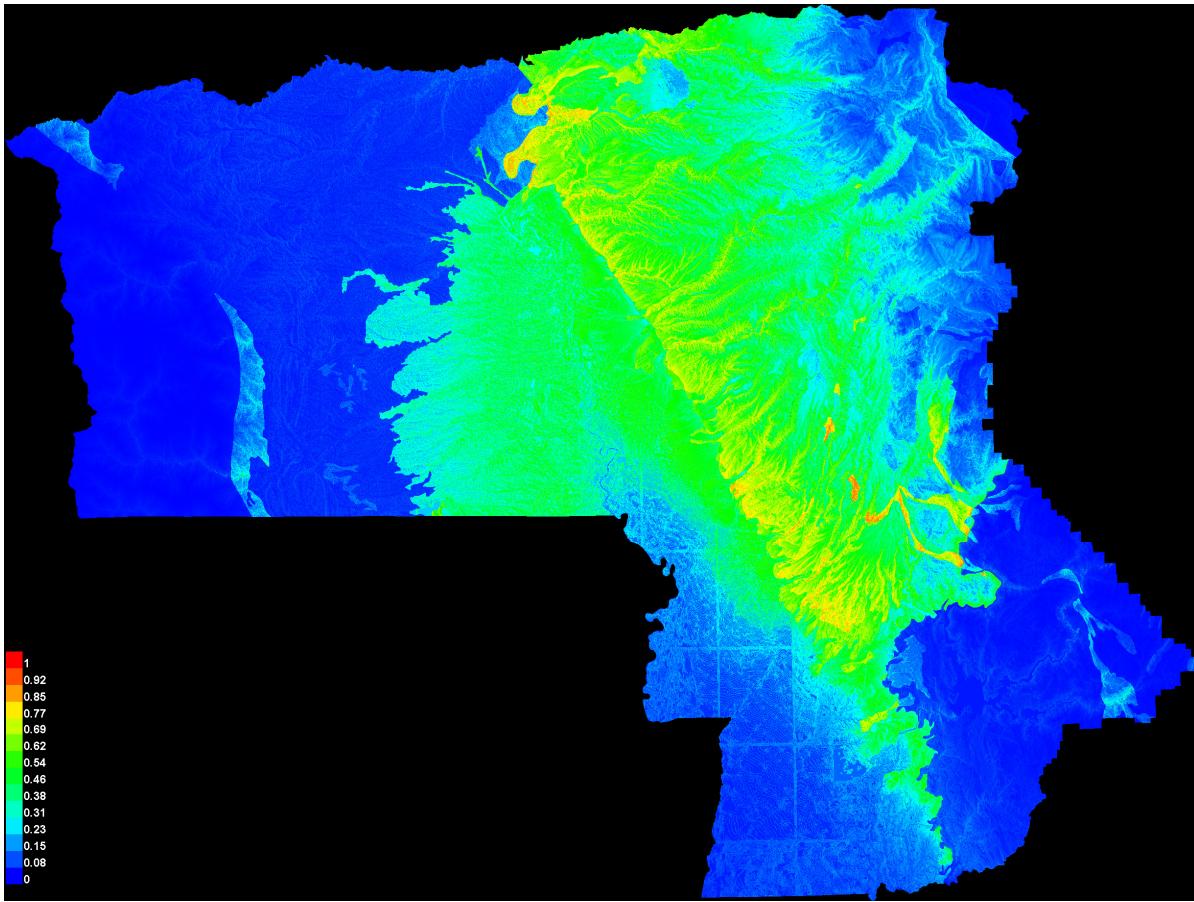


Figure 2. Mean probability distribution for the realized niche of *Mimulus glaucescens* in Butte and Tehama counties, California, USA, based on a MaxEnt model of elevation, slope, aspect, geologic substrates, and Jepson subecoregions. The probability of presence is depicted by a range of colors, where cooler colors give a range of probabilities from 0 (dark blue; most likely absent) to 0.62 (light green), and warmer colors predict a presence likelihood of 0.69 (light yellow) to 1 (red; most likely present).

potential gene flow to *M. glaucescens* from *M. guttatus* (i.e., RI = 0.45 for *M. glaucescens*) and 55% of gene flow in the reverse (RI = 0.55 for *M. guttatus*). In restricting the environmental extent to Butte County, and with the addition of soil series descriptions, the resulting niche overlap model (Figure 7) depicted 407.8 km² of suitable habitat for *M. guttatus*, 309.4 km² of suitable habitat specific to *M. glaucescens*, and relegated 93.3 km² of potential habitat overlap of both species. Thus, the habitat preferences, including soil

Table 3. Mean percent contribution and permutation importance of environmental variables to explaining *M. glaucescens* distribution in Butte and Tehama counties, California, USA based on a MaxEnt model.

Variable	Percent contribution	Permutation importance
Jepson subecoregions	45.3	41.3
Elevation	27.3	30.1
Geology	17.1	13.6
Slope	5.8	8.2
Aspect	4.5	6.8

distribution, suggest that potential gene flow to *M. guttatus* from *M. glaucescens* is restricted by approximately 81% ($RI = 0.81$ for *M. guttatus*) and approximately 77% in the reverse ($RI = 0.77$ for *M. glaucescens*).

Intrinsic Postzygotic Isolation

F₁ seed set. Mean seed set for *M. guttatus* did not significantly differ among cross types (Table 7). In contrast, crosses performed within populations of *M. glaucescens* yielded 37.5 % higher seed set than hybrids (Table 7). As a consequence, reduction in hybrid seed set (female fitness) contributed to reproductive isolation for *M. glaucescens* ($RI_{Seeds} = 0.24$), but not for *M. guttatus* ($RI_{Seeds} = 0$).

Seed germination. Mean germination rates among intraspecific and interspecific offspring did not significantly differ among cross types within *M. glaucescens* (Table 7) or *M. guttatus* (Table 7). Thus, germination of F₁ seeds does not appear to impose significant postzygotic reproductive isolation for either species.

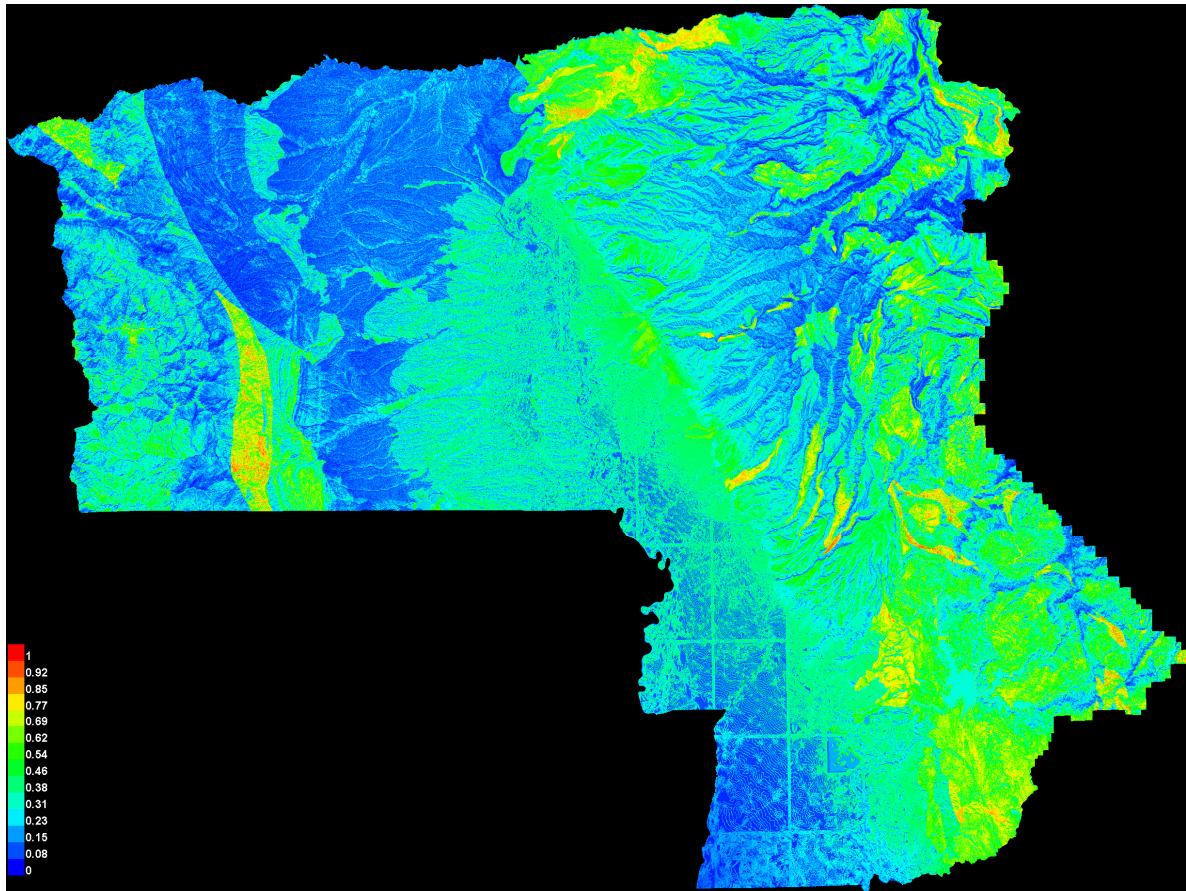


Figure 3. Mean probability distribution for the realized niche of *Mimulus guttatus* in Butte & Tehama counties, California, USA based on a MaxEnt model of elevation, aspect, slope, geologic substrates, and Jepson subecoregions. The probability of presence is depicted by a range of colors, where cooler colors give a range of probabilities from 0 (dark blue; most likely absent) to 0.62 (light green), and warmer colors predict a presence likelihood of 0.69 (light yellow) to 1 (red; most likely present).

Reproductive phenology. For hybrid plants whose maternal parent was *M. glaucescens*, the mean number of days before the first flower opened did not differ among cross types (Table 7). For *M. guttatus* hybrids, on the other hand, the timing of reproduction in interspecific crosses was delayed by 21.7%, relative to within-species crosses (Table 7). Therefore, delayed flowering phenology in *M. guttatus* hybrids may contribute to reproductive isolation from *M. glaucescens* ($RI_{Days} = 0.15$).

Table 4. Mean percent contribution and permutation importance of environmental variables to explaining *M. guttatus* distribution in Butte and Tehama counties, California, USA based on a MaxEnt model.

Variable	Percent contribution	Permutation importance
Geology	43.2	36.7
Jepson subecoregions	19.3	14.8
Slope	14.9	23.4
Aspect	13.6	12.1
Elevation	9	13.1

Total flower production. The mean number of flowers produced did not differ among cross types in which either *M. glaucescens* or *M. guttatus* was maternal parent (Table 7). Postzygotic fertility in terms of flower production was therefore unlikely to contribute to reproductive isolation in either species.

Biomass. Mean biomass of offspring did not differ among cross types for either *M. glaucescens* (Table 7) or *M. guttatus* (Table 7) parents. Therefore, differences in hybrid performance reflected in biomass were unlikely to contribute to intrinsic postzygotic reproductive isolation for either species.

Pollen viability. Mean proportion of viable pollen did not differ among cross types when either *M. glaucescens* (Table 7) or *M. guttatus* (Table 7) served as maternal parent. Pollen viability therefore was unlikely to contribute to postzygotic reproductive isolation between species.

Ovule production. Mean ovule production did not differ among cross types in which *M. glaucescens* served as the maternal parent (Table 7). However, 13.2% fewer ovules were produced in flowers of hybrid offspring for which the maternal parent was *Mimulus guttatus* (Table 7). Thus, female fertility as reflected in ovule production may

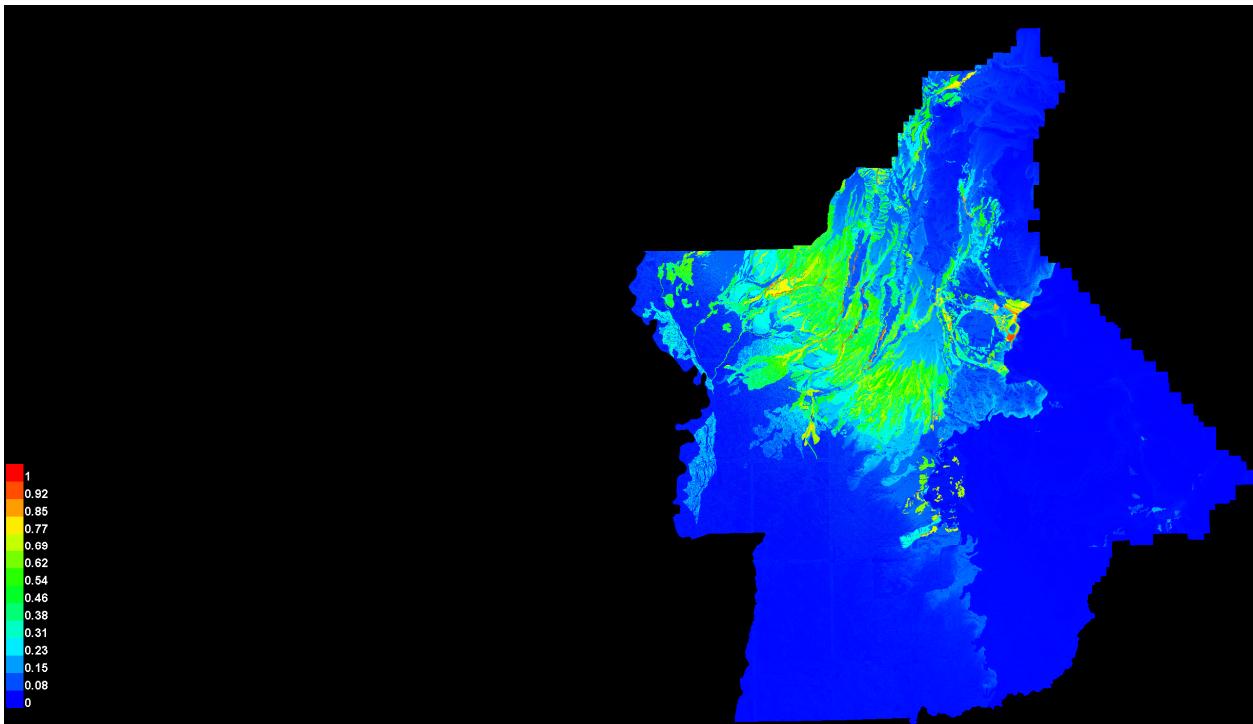


Figure 4. Mean probability distribution for the realized niche of *M. glaucescens* in Butte County, California, USA based on a MaxEnt model of elevation, aspect, slope, geologic substrates, Jepson subecoregions, and soil series. The probability of presence is depicted by a range of colors, where cooler colors give a range of probabilities from 0 (dark blue; most likely absent) to 0.62 (light green), and warmer colors predict a presence likelihood of 0.69 (light yellow) to 1 (red; most likely present).

contribute to intrinsic postzygotic isolation for *M. guttatus* ($\text{RI}_{\text{Ovules}} = 0.06$).

Table 5. Mean percent contribution and permutation importance of environmental variables to explaining *M. glaucescens* distribution in Butte County, California, USA based on a MaxEnt model.

Variable	Percent contribution	Permutation importance
Soils	42.8	44.1
Jepson subecoregions	24.3	31.5
Elevation	17.4	12.3
Geology	13	5.9
Aspect	1.7	4.4
Slope	0.8	1.8

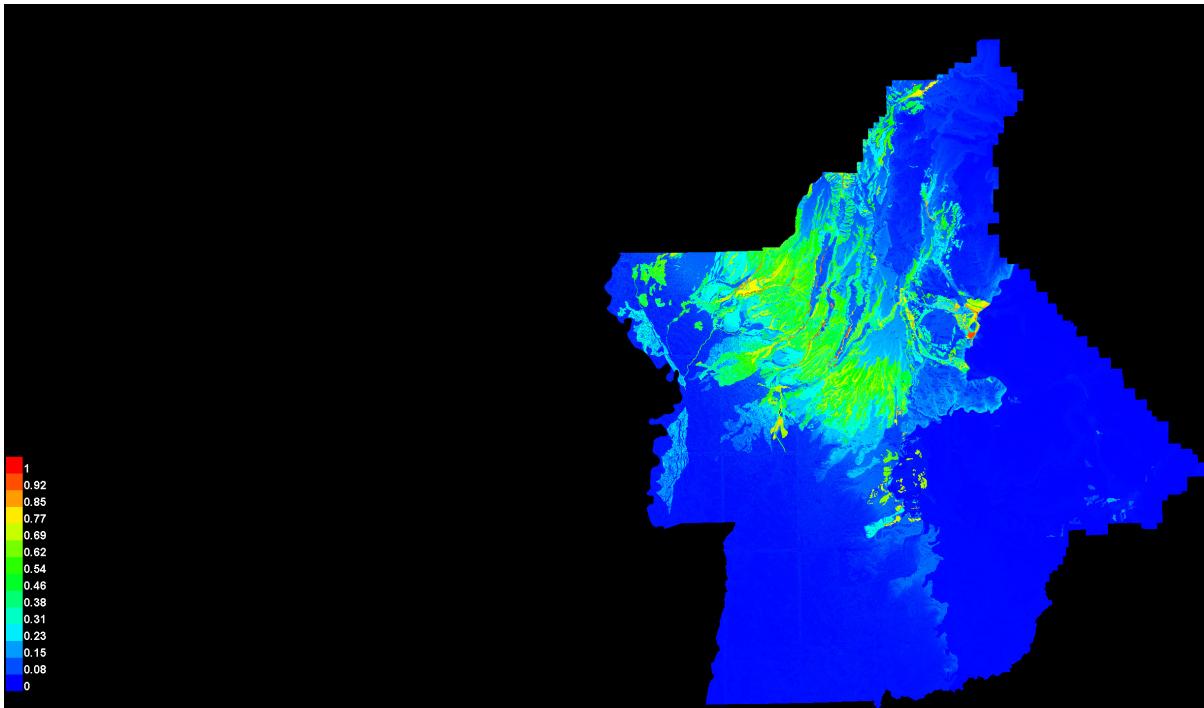


Figure 5. Mean probability distribution for the realized niche of *M. guttatus* in Butte County, California, USA based on a MaxEnt model of elevation, aspect, slope, geologic substrates, Jepson subecoregions, and soil series. The probability of presence is depicted by a range of colors, where cooler colors give a range of probabilities from 0 (dark blue; most likely absent) to 0.62 (light green), and warmer colors predict a presence likelihood of 0.69 (light yellow) to 1 (red; most likely present).

Table 6. Mean percent contribution and permutation importance of environmental variables to explaining *M. guttatus* distribution in Butte County, California, USA based on a MaxEnt model.

Variable	Percent contribution	Permutation importance
Soils	75.1	72.6
Slope	9.5	9.7
Geology	9.4	6
Elevation	4.8	11.3
Aspect	1	0.3
Jepson subecoregions	0.2	0.1

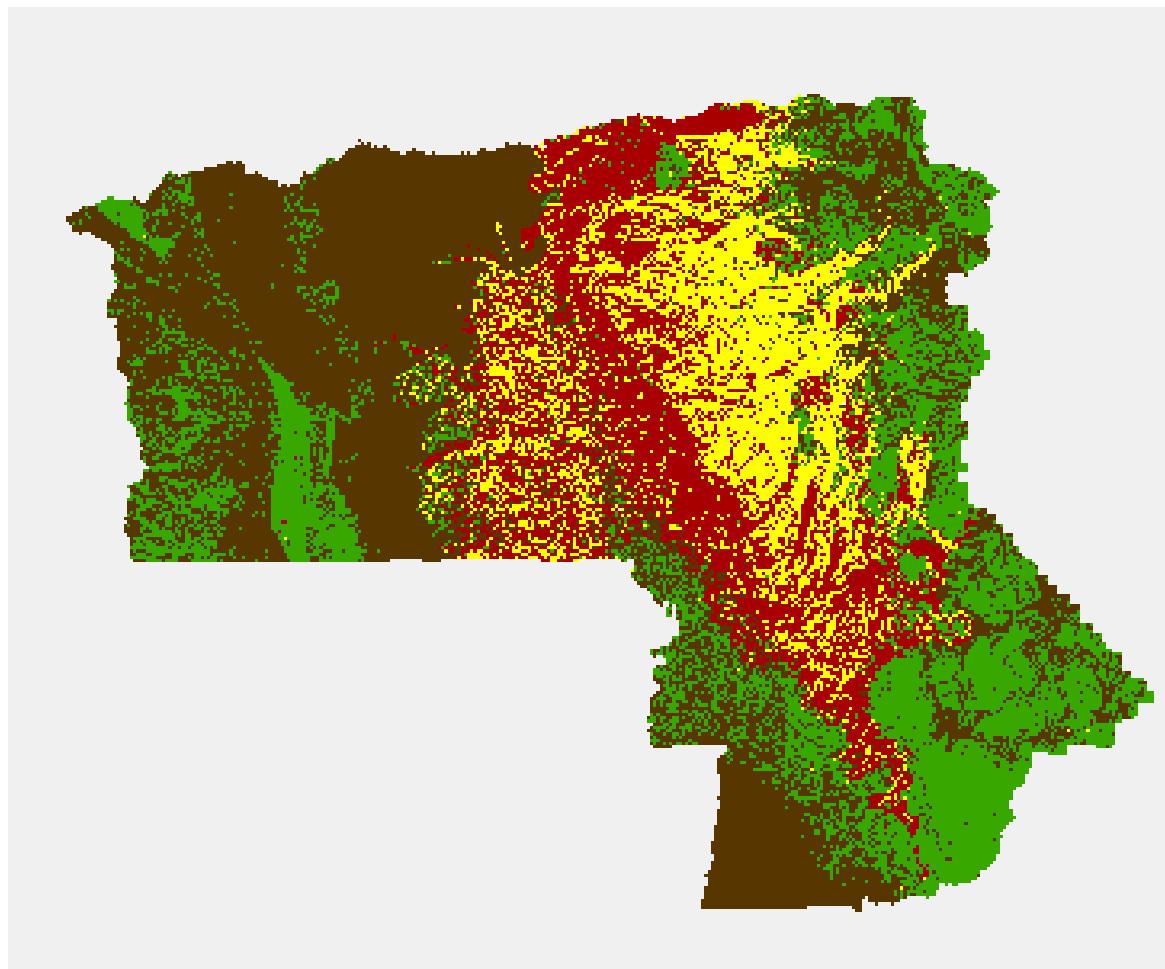


Figure 6. Distributions of predicted suitable habitat for *Mimulus guttatus* (green), suitable habitat for *M. glaucescens* (yellow), and potential niche overlap of both species (red) in Butte and Tehama counties, California, USA, based on a MaxEnt model including slope, aspect, elevation, geologic substrates and Jepson subecoregions. Unsuitable habitat for both species is indicated in brown.

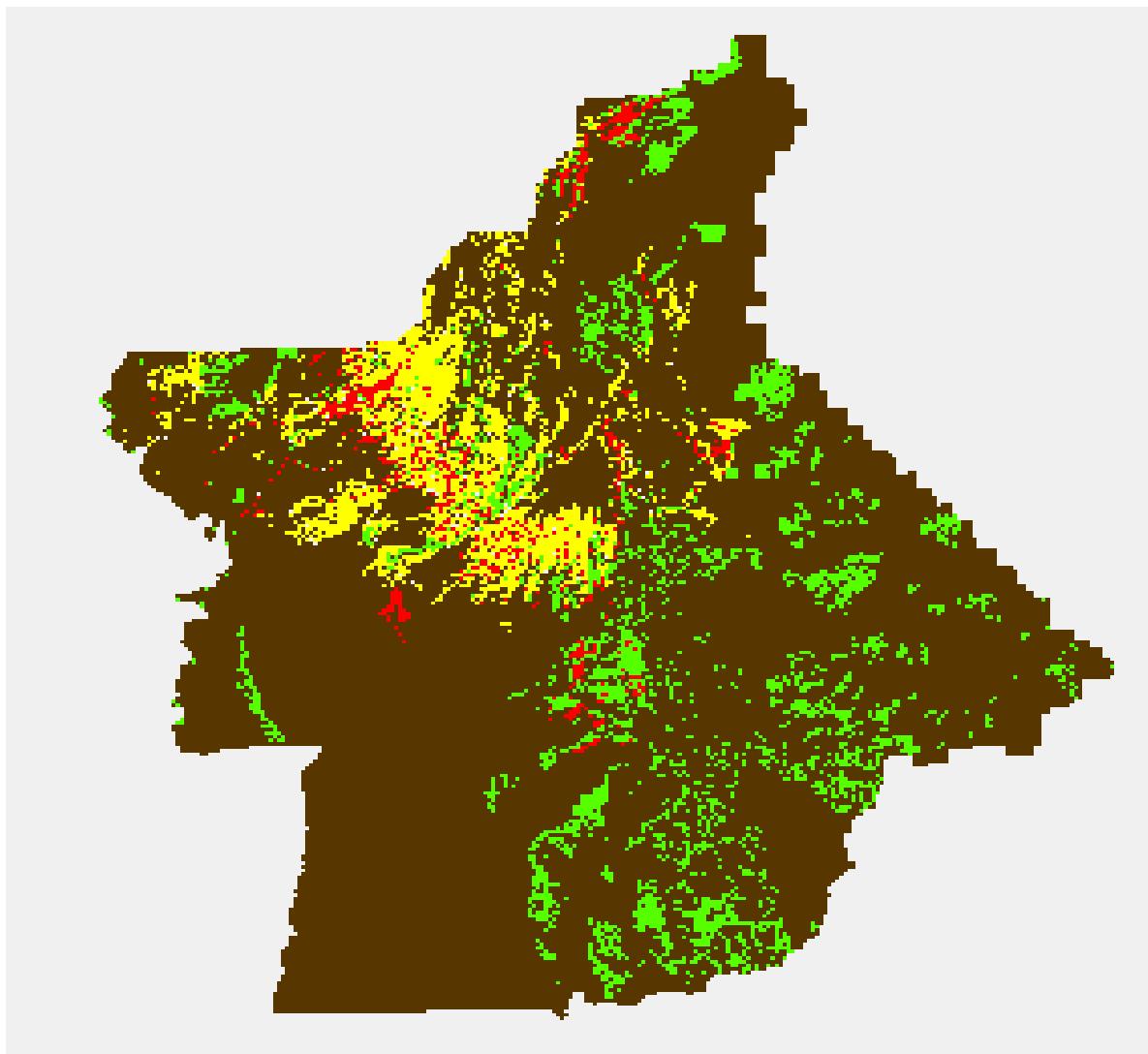


Figure 7. The resulting raster predicting suitable habitat for *M. glaucescens* (yellow), suitable habitat for *M. guttatus* (green), potential habitat overlap of both species (red), and unsuitable habitat for both species (brown) in Butte County, California, USA, based on a MaxEnt model, including slope, aspect, elevation, geologic substrates, Jepson subecoregions, and soil series descriptions.

Table 7. Least-squares mean (SE) performance (based on untransformed variables) of offspring from hand-pollinations of *Mimulus glaucescens* and *M. guttatus* collected from Butte county, California grown in the greenhouse, and results from one-way ANOVA (using transformed variables – see Methods) comparing means for each trait within each species. Means sharing a superscripted letter within a row do not significantly differ at $\alpha = 0.05$ by a Tukey's post-hoc test.

Component	Species	F	P	Cross		
				Within-population	Between-population	Hybrid
Seed Set (#)	<i>M. glaucescens</i>	$F_{2, 116} = 4.0$	0.02	357.8 (37.0) ^A	317.7 (53.0) ^{AB}	223.9 (31.1) ^B
	<i>M. guttatus</i>	$F_{2, 181} = 0.20$	0.1	660.3 (51.1)	481.9 (71.2)	552.7 (42.4)
Germination (#/20)	<i>M. glaucescens</i>	$F_{2, 91} = 0.10$	0.9	0.45 (0.16)	0.49 (0.17)	0.45 (0.16)
	<i>M. guttatus</i>	$F_{2, 123} = 2.5$	0.09	0.46 (0.15)	0.58 (0.16)	0.60 (0.15)
Days to First Flower (#)	<i>M. glaucescens</i>	$F_{2, 50} = 0.22$	0.8	32.3 (5.9)	28.4 (6.6)	32.0 (5.6)
	<i>M. guttatus</i>	$F_{2, 100} = 5.1$	0.008	28.9 (3.1) ^B	32.7 (3.3) ^{AB}	35.4 (2.9) ^A
Total Flowers (#)	<i>M. glaucescens</i>	$F_{2, 87} = 2.4$	0.1	42.4 (37.7)	53.9 (38.1)	44.8 (37.7)
	<i>M. guttatus</i>	$F_{2, 120} = 2.9$	0.06	41.4 (30.2)	68.2 (30.9)	58.6 (30.1)
Biomass (g)	<i>M. glaucescens</i>	$F_{2, 62} = 1.8$	0.2	1.41 (0.85)	1.80 (0.86)	1.76 (0.83)
	<i>M. guttatus</i>	$F_{2, 113} = 2.3$	0.1	1.66 (0.71)	2.29 (0.70)	1.96 (0.69)
Pollen Viability (#/100)	<i>M. glaucescens</i>	$F_{2, 25} = 0.8$	0.8	0.86 (0.06)	0.93 (0.08)	0.85 (0.05)
	<i>M. guttatus</i>	$F_{2, 25} = 0.12$	0.9	0.83 (0.04)	0.86 (0.04)	0.85 (0.03)
Ovules (#)	<i>M. glaucescens</i>	$F_{2, 50} = 1.8$	0.2	516.4 (100.0)	477.1 (105.9)	581.5 (96.6)
	<i>M. guttatus</i>	$F_{2, 100} = 4.3$	0.01	744.6 (128.6) ^A	743.8 (127.6) ^{AB}	658.7 (126.4) ^B

CHAPTER V

DISCUSSION

General Overview

Comparison of the strength of barriers to reproduction is necessary to distinguish those likely to be involved in speciation (Schemske 2000, Coyne & Orr 2004). Although prezygotic isolation barriers are important for prevention of interbreeding between populations, postzygotic isolation can also contribute to the formation of species boundaries (Coyne and Orr 2004, Rieseberg and Willis 2007). Ecogeographical isolation, a prezygotic barrier, is expected to be common in the radiation of young species evolving allopatrically (Behm *et al.* 2010). Because ecogeographical isolation can be strong, it should be included in reproductive isolation studies to determine its prevalence and relative strength compared to other barriers (Sobel *et al.* 2010). This barrier alone limits a minimum of 45% of the gene flow between *M. glaucescens* and *M. guttatus*. Intrinsic postzygotic isolation factors are also present, and limit a minimum 21% of the gene flow between these species.

Ecogeographical Isolation

Ecological modeling. While purely spatial geographical isolation could be important for limiting hybridization between species, it is not solely responsible for maintaining species barriers in most cases (Dobzhansky 1951, Mayr 1942, Stebbins 1950). More subtle ecological differences in habitat often permit coexistence of sympatric relatives

(Coyne and Orr 1998), and local adaptation can cause reproductive isolation (Coyne and Orr 2004, Martin and Willis 2007, Sobel *et al.* 2010). In some instances, adaptation to divergent habitats has prevented viable hybrids between closely related species in field experiments. For instance, no natural hybrids of stickleback fishes (*Gasterosteus* spp.) were found in a study of sympatric benthic and limnetic lake species, which displayed differences in habitat utilization and body morphology (Hatfield and Schluter 1999). The species readily produced viable hybrids in a controlled laboratory setting, but the hybrids grew slower than their parents when transplanted into the home lake of their parents (Hatfield and Schluter 1999).

Detail on species distributions has become increasingly accessible with the widespread availability of georeferenced species collection records (Graham *et al.* 2004, Phillips *et al.* 2006), which has facilitated our understanding of how distribution patterns contribute to reproductive isolation. Sample selection bias due to a higher frequency of records collected in easily accessed areas may be a concern in interpreting model results (Barry and Elith 2006). Despite this, Ramsey *et al.* (2003), found significant differences in elevation between *Mimulus cardinalis* and *Mimulus lewisii* based on herbaria records. The magnitude of sample selection bias was not estimated for *M. glaucescens* and *M. guttatus*, but the Butte-Tehama county area has a substantial collection history throughout, so bias should be minimal.

Others have used ecological niche modeling of collection records with environmental spatial data to delineate ecogeographic distributions of close relatives (Graham *et al.* 2004, Rissler and Apodaca 2007, Nakazato *et al.* 2010, Sobel 2010). Uncertainty caused by lack of important environmental variables (Barry and Elith 2006),

possible collinearity among them (Flory 2010, Gaston and García-Viñas 2011), and their grain sizes (Phillips *et al.* 2006) may all effect modeling of species distributions. Also, pseudo-absences generated in MaxEnt (Phillips *et al.* 2006) may render some areas of suitable habitat as unsuitable (Barry and Elith 2006). All concerns listed above are common for use with modeling, but should not prevent interpretation of model results as a preliminary step in understanding the evolution of reproductive barriers between species. Nakazato *et al.* (2010), for example, modeled the distribution of four sister species pairs of wild Andean tomatoes (*Solanum* spp.) to conclude three out of four pairs utilized significantly different habitat niches. In comparing the distribution of *M. glaucescens* and *M. guttatus*, collinearity among environmental variables of the Jepson subecoregions, geology, and elevation may have influenced results, although all of these variables ranked high in the models. A grain size of 10 m² may not have been appropriate for broader polygon descriptions such as the Jepson subecoregions and geology data, yet the suggested results are interesting (see below). The exclusion of detailed climate variables may have also caused erroneous predictions, although microclimate intuitive of slope and aspect ranked low.

Model interpretation. The geographic diversity of the California Floristic Province has likely contributed to its high plant diversity (Raven and Axelrod 1978). Evidence of this is reflected in the endemic range of *Mimulus glaucescens*, which approximates the distribution of the Tuscan formation depicted in Lydon (1969). *Mimulus guttatus* populations are dispersed throughout the Cascade and the Sierra Nevada mountain ranges, but are less frequently observed within areas covered by the Tuscan formation. The central Tuscan lahars—landslides, or mudflow, in this case—formed within the last one

million years upon the eruption of Mt. Yana, the southernmost volcano of the Cascade Range (Lydon 1969). Multiple examples of plant species restricted to single volcanoes have been reported from the Cascade Range (Kruckeberg and Rabinowitz 1985), and dispersal into the devastating Tuscan mudflow (Lydon 1969) by the ancestor of present-day *M. glaucescens* populations may have been a catalyst for its divergence from a *guttatus*-like ancestor.

Because the Tuscan substrates have been recolonized by plant species that are extant and geographically widespread (Hickman 1993), the Jepson subecoregions are unlikely to currently impose sufficiently strong selection to exclude *Mimulus guttatus* (see Table 4). Instead, initial ecological aspects shaped by the inhospitable volcanic event are predicted to have played a role in the speciation of *Mimulus glaucescens*, as insinuated in the Jepson subecoregions, in which elevation and geology are already imbedded (an issue of collinearity; see Table 3). The Butte-Tehama MaxEnt models suggest a geologically-directed distribution of the species on a landscape-level scale, while the models restricted to the extent of Butte County explain a finer ecological scale in which the species' distributions are edaphically-directed. In an attempt to understand the species' distributions on soils, information on populations of *M. guttatus* and *M. glaucescens* and the soils at collection locations in Butte County were extracted via a polygon overlay in ArcGIS. No significant differences were found between the locations of *Mimulus glaucescens* and *M. guttatus* populations, according to soils organized into great group types for a chi-square test of independence ($\chi^2 = 1.406$, $df = 1$, $P = 0.236$), but these soils may have been categorized too broadly.

One of the main distinguishing traits of *M. glaucescens* is the presence of glaucous, peduncular bracts subtending the inflorescence. In other taxa, glaucousness has been suggested to attenuate loss of water and to protect from UV radiation (Reicosky and Hanover 1978). Similarly, glaucous leaves are a trait targeted in the breeding of drought-tolerant wheat for the Mediterranean climate of southern Australia (Bennett *et al.* 2012). Although the adaptive significance of glaucous bracts in *M. glaucescens* remains to be tested, this is at least consistent with the observations of Banchero (1987), who suggested *M. glaucescens* tolerates dryer habitats than *M. guttatus*. Other studies have suggested that contrasting drought adaptation strategies have likely contributed to divergence among other members of the *M. guttatus* species complex (Lowry *et al.* 2008a, Wu *et al.* 2010, Ivey and Carr 2012). Thus, the hypothesis that genetic and morphological divergence in *M. glaucescens* reflects selection based in part on water availability may coincide with its ecogeographical isolation of *M. guttatus* in water retention properties of soils, and warrants further consideration.

Intrinsic Postzygotic Isolation

Hybrid seeds may form when prezygotic barriers are successfully evaded, but they face potential fitness disadvantages compared to seeds from conspecific matings (Dobzhansky 1951, Mayr 1942, Stebbins 1950, Coyne and Orr 2004, Ramsey *et al.* 2003). Measures of hybrid fitness were conducted in a controlled laboratory as indicators of intrinsic postzygotic isolation, estimating asymmetric gene flow, and thus, the existence of different isolating barriers between the species.

Genetic differences in developmental timing may be concluded from the delayed onset of flowering in *Mimulus guttatus* hybrids in the laboratory. The intrinsic occurrence of phenological shifts in the lab suggests the accumulation of genetic incompatibilities that may reduce the likelihood that hybrids would mate with their parent species (Stebbins 1950, Coyne and Orr 2004); thus, this seems likely to be an effective mechanism of reducing gene flow from *M. glaucescens*. Based on the data collected, *Mimulus guttatus* hybrids also produced 6 % fewer ovules, indicating a reduction in female fertility. Seed set was reduced 24% in *Mimulus glaucescens* flowers pollinated by hybrid pollen, which was comparable to the findings of Vickery (1964), who found seed set to be reduced by 50% or more in his F₁ and F₂ crosses of *Mimulus glaucescens* plants with pollen from *M. guttatus*. The presence of these isolating barriers suggests the existence of genes that reduce fertility of hybrids between these species. Additional components of male and female fertility (Ramsey *et al.* 2003, Coyne and Orr 2004), as well as advanced generation hybrid crossings and backcrosses (Rundle and Whitlock 2001), could be considered for future studies.

Although reduced fertility was observed in hybrids of both species, there were no differences in other measurements of hybrid fitness (germination rate, total flowers, biomass, and pollen viability), indicating that the strength of reproductive isolation conferred by intrinsic postzygotic isolation is uniform across all possible barriers. Similar results were found in a study by Ramsey *et al.* (2003), who reported that both seed mass and pollen viability in hybrids of *Mimulus cardinalis* and *Mimulus lewisii* were about one-third of their parents, but that the hybrids survived and grew well.

Total Reproductive Isolation

Coyne and Orr (1989, 1997) pointed out that barriers to reproduction act sequentially, based on the organisms' life histories, to filter opportunities for gene flow, and that earlier-acting barriers reduce the magnitude of gene flow available for later-acting barriers to act upon. This hierarchy must be considered when comparing the strength of barriers acting at different stages. Thus, Coyne and Orr (1989, 1997) and others (Ramsey *et al.* 2003, Sobel and Chen *in review*) have suggested a quantitative framework for comparing the strength of reproductive isolation (RI) or potential gene flow interruption imposed individual barriers, and for considering how these act together to limit total gene flow (T). In essence, the comparison is based on the restriction of total gene flow imposed by each barrier after gene flow is filtered from earlier-acting barriers, or actual contribution (AC) of each barrier. The proportion of isolation conferred by each barrier in turn, or relative contribution (RC), could then be calculated, which would be equal to each barrier's actual contribution (AC) if the total isolation (T) between species was complete (or equal to an index of 1) (see Coyne and Orr 1989, Coyne and Orr 1997, Ramsey *et al.* 2003 for more on this approach). This protocol was utilized by Ramsey *et al.* (2003) to compare prezygotic and postzygotic barriers between *Mimulus cardinalis* and *M. lewisii*. They found prezygotic barriers to contribute more than 99% to total isolation between these species.

In a related study of *M. glaucescens* and *M. guttatus*, Bergmann (2012) estimated the strength of six potential prezygotic barriers, including floral phenology, pollinator specificity, floral morphology, and specificity in three post-pollination, prezygotic traits (pollen adhesion, pollen germination, and pollen tube growth rate). Among these barriers, he

found that offset floral phenology potentially limited gene flow from *M. glaucescens* to *M. guttatus* (Bergmann 2012). To compare the relative strength of all barriers to reproduction between *M. glaucescens* and *M. guttatus* using the approach described by Coyne and Orr (1989, 1997), Bergmann's (2012) prezygotic estimates were incorporated with those from the current study (Table 8 for Butte-Tehama County; Table 9 for Butte County).

Based on this summary, ecogeographical isolation is the strongest barrier preventing reproduction between *M. glaucescens* and *M. guttatus*. Furthermore, it appears to be more important for maintaining species boundaries than any of the intrinsic postzygotic isolating barriers estimated, or Bergmann's (2012) estimates. Each species had reduced fertility in hybrid offspring, indicating postzygotic isolation, but all hybrids had some fertility. Estimates of total reproductive isolation suggest gene flow may occur between these closely related species, although additional barriers to reproduction may exist that have not yet been measured.

Table 8. Barriers contributing to reproductive isolation between *Mimulus guttatus* and *M. glaucescens* based on experiments described by Bergmann (2012) and herein for the Butte-Tehama County extent. Shown is reproductive isolation index or potential amount of gene flow affected by the barrier (RI), actual contribution to reproductive isolation relative to the gene flow filtered from previous barriers (AC), relative contribution compared to the isolating barriers explored (RC), and total isolation of the barriers combined (T). See Sobel and Chen (*in review*) for details on calculations.

<i>Species</i>	<i>Barrier</i>	<i>RI</i>	<i>AC</i>	<i>RC</i>	<i>T</i>
<i>M. glaucescens</i>					
<u>Prezygotic</u>					
	Ecogeographic	0.45	0.45	0.72	
<u>Postzygotic</u>					
	Seed Set	0.24	0.17	0.28	
					0.62
<i>M. guttatus</i>					
<u>Prezygotic</u>					
	Ecogeographic	0.55	0.55	0.72	
	Phenology	0.26	0.12	0.15	
<u>Postzygotic</u>					
	Lab Phenology	0.15	0.08	0.10	
	Ovules	0.06	0.03	0.03	
					0.77

Table 9. Barriers contributing to reproductive isolation between *Mimulus guttatus* and *M. glaucescens* based on experiments described by Bergmann (2012) and herein for Butte County. Shown is reproductive isolation index or potential amount of gene flow affected by the barrier (RI), actual contribution to reproductive isolation relative to the gene flow filtered from previous barriers (AC), relative contribution compared to the isolating barriers explored (RC), and total isolation of the barriers combined (T). See Sobel and Chen (*in review*) for details on calculations.

<i>Species</i>	<i>Barrier</i>	<i>RI</i>	<i>AC</i>	<i>RC</i>	<i>T</i>
<i>M. glaucescens</i>					
<u>Prezygotic</u>					
	Ecogeographic	0.77	0.77	0.90	
<u>Postzygotic</u>					
	Seed Set	0.24	0.08	0.10	
					0.85
<i>M. guttatus</i>					
<u>Prezygotic</u>					
	Ecogeographic	0.81	0.81	0.89	
	Phenology	0.26	0.05	0.06	
<u>Postzygotic</u>					
	Lab Phenology	0.15	0.03	0.04	
	Ovules	0.06	0.01	0.01	
					0.91

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

Habitat differences appear to be important to maintaining species boundaries between *Mimulus guttatus* and *M. glaucescens*. Genes contributing to their postzygotic isolation may have evolved allopatrically in different habitats, given that estimates of prezygotic barriers other than ecogeographic isolation are weak or absent (Bergmann 2012, Coyne and Orr 1998, Coyne and Orr 2004). Moreover, Vickery (1964) reported putative hybrids between these taxa in the field, so the common observation of distinct populations (e.g., Hickman 1993), especially in their areas of sympatry, suggests some mechanism maintaining species boundaries (Jiggins *et al.* 2001, Coyne and Orr 2004). Local adaptation to microhabitats of prevailing moisture conditions may be responsible for the divergence of these species, and fine-scale characterization of local habitat would be necessary to test that hypothesis. Reciprocal transplants of parental and hybrid plants between parental populations would also clarify the extent to which distributions are based on adaptation to local ecological conditions (showing extrinsic postzygotic isolation) (Stebbins 1950, Rundle and Whitlock 2001, Coyne and Orr 2004, Rieseberg and Willis 2007, Martin and Willis 2007, Sobel *et al.* 2010).

Although fourteen potential mechanisms of reproductive isolation between *M. guttatus* and *M. glaucescens* have been estimated, other possible barriers may remain to be

characterized. Some prezygotic barriers, such as non-overlapping phenology (Bergmann 2012), can be favored by natural selection, a phenomenon known as reinforcement (Schluter 2000, Coyne and Orr 2004, Kay and Schemske 2008, Lowry *et al.* 2008b). However, the postzygotic isolation characterized herein may not be sufficient for reinforcement to occur (Behm *et al.* 2010). Furthermore, if isolation is less than complete, as these data suggest (Table 6), then some introgression is likely. Rapidly developing molecular tools (Wu *et al.* 2008) should facilitate characterization of the extent of introgression between these closely related taxa (Sweigart and Willis 2003). Nonetheless, based on these results it seems safe to consider *Mimulus glaucescens* and *Mimulus guttatus* as distinct biological species (*sensu* Mayr 1942).

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