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Evolutionary Differences between Two Lupines with Differing Mating Systems

By
Jessica Berta Shade

A dissertation submitted in partial satisfaction of the requirements for the degree of
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Evolutionary Differences between Two Lupines with Differing Mating Systems

by

Jessica Berta Shade

Abstract

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Doctor of Philosophy in Integrative Biology
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The shift from outcrossing to selfing is one of the most common and evolutionarily significant transitions in flowering plants. Breeding system affects several evolutionary processes, which result in important differences between closely related selfing and outcrossing taxa. However, while the genetic and phenotypic differences between selfers and outcrossers have been documented often, the adaptive processes responsible for these changes are still not well understood.

In this dissertation, I compare several populations of the outcrossing *Lupinus nanus* and the selfing *L. bicolor*, to investigate the genetic, selective, phenotypic, and adaptive consequences of mating system. I predicted that the selfing mating system of *L. bicolor* would result in low levels of genetic diversity and thus an inability to respond to selective pressures. The outcrossing nature *L. nanus*, on the other hand, should maintain sufficient intrapopulation genetic diversity to allow for natural selection to take place.

First, I used microsatellites to examine genetic diversity and population differentiation in both species to test the hypothesis that *L. bicolor* had less intrapopulation diversity and more interpopulation isolation than *L. nanus*. Second, I calculated the selective landscapes of both species and their ability to respond to selection. Third, I measured local adaptation in *L. nanus* and *L. bicolor* to answer the question: Do *L. nanus* or *L. bicolor* populations exhibit higher levels of local adaptation? Finally, I compared variance-covariance matrices to examine past evolutionary trajectories.

I found that *L. bicolor* has consistently lower intrapopulation genetic diversity and higher levels of isolation by distance than *L. nanus*. Additionally, *L. nanus* showed a consistent floral shift in the direction predicted by selection whereas *L. bicolor* did not, suggesting that *L. bicolor* is unable to respond to selective pressures due to its lack of genetic diversity. My measurements of local adaptation and home-site advantage were consistent with this finding, as all *L. nanus* populations showed evidence of home-site advantage, and three of the four populations were locally adapted, while *L. bicolor* showed neither home-site advantage nor local adaptation for any of my study populations. The variance-covariance matrix comparisons were less conclusive, showing moderate similarities among floral suites of traits for both *L. nanus* and *L. bicolor*.

We conclude that, because *L. bicolor* is strongly selfing, it lacks the genetic diversity needed for selection to work on. Evolution in this species is likely dominated by neutral evolution, such as genetic drift. *L. nanus*, on the other hand, is not genetically depauperate and thus can respond to selective pressures. We also discuss the conservation and restoration implications of these findings.

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Chapter 1. Conservation implications of genetic variation and population differentiation in two species of *Lupinus* with different mating systems

1.1 INTRODUCTION

For over a decade, much attention has focused on genetic issues in conservation and restoration. Investigations on the partitioning of genetic variation in native species populations are especially important for management and restoration seed source selection, because intrapopulation genetic diversity and interpopulation gene flow are linked to population persistence and extinction rates (Fisher 1930, Falk and Holsinger 1991, Ellstrand and Elam 1993, Frankham 2005). One factor that has been identified as having an important effect on patterns of genetic diversity in plants is mating system (Allard et al. 1968, Jain 1976, Abbott and Gomes 1989, Schoen et al. 1996, Viard et al. 1997, Szarowska et al. 2003, Oja 2005, Hensen et al. 2010, Koelling et al. 2011). However, the extent to which mating systems affect conservation and restoration success is still not well understood.

The shift from outcrossing to selfing, in particular, causes changes in how genetic variation is partitioned within and among populations and therefore could have important conservation implications (Wright 1965, Pollak 1987, Schoen and Brown 1991, Hamrick and Godt 1996, Nybom 2004, Glemin et al. 2006, Koelling et al. 2011). In particular, selfing drives neutral evolution (i.e., genetic drift) by reducing effective population size (Schoen and Brown 1991) and increasing population extinction and recolonization rates (Ingvarsson 2002). These processes decrease heterozygosity and within-population genetic diversity (Koelling et al. 2011). For example, Glover and Barrett (1987) found that genetic diversity and heterozygosity were significantly correlated with outcrossing rate in *Eichhornia paniculata* (Glover and Barrett 1987). Hamrick and Godt (1996) supported these results with their comprehensive study of over 500 selfing and outcrossing species, finding that outcrossing species had consistently higher intrapopulation genetic diversity.

The low levels of within-population genetic diversity found in selfing plants could indicate an increased need for conservation of these species due to species vulnerability. Reduced intrapopulation genetic diversity levels have been associated with decreases in fitness due to reduced growth rates, survival, fecundity, and resistance to pathogens and herbivores (Mitton 1984, Huenneke 1991), as well as an increased risk for extinction (Reed and Frankham 2003, Frankham 2005). For example, Newman and Pilson (1997) found that decreased genetic variation in *Clarkia pulchella* lowered population survival probability (Newman and Pilson 1997). Speilman *et al.* (2004) also examined the relationship between genetic diversity and extinction rate, and found that reduced heterozygosity was associated with higher extinction risk in the wild (Spielman et al. 2004).

Selfing species may also be particularly threatened by climate change, because another consequence of low genetic diversity levels is a reduced ability for populations to adapt to changing environmental conditions (Frankham 1980, Soule 1980, Simberloff 1988). Because the ability of a species to respond to novel selective pressures is proportional to the amount of genetic variation in that species, selfing species with low levels of genetic diversity may be unable to evolve in response to selection imposed by climate change (Falconer 1989).

Populations with high self-pollination rates receive little gene flow via pollen from nearby populations, while outcrossing populations, on the other hand, have high levels of pollinator-mediated gene flow (Gottlieb 1984, Abbott and Gomes 1989, Williams et al. 2001, Oja 2005, Hensen et al. 2010). Reduced gene flow among selfing populations decreases the genetic cohesion of the species and increases population differentiation in comparison to outcrossing species with pollination-vector-mediated gene flow (Hamrick and Godt 1996, Nybom 2004). Several plant studies have supported this, such as Williams *et al.* (2001) who found levels of inter-population genetic differentiation ten times greater in the partially selfing *Delphinium barbeyi* than in its predominantly outcrossing congener, *D. nuttallianum*.

The differences in selfing and outcrossing inter-population gene flow can affect long-term population health. Gene flow protects against intrapopulation depletion of genetic variation, thus preventing inbreeding depression associated with genetic drift (Allendorf 1983, Huenneke 1991). However, gene flow also has the potential to reduce local population fitness by acting against local adaptation (Antonovics 1976, Simberloff 1988, Waser and Price 1989). Because gene flow is restricted by geographic distance and environmental barriers, distant populations are able to evolve in response to differing selection gradients, thus adapting to their local environments (Wright 1946). Patterns of genetic differentiation such as isolation by distance can therefore enable higher overall species fitness because, if a population in one location receives immigrant genes from a population adapted to a different environment, it could disrupt local adaptation and reduce mean fitness in the population (Ellstrand 1992). Understanding patterns of genetic differentiation in local populations is thus critical for understanding the forces affecting fitness in native populations.

Few studies have compared how mating system affects patterns of population differentiation. Some studies have suggested that the extreme restriction of gene flow among selfing populations could isolate even neighboring populations. In this case, patterns of genetic differentiation in selfing plants may be dominated by founder effects, time since colonization, and bottlenecks due to frequent extinction and recolonization events rather than gene flow limitations such as distance or geographic barriers (Gold et al. 1999, Kark et al. 1999, Koljonen et al. 1999, McLean et al. 1999, Shaffer et al. 2000, Caizergues et al. 2001).

However, recent studies have predicted that even extremely limited gene dispersal among selfing populations could swamp out these effects. In fact, the restriction of gene flow among populations of a selfing species could magnify the rate that genetic differentiation increases as a function of distance and produce a well defined structure of isolation by distance (Vekemans and Hardy 2004, Crispo and Hendry 2005, Epperson 2007). Vekemans and Hardy (2004) supported this prediction by showing that fine-scale spatial genetic structure was higher for selfing species than outcrossing species.

Isolation by distance has also been documented in outcrossing species, because distance also constrains gene flow via pollen (Cuenca et al. 2003, Van Puyvelde and Triest 2007, Chybicki et al. 2009, Le Guen et al. 2009, Logossa et al. 2011). However, many studies of outcrossing species have found little to no evidence for isolation by distance (Campbell and Dooley 1992, Waser 1993, LeCorre et al. 1997). One hypotheses that has been advanced to explain this result is that high gene flow among populations might override any distance barrier (Seppa and Laurila 1999). This would result in low levels of genetic differentiation among all outcrossing populations, regardless of the distance separating them. In this case, high gene flow among populations could be detrimental to overall species fitness if it prevents or disrupts local adaptation.

Despite the importance of understanding how mating systems affect genetic patterns in native species, few studies have compared genetic partitioning in native plants with differing breeding systems (Williams et al. 2001, Moyle 2006, Koelling et al. 2011). One reason these studies are uncommon is because study system requirements are stringent, and most organisms do not meet the criteria necessary for among-population comparisons of a selfing and an outcrossing species. For instance, the two study organisms must be closely related to avoid differences due to phylogeny. They must also grow sympatrically and bloom simultaneously to avoid differences due to varying environmental conditions across seasons. Moreover, the populations under consideration must be distinct to avoid pseudoreplication.

Lupinus nanus and its selfing congener *L. bicolor* are ideal species for studying differences in genetic variation partitioning because they are closely related, grow sympatrically, and have overlapping anthesis periods (Smith 1923b, a, 1924, Dunn 1956, Harding et al. 1974, Karoly 1992). Additionally, both species grow in patches throughout California, allowing for comparisons across both short and long distances. These species are two of the most commonly used species in restoration efforts throughout California, but there has been little, if any, genetic work examining their patterns of genetic variation, thus precluding predictions about species restoration potential or conservation necessity (Caltrans 2003, Ashly 2005, Caltrans 2008, Balzer and Robinson 2011).

This study examines the population genetics of *L. nanus* and *L. bicolor* to determine how breeding system affects differences in partitioning of genetic variation and to provide a genetic platform for estimates of conservation need and restoration potential. Specifically, we investigate the following predictions: (1) Because selfing species experience reduced effective populations sizes and frequent extinction and recolonization events, *L. bicolor* should have lower within-site genetic diversity than *L. nanus*. (2) Selfing species have lower levels of gene flow among populations than outcrossing species, so *L. bicolor* should exhibit higher levels of genetic variation among populations than *L. nanus*. (3) This restriction of gene flow in selfing species also increases levels of isolation by distance; therefore, *L. bicolor* should exhibit a stronger regression pattern between population pair genetic isolation and physical distance than *L. nanus*.

Because previous studies of these species have found variable outcrossing rates, hybridization viability, and population sizes (Dunn 1956, Elliot et al. 1974, Harding et al. 1974), we first test the following three assumptions: 1) *L. bicolor* is selfing; *L. nanus* is outcrossing, 2) *L. bicolor* and *L. nanus* do not hybridize in the wild, and 3) our study populations of both species are distinct. We then examine genetic variation and differentiation of sympatric populations of *L. nanus* and *L. bicolor* to test the three predictions made above.

1.2 METHODS

Study System

Lupinus nanus and *L. bicolor* are annual legumes in the subfamily Papilionoideae endemic to western North America. *Lupinus nanus* is limited to California, Nevada, and Oregon, but *L. bicolor* has a range that extends from Arizona to British Columbia (Baldwin et al. 2012). Both species grow in similar habitats and can often be found growing sympatrically in coastal scrub, foothill woodland, coastal prairie, and valley grassland communities. While studies have found variable rates of outcrossing for both species, *L. bicolor* generally has higher rates of selfing than *L. nanus* and sets seeds readily in the absence of pollinators (Elliot et al. 1974, Harding et al. 1974, Karoly 1992). Additionally, bees have been documented as the main pollinators of *L. nanus*, but *L. bicolor* flowers are rarely visited by insects (Dunn 1956).

We selected ten sites at which populations of *L. nanus* and *L. bicolor* occur sympatrically. The distances between these locations ranged from 4km to 617km (Fig. 1). From north to south, our sites were: Dye Creek Reserve (DC), Corning (CO), Pepperwood Creekside (PWC), Pepperwood Borrow Pit (PWP), Hicks Valley Rd. (HV), Lucas Valley Rd. (LV), Sibley Volcanic Regional Park (SV), Toro Park (TP), Fort Ord (FO), and Tejon Ranch (TR).

Soils at the DC and CO sites consist of over 80% sand, with little organic matter (Holmes 2008), and dry quickly during the Mediterranean-like summer. Lupine populations at these sites are dense. Sites PWC and PWP are both located on Pepperwood Preserve, but have different site characteristics. PWC is located along Martin Creek; it therefore has moist soil until late in the growing season. Lupine plants at this location grow in dense clumps in the loamy soil around the creek and have a longer flowering season than other populations due to the late season water availability (USDA_Soil_Conservation_Service 1978). PWP, on the other hand, is located in a gravel borrow pit 4.17km away from PWC. Plants at PWP grow sporadically throughout the pit and have a short life cycle due to the poor soil quality and low water availability. Both HV and LV have high soil clay content, allowing for some water retention in the late spring or early summer (Buck and Evens 2010). The basalt rocks that are common in SV keep soil acidity low, and the coastal fog keeps temperatures cool in the summer (Sloan 2006). TP and FO also experience summer coastal fog, but while TP has coarse clay loam soil, our site at FO has loamy sand soils (Cook 1978). The TR site is composed of gravely sandy loam with alluvium derived mostly from sandstone and shale (USDA_Soil_Conservation_Service 1980).

Plant Material Collection

To ensure correct species identification plant samples were collected while in flower. We accommodated the broad range of flowering times among populations spread throughout California by collecting tissue between April and June of 2009. We collected five immature leaves from 30 individuals of each species (*L. nanus* and *L. bicolor*) at each site (CO, DC, FO, HV, LV, PWC, PWP, SV, TP, and TR). We selected plants in a 100m by 100m quadrat by generating two sets of random numbers between zero and 100: one set of x coordinates and one set of y coordinates. We then sampled the plant located within the quadrat at the specified x and y coordinates. Leaves were oven dried at 50°C for four days and stored in coin envelops at -20°C.

Microsatellite Analysis

We extracted total genomic DNA from 100mg of each leaf sample using DNeasy Plant Mini kits (Qiagen, Valencia, CA). We then diluted DNA by 1:20 before amplifying the microsatellite loci Luna1-Luna6, Luna8- Luna9, Luna12- Luna13, Luna15- Luna18, and Luna20 using the protocol described in Molecular Ecology Resources Primer Development *et al.* (2012). Briefly, we performed a 25µl PCR reaction on the following mixture: 2.5µl PCR buffer (10X, - $MgCl_2$) (NEB final concentration 10 mM Tris-HCl, 50 mM KCl, pH 8.3), 25 µg/ml BSA, 4 mM $MgCl_2$, 0.52 mM of each primer, 150 µM of each dNTP, 1 unit *Taq* DNA Polymerase (NEB), and 2.5 µl of a 1:20 dilution of extracted DNA. PCR started with a 10-min activation cycle at 95°C followed by two cycles of a 1-min denaturing step at 94°C, a 1-min annealing step at 60°C and a 35-sec extension at 70°C. This was followed by 18 cycles consisting of 45 sec of denaturing at 93°C, a 45-sec annealing step, which started at 59°C and stepped down 0.5°C per cycle, and a 45-sec extension at 70°C. Then there were 20 cycles, each consisting of 30 sec of denaturing at 92°C, 30 sec of annealing at 50°C and a 1 min extension at 70°C. This was followed by a final extension for 5 min at 72°C. One-half µl of PCR product was mixed with a solution of 9 µl of formamide and 0.5 µl of ROX 500 size standard (Applied Biosystems) and sent to the UC Berkeley Sequencing Facility for electrophoresis. Results were analyzed with Peak Scanner Software v1.0 (Applied Biosystems).

Population departure from Hardy-Weinberg expectations and genotypic linkage disequilibrium within each population for each pair of loci was tested using Fisher's exact test with GENEPOP version 4.1 (Raymond and Rousset 1995). We also used GENEPOP to estimate observed heterozygosity and Nei's Gene Diversity rates. To examine inbreeding levels of *L. nanus* and *L. bicolor*, we calculated F_{is} values with 95% confidence and allelic richness intervals using HIERFSTAT (Goudet 2005, R Development Core Team 2011).

We used ARLEQUIN version 3.11 (Excoffier and Lischer 2010) to perform separate Analyses of Molecular Variances (AMOVA) on each species to partition total genetic variation into within-species, within-site, and within-individual (i.e., heterozygosity) components. To further explore the effects of site and species on the genetic structure of *L. bicolor* and *L. nanus*, we used HIERFSTAT to test for the significance of site and species effects (Goudet 2005, R Development Core Team 2011).

To examine admixture within populations we used the Bayesian program STRUCTURE (Pritchard et al. 2000, Falush et al. 2007, Hubisz et al. 2009). STRUCTURE assigns individuals to a specified number (K) of populations, each characterized by a set of alleles. The probability that individuals from a metapopulation belong to a specific subpopulation is estimated using a Markov chain Monte Carlo (MCMC) approach, which analyzes the data by using previous population sample values to randomly generate the next sample value at each step of the chain. Only the most recent sample value affects the transitional probabilities between the two sample values; earlier sample values are not considered. If an individual possesses a genotype that is characteristic of more than one population, that individual can be jointly assigned to two or more populations, indicating admixture. In implementing this method, we used a burn-in of 50,000 iterations and repeated twenty runs with 500,000 iterations with K values set from 1 to 50. We chose a wide array of population numbers because a single population could be possible if gene flow were high and there was little differentiation among species, while a larger number of populations could exist if cryptic structure existed within populations. Selfing species, in particular, can have sites that house multiple populations because when gene flow is restricted within family lines it is possible for structure to exist within what looks like a cohesive population.

Because STRUCTURE sometimes has difficulties determining the appropriate number of population clusters we used the program STRUCTURAMA to verify the total number of distinct *L. nanus* and *L. bicolor* populations (Pritchard et al. 2000, Pella and Masuda 2006, Huelsenbeck and Andolfatto 2007, Huelsenbeck et al. 2011). This program allows both the number of clusters and the assignment of individuals to each cluster to be random variables that follow a Dirichlet process prior. The Dirichlet process prior is a model assigning individuals to one of an infinite number of possible populations, with the probability of assignment increasing with the number of individuals already assigned to that population, and the probability of assignment to a new population proportional to alpha (a positive scaling parameter). In our model, alpha was assigned as a random variable drawn from a gamma distribution (shape parameter = 2.5, scale parameter = 0.5) (Pella and Masuda 2006). STRUCTURAMA implements the Dirichlet process model in a Bayesian MCMC to determine the probability that the data is clustered into each possible population number. We ran the MCMC with 150,000 cycles, which were sampled every 100th cycle, with the first 50,000 samples discarded as burn-in.

Both STRUCTURE and STRUCTURAMA rely on the assumption of Hardy-Weinberg and linkage equilibrium. However, because both *L. bicolor* and *L. nanus* are self-compatible their populations may not meet these assumptions (Harding et al. 1974, Karoly 1992). Departure from Hardy-Weinberg or linkage equilibrium may result in the number of populations being overestimated (Falush et al. 2003), so we confirmed the independence of our predicted populations by performing exact tests of population differentiation in ARLEQUIN version 3.11 (Excoffier and Lischer 2010)

Because relative measures of among-population divergence, such as F_{st} and G_{st} values, are dependent on within-population diversity levels (Nei and Chesser 1983), F_{st} and G_{st} are not good measures of differentiation in populations with high or variable levels of inbreeding (and therefore extremely low levels of within-site genetic diversity) (Hendrick 2005). This is especially pertinent to our study, because while *L. nanus* is predominantly outcrossing, it is self-compatible, and therefore may have different levels of inbreeding among sites. Additionally, we predicted our selfing populations would have significant deviation from Hardy-Weinberg equilibrium and low within-site variation levels. Therefore, we used an estimate of actual differentiation (D_{est}), as described in Jost (2008), to estimate genetic differentiation among populations. Ninety-five percent confidence intervals for D_{est} measurements were calculated using 1,000 bootstrap replicates. All D_{est} estimates were calculated using SMOGD (Crawford 2010). We performed full-model T-tests on the average D_{est} values of each species to determine if *L. nanus* populations were less genetically differentiated than were *L. bicolor* populations.

We established whether populations exhibited isolation by distance by using species-specific Mantel tests to determine if the matrix of pairwise distances between sites and the matrix of pairwise D_{est} values were correlated (Mantel 1967). To examine the strength of isolation by distance patterns we then calculated the slope and intercept from Reduced Major Axis (RMA) regressions. RMA regression is more appropriate for examining isolation by distance than Ordinary Least-Squares regression (OLS), because RMA is less biased when both the dependent and independent variables are measured with error (McArdle 1988, LaBarbera 1989). Because the independent variable used in isolation by distance analyses (geographic distance) is an approximation of gene flow barriers, rather than a direct measurement without error, RMA provides a better estimate of the relationship between the two variables. In his study examining populations of *Balanophyllia elegans*, Hellberg (1994) used simulations to confirm that RMA was a more accurate tool for isolation by distance than OLS. To properly account for each population being applied to multiple pairwise contrasts we used bootstrapping over independent population pairs to estimate the regression error. All RMA calculations were done using the Isolation by Distance Web Service (Jensen et al. 2005). Our D_{est} data violated assumptions of normality of residuals, so we log transformed D_{est} for these calculations.

1.3 RESULTS

All *L. bicolor* populations deviated from Hardy-Weinberg Equilibrium, as did the HV and PWP *L.nanus* populations. We found linkage disequilibrium at all sites that departed from Hardy-Weinberg expectations (Appendix 1).

Populations of *L. bicolor* averaged higher F_{is} values (0.92) than did *L. nanus* populations (0.20) (Fig. 2), but values varied more among populations of *L. nanus* than *L. bicolor* (Table 1). For example, at PWP, the F_{is} of *L. nanus* was 0.70 with a 95% confidence interval of 0.14, indicating an intermediate level of inbreeding, while at TP the F_{is} of *L. nanus* was -0.09 with a 95% confidence interval of 0.09, indicating random mating to slight assortative mating. Populations of *L. bicolor* had consistently high F_{is} values, although values at the PWP and TP populations suggested intermediate rather than high inbreeding.

Averaged across all populations, *L. bicolor* had lower intra-population gene diversity ($p= 0.01$) and allelic richness ($p=0.03$) than *L. nanus* populations (Fig. 3). Within each site, *L. nanus* exhibited consistently higher observed heterozygosity rates than did *L. bicolor*, which often had completely homozygous populations (Table 2).

In partitioning the total genetic variation of *L. bicolor*, AMOVA (Table 3) identified that a very small proportion of variation (1.68%) was explained within individuals and some variation was explained among individuals within sites (38.28%). Differences among sites accounted for the largest proportion of variation (60.04%) within this species. In contrast, 37.31% of total genetic variation was explained within *L. nanus* individuals and 11.97% was explained by individuals within sites. Differences among sites accounted for half of the total variation (Table 4).

Analysis by STRUCTURE showed the independence of both site and species. Posterior probabilities of K reached an asymptote at 20 populations (pooling data across species), suggesting that each species population at each site is separate from all others (Fig. 4). We also did not find significant admixture, either between sympatric populations of *L. nanus* and *L. bicolor* or among sites within species (Fig. 5). Analysis by STRUCTURAMA confirmed the existence of 20 discrete populations, with a probability of 0.90. All exact probabilities of population differentiation across loci were less than 0.01, thus confirming the independence of all 20 populations.

Values of D_{est} ranged from 0.19 to 1 for *L. bicolor*, and all D_{est} values were significantly different from zero. *Lupinus nanus* D_{est} values ranged from 0.04 to 0.98 and 37 out of the 45 pairwise comparisons were significantly different from zero. Average among-site genetic differentiation in *L. bicolor* was 0.63, which was significantly higher than that of *L. nanus* (0.42; $t_{d.f.}$ =4.27, $p>0.01$; Fig. 6).

Mantel tests showed that the geographic distance matrix was correlated with the D_{est} matrix of *L. bicolor* ($r=0.53$, $p<0.01$) but not *L. nanus* ($r=-0.12$, $p=0.60$). The Reduced Major Axis regression of log D_{est} versus distance (Fig. 7) showed that *L. bicolor* had a slope of 1.0×10^{-3} (95% CI 6.5×10^{-4} to 1.4×10^{-3}) and an r^2 value of 0.28 (95% CI 0.07 to 0.51) while *L. nanus* had a slope of -2.7×10^{-3} (95% CI -6.4×10^{-3} to 5.5×10^{-3}) and an r^2 value of 6.1×10^{-3} (95% CI 0 to 0.87). The 95% confidence intervals for the *L. bicolor* and *L. nanus* intercept ranged from -0.54 to -0.34 and -1.76 to 0.70, respectively.

1.4 DISCUSSION

Selfing tendencies

As predicted from the small flowers and lack of pollinator visits of *L. bicolor* (Dunn 1956, Elliot et al. 1974, Harding et al. 1974), high F_{is} levels suggest that all of its sampled populations exhibit high to complete selfing (Fyfe and Bailey 1951). Although F_{is} values obtained from microsatellites can sometimes overestimate the level of selfing in a population if null alleles and/or partial dominance result in the mis-scoring of heterozygotes, the consistency of values across all loci at all populations suggests that *L. bicolor* is highly selfing (Wattier et al. 1998, Bonin et al. 2004, Hoffman and Amos 2005). Non-random mating, such as self-pollination, violates Hardy-Weinberg assumptions, so high selfing would explain the deviation from Hardy-Weinberg equilibrium we observed in all *L. bicolor* populations.

Whereas *L. bicolor* populations consistently exhibited high F_{is}, values ranged widely across *L. nanus* populations, suggesting variable outcrossing rates. At most sites F_{is} values of *L. nanus* populations were not significantly different from zero and were in Hardy-Weinberg equilibrium, suggesting heterozygosity levels consistent with random mating. However, HV and PWP had positive F_{is} values, departure from Hardy-Weinberg equilibrium, and linkage disequilibrium, suggesting higher levels of selfing (Nordborg 2000, Flint-Garcia et al. 2003). Such variation in selfing level is consistent with the findings of previous studies, predicting that *L. nanus* is a facultative selfer with variable outcrossing rates (Dunn 1956, Harding 1970, Horovitz and Harding 1972, Harding et al. 1974, Karoly 1992). *Lupinus nanus* plants are self-compatible; self-pollination is inhibited by protandry and a collar of peristigmatic hairs. However, these hairs eventually wilt and allow self-pollination in flowers that have not been yet pollinated (Juncosa and Webster 1989). Therefore, *L. nanus* individuals growing in circumstances where pollinators are limited will have higher selfing rates due to facultative selfing. Pollinator species composition may also affect the selfing rate of a population. For example, Horovitz and Thorp (1970) found higher inbreeding rates in *L. nanus* populations that were visited by honey bees than in those visited primarily by bumble-bees. Differences in pollinator abundance and species composition could therefore explain the variation in F_{is} values among the *L. nanus* populations studied here (Horovitz and Thorp 1970).

Hybridization and Reproductive Isolation

Because these species are reported to be interfertile in greenhouse studies (Elliot et al. 1974), we wondered if hybridization might occur in the field. However, we found no instances of this in our study populations. Lack of hybridization is consistent with the nearly complete selfing in *L. bicolor* that was suggested by the high F_{is} values of each population. Selfing is an important pre-zygotic mechanism preventing hybridization between closely related species (Jacquemart and Thompson 1996, Wendt et al. 2002, Dubois et al. 2003, Drummond and Hamilton 2007). Although other pre-zygotic isolating mechanisms, such as geographic separation and flowering asynchrony can account for a large proportion of species isolation (Chari and Wilson 2001, Husband and Sabra 2004, Kay 2006), because *L. nanus* and *L. bicolor* grow sympatrically and have overlapping anthesis periods these isolation methods can be ruled out in this system.

Pollinator choice also plays an important role in preventing species hybridization. Whereas *L. nanus* inflorescences are often visited by bees, *L. bicolor* flowers are rarely observed to have insect visitors, which may be due to their small size (Dunn 1956). The lack of shared pollinators can add to the isolation of these two species by preventing *L. bicolor* pollen from reaching *L. nanus* flowers. Because *L. nanus* and *L. bicolor* are not geographically separated and do not exhibit flowering asynchrony we predict that self-pollination and differences in pollinator preference explain the lack of hybridization found between these two species in nature.

Intrapopulation Genetic Variation

This study supported our prediction that the selfing *L. bicolor* has less intrasite genetic variation than the outcrossing *L. nanus*. Several other studies have found similar results in comparisons among selfing and outcrossing populations. For instance, Holtsford and Ellstrand (1989) examined populations of *Clarkia tembloriensis* with variable outcrossing rates and found that populations with higher outcrossing rates also had higher intrapopulation genetic diversity (Holtsford and Ellstrand 1989). Similarly, Schoen (1982) examined groups of selfing and outcrossing populations of *Gilia achilleifolia*, and found lower genetic diversity within populations of selfing plants (Schoen 1982).

These studies also coincide with our findings that a smaller component of genetic variation is explained within *L. bicolor* individuals than *L. nanus* individuals. Small within-individual variation components suggests a history of genetic drift and frequent population bottle-necks or recolonization events in combination with low levels of gene flow among populations, all of which are common in selfing species (Hamrick and Godt 1996, Michalski and Durka 2007).

Because of the low intra-population genetic diversity levels we found in *L. bicolor* it is important that multiple populations of this species be maintained (Ellstrand and Elam 1993, Neel et al. 2001). Low levels of genetic diversity can increase the probability of population extinction, which subsequently results in further loss of species diversity, so numerous populations are required to support the genetic viability of *L. bicolor* (van Treuren et al. 1993, Roze and Rousset 2003). The low levels of genetic diversity we found within *L. bicolor* could also mean that this species is more vulnerable to climate change. If *L. bicolor* does not have enough genetic variation to respond to novel selective pressures caused by a changing climate, it may be in greater danger of extinction. Vrijenhoek (1994) found that the loss of genetic variation caused by multiple extinction and recolonization events was associated with a decrease in tolerance of physical extremes, decreased competitive ability, and an increased susceptibility to parasites. These and other traits may be important to species success in a changing climate, and could cause a cascading cycle of population extinctions followed by decreases in genetic diversity. Therefore, the preservation of numerous wide-spread populations of *L. bicolor* may be essential for the long term persistence of the species.

The high levels of genetic diversity and heterozygosity we found in *L. nanus*, on the other hand, may make it an excellent restoration species. Heterozygosity and genetic diversity are associated with population fitness and resistance to disturbances (Reed and Frankham 2003, Hughes and Stachowicz 2004), and are essential for a population's ability to respond to the novel environmental selection pressures of a restoration site (Falconer 1989). However, collecting plants from distant gene pools to provide restoration seed mixes with high levels of genetic diversity can have unintended consequences such as outbreeding depression (Fenster and Dudash 1994). Our results show that when *L. nanus* is used in restoration projects, genetically diverse seeds could be collected from a single population, thus avoiding negative effects of gene mixing while providing restoration populations with the genetic variation that may enhance long-term survival of the population.

Interpopulation Genetic Differentiation

Lupinus nanus exhibited significantly lower average differentiation among sites than did *L. bicolor*, supporting our prediction that outcrossing populations are more integrated than selfing populations. This is consistent with other studies that have compared genetic differentiation in outcrossing versus selfing species (Layton and Ganders 1984, Wolff 1991, Loos 1993, Hamrick and Godt 1996). Loos (1993), for example, compared three outcrossing *Lolium* species with four of their selfing congeners, and found that all of the outcrossing species had lower genetic differentiation among populations than the selfing species. Wolff (1991) also found higher genetic differentiation among selfing populations in his study examining *Plantago* species with differing breeding systems.

High levels of genetic differentiation could be detrimental to native *L. bicolor* populations. Because this species has low levels of intrapopulation genetic diversity a lack of gene flow would prevent new alleles from counteracting genetic drift. However, because we found evidence supporting our hypothesis that isolation by distance is present in *L. bicolor* we can conclude that, in near-by populations, there is a small amount of interpopulation genetic exchange. This interesting result suggests that selfing restricts, but does not entirely rule out gene flow. If selfing populations were completely genetically isolated from one another, then genetic drift, selection, and founder genotypes would be entirely responsible for population differentiation (Stebbins 1957). However, even a small amount of gene dispersal can create spatial gradients in genetic structure, leading to significant isolation by distance patterns (Crispo and Hendry 2005, Epperson 2007). While self-pollination limits gene flow, there are several examples of dispersal by pollen as well as propagules in highly selfing plants (Govindaraju 1989, Erickson et al. 2004). For example, Govindaraju (1989) documented instances of high and medium gene flow levels in 53 selfing plants. Our study suggests that, while gene flow in *L. bicolor* is below the level required for maintenance of polymorphisms, it does occur and is restricted by geographic distance.

Unlike *L. bicolor*, we did not find evidence of isolation by distance for *L. nanus* populations. Three possible explanations for the lack of isolation by distance in *L. nanus* populations are that 1) gene flow at the spatial scale measured in this study is too high to reveal isolation by distance, 2) *L. nanus* populations are experiencing isolation by environment rather than isolation by distance, or 3) interpopulational variation in selfing rates obscured the pattern of isolation by distance.

If gene flow among *L. nanus* populations is so high that it overcomes the spatial scale of this study we would not be able to detect isolation by distance among our observed *L. nanus* populations. In some outcrossing populations gene flow is high enough that it swamps out the effects of isolation by distance, but not high enough to unite populations entirely (Loveless and Hamrick 1984). Isolation by distance becomes less pronounced as gene flow increases, so the high outcrossing rates of *L. nanus* may be responsible for the lack of isolation by distance in our populations (Leblois et al. 2000, Crispo and Hendry 2005, Epperson 2007). In *L. nanus* populations, levels of gene flow high enough to swamp out patterns of genetic differentiation could be detrimental to local population fitness. If gene flow is happening among populations with differing environmental selection pressures, local adaptation may be prevented, thus preventing populations from reaching local fitness optima.

However, another possibility for why we did not detect isolation by distance in *L. nanus* is that environmental differences, rather than physical distances, are contributing to population divergence. Isolation by environment has been documented in several plant species (Bockelmann et al. 2003, Mooney et al. 2010, Tang et al. 2011) and can be detected with neutral markers (Li et al. 2000). Ortego *et al* (2012), for example, found that microsatellite genetic relatedness among *Quercus engelmannii* populations decreases with environmental differentiation among population growth sites using. In this case populations would experience, and be able to respond to, natural selection against foreign genotypes adapted to non-local environments, resulting in patterns of genetic structure explained by environmental conditions rather than geographic distance. Thus, if *L. nanus* populations are experiencing isolation by environment, local adaptation would still be possible for this species. We suggest further investigations on levels of local adaptation in *L. nanus* to determine how gene flow is affecting local population fitness.

Finally, the interpopulational variation in selfing rates could also explain why we did not find a pattern of isolation by distance for *L. nanus*. Low outcrossing rates increase differentiation among populations, so the high variation in F_{is} and heterozygosity levels among *L. nanus* populations could obscure a pattern of isolation by distance (Loveless and Hamrick 1984). However, the extent to which intraspecific levels of inbreeding affect patterns of isolation by distance is not well understood, so future studies addressing isolation by distance should be conducted among populations with similar inbreeding levels to control for differences in genetic variation due to selfing rates.

Despite high variation in *L. nanus* population differentiation levels, STRUCTURE and STRUCTURAMA identified each population as distinct: there has not been enough gene flow to unite even nearby populations with low genetic differentiation. This result might be somewhat surprising for an outcrossing species. Periodic long-distance dispersal among outcrossing populations has been documented frequently and is important in preventing the loss of polymorphisms and heterozygosity from populations (Depamphilis and Wyatt 1989, Chase et al. 1996, Mills and Allendorf 1996, Kamm et al. 2009). However, while one migrant per generation introduces enough genetic material to prevent genetic degradation, up to 10 migrants can be introduced to a population per generation before population structure begins to dissolve (Spieth 1974, Slatkin 1987, Mills and Allendorf 1996). This means that, while *L. nanus* may be exchanging some genetic material among nearby populations, the amount of gene flow is below the critical value for genetic homogenization.

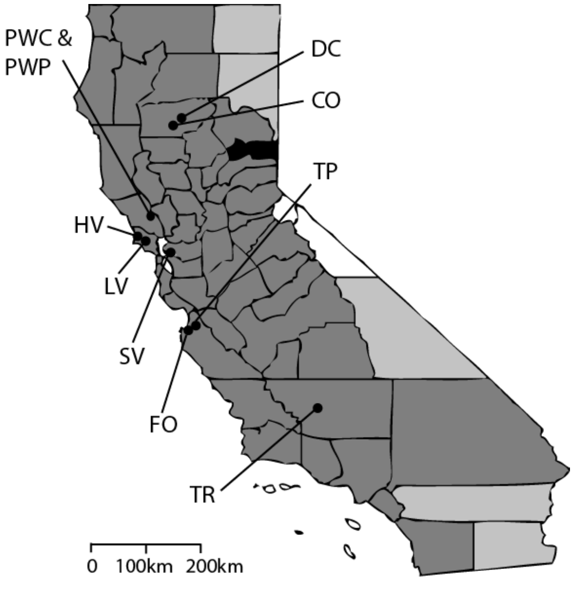
Conclusions and Environmental Implications

In summary, this study supports our predictions that the selfing *L. bicolor* has lower within-site genetic diversity, greater differentiation among populations, and a more distinct pattern of isolation by distance than the outcrossing *L. nanus*. The low levels of intrapopulation genetic diversity we found for *L. bicolor* suggest that this species may be at greater risk for inbreeding related fitness reductions and be more susceptible to climate change threats than *L. nanus*. Because differences among populations accounted for most of the species-level genetic variation in *L. bicolor*, rather than within-population diversity, conservation attempts should focus on maintaining multiple populations of *L. bicolor* to prevent genetic depletion.

While our genetic results did not indicate that *L. nanus* is in need of immediate conservation efforts, its varying levels of inerpopulation gene flow may affect local adaptation. Future studies should examine whether populations of this species are locally adapted, and how this local adaptation changes in relation to spatial or environmental differences. This is especially important for restoration efforts, because patterns of local adaptation can affect restoration population success rates. For example, if *L. nanus* experiences high levels of local adaptation it may be important to use local seed sources for restoration attempts, because the introduction of maladapted plants may reduce the success of the restored population (Mortlock 2000, Hamilton 2001, Krauss and Koch 2004). Additionally, the use of foreign seeds could disrupt any evolutionary structure that is present in these populations. Thus, the use of local plants in restoration efforts may be critical for protecting cryptic evolutionary lineages and avoiding the loss of biodiversity (Baldwin 2000).

1.5 FIGURES and TABLES

Figure 1: Locations of all ten field sites. Counties in which only *L. bicolor* grows are in light gray and the county in which only *L. nanus* grows is in black. The counties in which both species grow, and can be found in sympatry, are in dark gray. The coordinates of the sites and their abbreviations are listed in the table from north to south.



Site County	Site Name	Abbreviation	Coordinates
Tehama	Dye Creek	DC	40° 06' 16"N 122° 02' 38"W
Tehama	Corning	CO	39° 51' 16"N 122° 12' 01"W
Sonoma	Pepperwood Creekside	PWC	38° 36' 08"N 122° 44' 07"W
Sonoma	Pepperwood Borrow Pit	PWP	38° 34' 32"N 122° 42' 06"W
Marin	Hicks Valley Rd.	HV	38° 09' 04"N 122° 42' 28"W
Marin	Lucas Valley Rd.	LV	38° 02' 02"N 122° 34' 29"W
Contra Costa	Sibley Volcanic Regional Park	SV	37° 51' 09"N 122° 11' 26"W
Monterey	Toro Park	TP	36° 38' 19"N 121° 40' 04"W
Monterey	Fort Ord	FO	36° 36' 14"N 121° 43' 58"W
Kern	Tejon Ranch	TR	35° 12' 34"N 118° 44' 55" W

Figure 2: Box plot showing average F_{is} values for *L. bicolor* and *L. nanus* populations. The *L. nanus* Pepperwood Borrow Pit population is the only outlier, and F_{is} values for *L. bicolor* are significantly higher than *L. nanus* ($p < 0.01$).

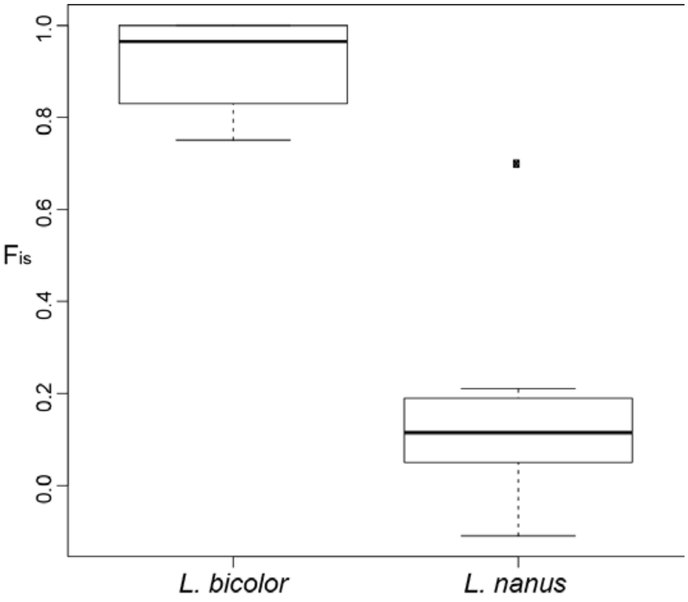


Table 1: F_{is} values with 95% confidence intervals (95% CI) for both species at each site. Abbreviations as described for Figure 1.

Site	DC	CO	PWC	PWP	HV	LV	SV	TP	FO	TR
L. bicolor	0.92	0.83	1	0.76	0.93	1	1	0.75	1	1
95% CI	0.01	0.17	0	0.24	0.05	0	0	0.25	0	0
L. nanus	0.05	0.05	0.19	0.70	0.21	-0.11	0.15	-0.09	0.17	0.08
95% CI	0.66	0.31	-0.20	0.14	0.16	0.53	0.50	0.09	0.15	0.08

Figure 3: Average gene diversity (H_{exp}) and allelic richness within *L. bicolor* and *L. nanus* populations. The line above a bar indicates one standard error. *-$p<0.05$ **-$p<0.01$

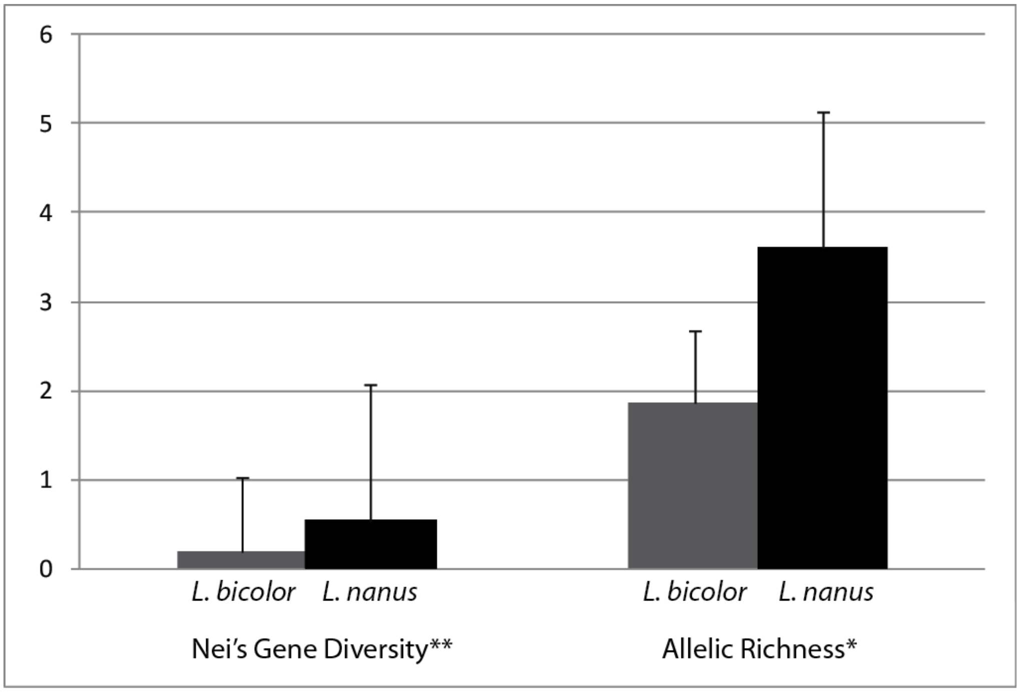


Table 2. Observed intra-population heterozygosity (H_{obs}), Nei's Gene Diversity (H_{exp}), and allelic richness values for both species at each site. Abbreviations as described for Figure 1.

site	DC	CO	PWC	PWP	HV	LV	SV	TP	FO	TR
L. bicolor										
H_{obs}	0.02	0.01	0	0.02	0.04	0	0	0.05	0	0
H_{exp}	0.07	0.07	0.30	0.29	0.45	0.15	0.10	0.16	0.01	0.12
Allelic Richness	1.25	1.40	2.26	2.10	3.28	1.40	1.33	1.60	1.06	1.36
L. nanus										
H_{obs}	0.08	0.60	0.63	0.12	0.54	0.26	0.08	0.82	0.62	0.70
H_{exp}	0.20	0.79	0.77	0.33	0.65	0.24	0.33	0.82	0.76	0.73
Allelic Richness	1.55	1.90	5.95	2.40	5.74	1.42	2.50	3.70	6.32	6.12

Table 3: AMOVA table from a model that partitions multilocus microsatellite variation of *L. bicolor* among sites, among individuals within sites, and within individuals. Degrees of freedom indicated as d.f.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation	P-value
Among Sites within *L. bicolor* Species	9	390.77	0.89	60.04	<0.01
Among *L. bicolor* Individuals within Sites	230	268.20	0.57	38.28	<0.01
Within *L. bicolor* Individuals	240	6	0.02	1.68	<0.01

Table 4: AMOVA table from a model that partitions multilocus microsatellite variation of *L. nanus* among sites, among individuals within sites, and within individuals. Degrees of freedom indicated as d.f.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation	P-value
Among Sites within *L. nanus* Species	9	964.13	2.21	50.72	<0.01
Among *L. nanus* Individuals within Sites	230	614.4	0.52	11.97	<0.01
Within *L. nanus* Individuals	240	390.5	1.63	37.31	<0.01

Figure 4: Posterior probabilities at different postulated population numbers (K), as calculated by STRUCTURE.

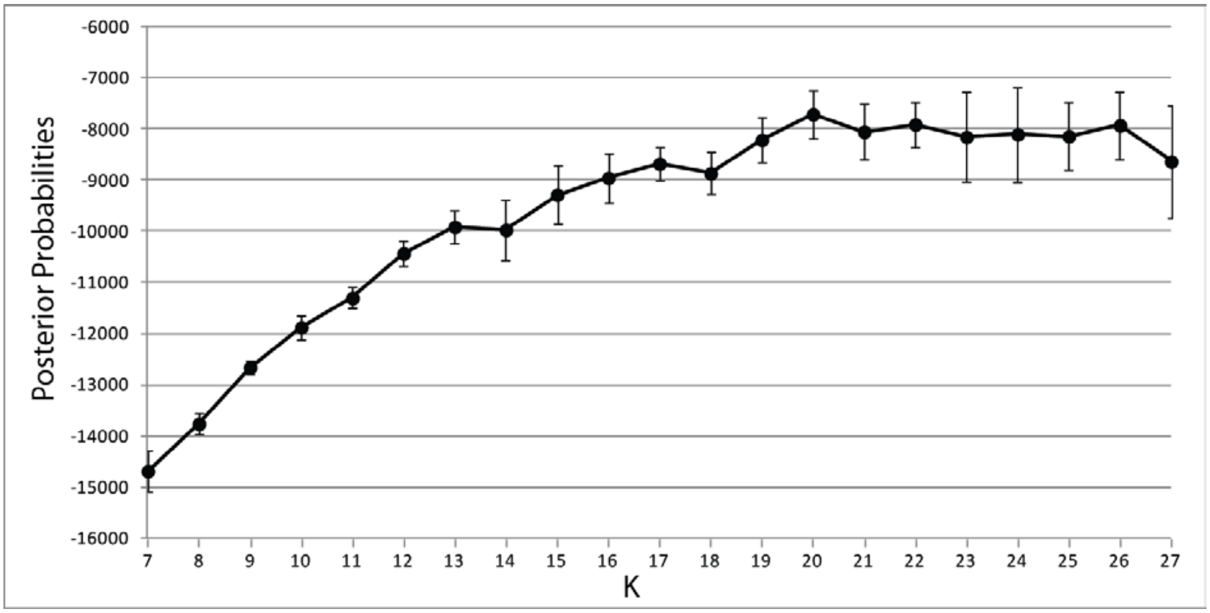


Figure 5: Bar plot of STRUCTURE population assignments for *L. nanus* and *L. bicolor* individuals. Individuals are arrayed along the abscissa and probability of population assignment along the ordinate. Different hues indicate different populations. This picture includes 20 unique colors, one for each independent population. Bars with more than one color indicate admixture; the respective length of a color indicates the proportion of alleles from that population. Abbreviations as described for Figure 1, except that a trailing "Lb" indicates *L. bicolor* and "Ln" indicates *L. nanus*.

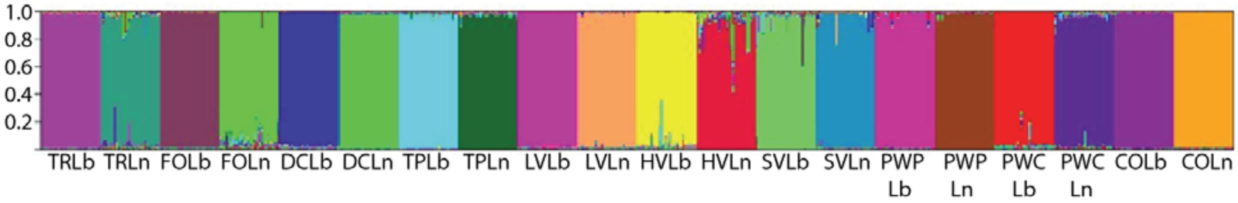


Figure 6: Among-population average D_{est} values for *L. bicolor* and *L. nanus*. The line above a bar indicates one standard error.

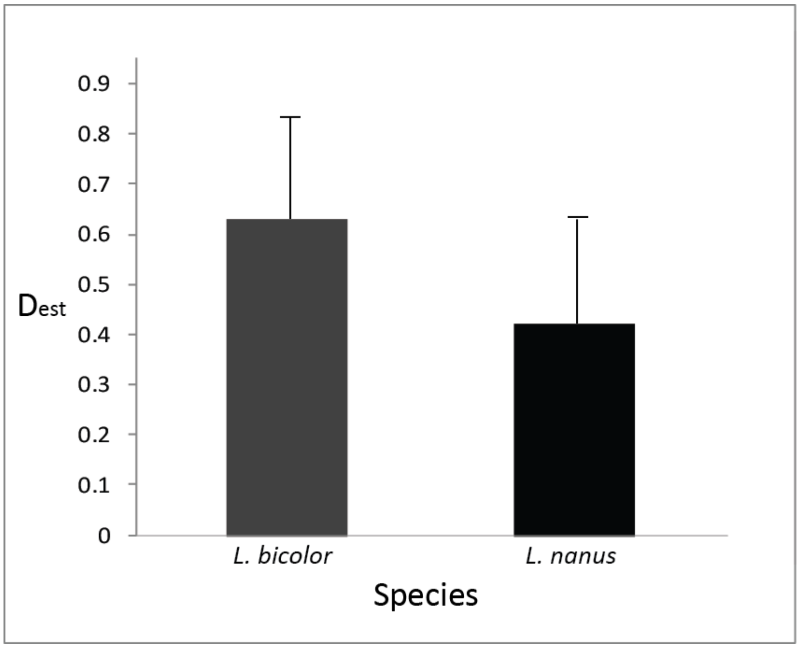
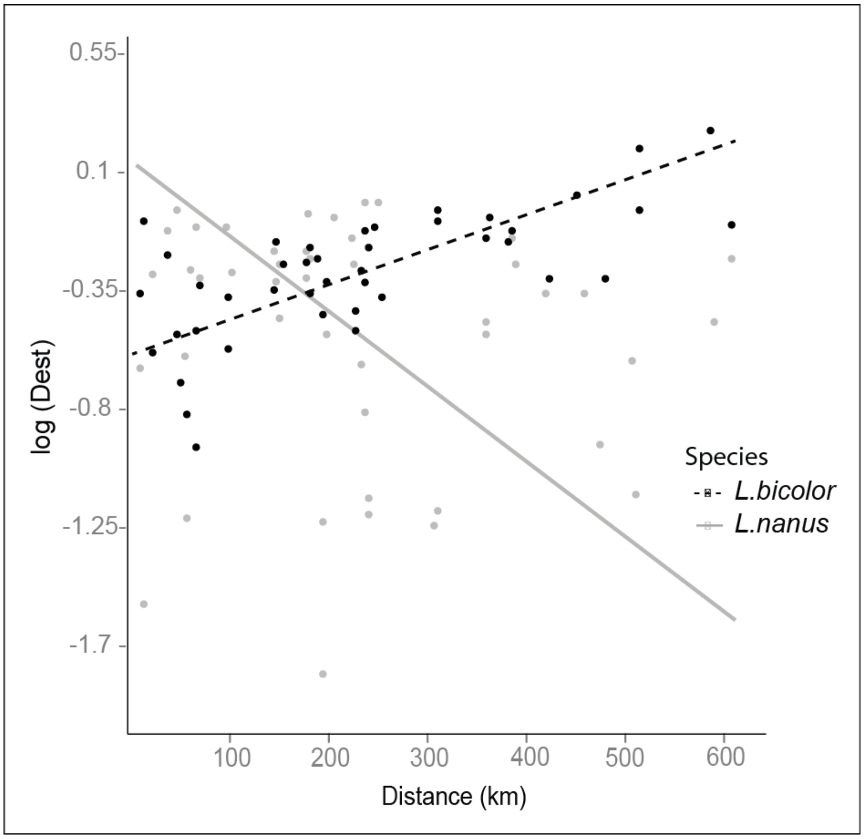


Figure 7: Pairwise log D_{est} versus geographic distance for L. *nanus* and L. *bicolor* populations using Reduced Major Axis regression. Each point indicates the log genetic differentiation (D_{est}) and geographic distance between a single pair of populations of either L. *bicolor* (dark circles) or L. *nanus* (light circles). Regression of D_{est} as a function of geographic distance (*L. bicolor*: b=1.0x10^{-3}, r^{2}=0.28; *L. nanus*: b= -2.7x10^{-3}, r^{2}=6.1x10^{-3}).



1.6 APPENDICES

Appendix 1: Table showing departure from Hardy-Weinberg Equilibrium p-values (HW p-value) and pairs of loci with significant linkage disequilibrium ($p<0.01$) (LD Pairs) for *Lupinus bicolor* and *L. nanus*.

Lupinus bicolor			*Lupinus nanus*		
Population	HW p-value	LD Pairs	Population	HW p-value	LD Pairs
Tejon Ranch	<0.01	Luna1-Luna8 Luna4-Luna6 Luna6-Luna16 Luna8-Luna12 Luna8-Luna16	Tejon Ranch	0.53	-
Fort Ord	<0.01	Luna8-Luna9 Luna8-Luna16	Fort Ord	0.65	-
Dye Creek	<0.01	Luna6-Luna12 Luna4-Luna6 Luna6-Luna17 Luna6-Luna18 Luna12-Luna17 Luna12-Luna18 Luna4-Luna12 Luna4-Luna17 Luna4-Luna18 Luna18-Luna18	Dye Creek	1	-
Toro Park	<0.01	Luna16-Luna18 Luna16-Luna17 Luna18-Luna20	Toro Park	1	-
Lucas Valley Rd.	<0.01	Luna6-Luna20 Luna19-Luna20	Lucas Valley Rd.	0.96	-
Hicks Valley Rd.	<0.01	Luna1-Luna6 Luna1-Luna8 Luna1-Luna9 Luna1-Luna12 Luna1-Luna18 Luna1-Luna20 Luna6-Luna8 Luna6-Luna12 Luna6-Luna18 Luna8-Luna9 Luna8-Luna18 Luna19-Luna20	Hicks Valley Rd.	<0.01	Luna1-Luna8 Luna1-Luna4 Luna2-Luna16 Luna8-Luna16 Luna3-Luna4
Sibley Volcanic Regional Park	<0.001	Luna1-Luna2 Luna1-Luna5 Luna1-Luna6 Luna6-Luna8 Luna8-Luna9 Luna8-Luna11 Luna8-Luna12 Luna8-Luna20 Luna12-Luna17 Luna12-Luna18 Luna18-Luna20	Sibley Volcanic Regional Park	0.37	-
Pepperwood Borrow Pit	<0.01	Luna1-Luna8 Luna8-Luna12 Luna6-Luna8	Pepperwood Borrow Pit	<0.01	Luna1-Luna8 Luna1-Luna9 Luna8-Luna9 Luna12-Luna16
Pepperwood Creekside	<0.01	Luna1-Luna6 Luna1-Luna9 Luna1-Luna12 Luna17-Luna20	Pepperwood Creekside	0.06	-
Corning	<0.01	Luna6-Luna8	Corning	0.76	-

Chapter 2. Floral evolution in lupines: Responses to selection in outcrossing and selfing populations

2.1 INTRODUCTION

Pollination systems affect the evolutionary trajectory of plants by altering both selection gradients and neutral evolutionary processes, resulting in predictable differences between closely related taxa with differing breeding systems. The shift from outcrossing to selfing, for instance, is accompanied by floral phenotypic changes including decreased pollen-ovule ratio, time to anthesis, and flower size (Schemske and Lande 1985, Gallardo et al. 1994, Damgaard and Abbott 1995, Eckhart and Geber 1999, Runions and Geber 2000, Dudley et al. 2007). However, while these phenotypic differences between selfers and outcrossers are well documented, the evolutionary processes responsible for these changes are still not well understood, nor is the degree to which selective forces versus neutral processes contribute to these changes.

Selfing plants have a history of inbreeding combined with multiple extinction and recolonization events, so they are more likely than outcrossing plants to have neutral evolutionary processes overpower their adaptive responses to selection (Takebayashi and Morrell 2001, Dudash et al. 2005). Highly inbred progeny have a higher probability of inheriting identical ancestral genetic sequences from their parents, resulting in lower levels of intrapopulation genetic diversity than outcrossed progeny. Selfing populations also experience high extinction and recolonization rates, which can further accelerate genetic drift because of associated genetic bottlenecks (Viard et al. 1997, Lavigne et al. 2001, Szarowska et al. 2003). The ability of a species to respond to selection is proportional to the amount of genetic variance in that species, so these low levels of within-population genetic diversity could result in selfing species being unable to respond to selection (Lande and Schemske 1985, Charlesworth and Charlesworth 1998, Kirkpatrick and Jarne 2000, Figueroa-Castro and Holtsford 2010).

However, it is still important to examine selection pressures in selfing populations, because, although selfing plants generally have lower genetic diversity than outcrossing plants, some studies suggest that selfers may still exhibit sufficient genetic variance to respond to environmental selection pressures (Dudley 1996). For example, because reproductive success in selfers depends on within-flower fertilization rather than pollinator preference, selfing plants may experience direct selection for an optimal floral shape that maximizes anther-stigma contact while minimizing floral size and concomitant resource expenditures (Ushimaru and Nakata 2002, Tang and Huang 2007).

In outcrossing plants, on the other hand, floral morphology should evolve in response to pollinator preference (Chittka and Menzel 1992, Podolsky 1992, Schemske and Bradshaw 1999, Fenster et al. 2004, Fishman and Willis 2008, Gong and Huang 2009, Nattero et al. 2010b), which imposes direct selection for optima in both size and shape. Even within a single plant species, adaptive responses to local abundances and preferences of effective pollinator classes can yield floral ecotypic differentiation among sites (Schmidt and Levin 1985, van Tienderen and van der Toorn 1991, Kawecki and Ebert 2004, Geber and Eckhart 2005, Abdala-Roberts and Marquis 2007, Anderson and Geber 2010), which indicates that floral sizes and shapes in outcrossing populations can respond to direct selection imposed by pollinators.

Floral traits may also change over time due to indirect selection on vegetative traits. Because floral organs are derived from leaves (Steeves and Sussex 1989) they share some genetic components, so that floral traits often covary with leaf traits (Berg 1959, Brock and Weinig 2007). Thus, floral traits might evolve as correlated responses to spatially heterogeneous selection for locally beneficial vegetative traits, such as leaf size, rather than as direct responses to floral selection (Doyle and Myers 1982, Campbell et al. 1994, Armbruster 2002). High correlation between floral and vegetative traits is particularly likely to occur in selfers, because selfing plants do not require pollinators for propagation and thus have reduced pressure for independent evolution of leaf and floral characters. Therefore, in selfing species, these characters are likely to be inter-correlated across sites, as floral characters evolve in response to site-specific selection on vegetative characters.

Unlike selfing species, outcrossing plants with specialized pollination systems could experience selection that breaks down correlations between floral and vegetative traits(Berg 1960, Armbruster et al. 1999). If floral traits are tightly correlated with vegetative traits, then, as vegetative traits evolve in response to environmental variation, correlated responses by floral traits may shift them away from their selective optima. In outcrossing plants this scenario could result in pollen being placed ineffectively on pollinators, or stigma/pollinator contact occurring outside of the pollen coated area, which would decrease successful fertilization (Herrera et al. 2002, Chalcoff et al. 2008, Meng et al. 2008). Outcrossing plants that retain stable flower shapes and sizes in the face of environmental variation in vegetative traits should therefore have higher seed set than plants with high correlations between floral and vegetative traits, resulting in selection toward decoupling these two suites of traits. Therefore, outcrossed plants should exhibit less evidence for indirect evolution of floral traits in response to selection on leaf traits than selfing plants.

Understanding the type of selection (direct vs. indirect) imposed on floral traits in outcrossing versus selfing plants, and the relative ability of these traits to respond to these selection gradients is important not only because it can elucidate the evolutionary processes behind current patterns of floral phenotypes, but also because it can be used to predict responses to future changes in the selection environment. This type of information could be especially valuable in the field of conservation biology, because how populations respond to selection may have important consequences for species conservation and restoration. For example, if a species is unable to respond to novel selective forces due to genetic depletion it may be at greater risk of extinction due to climate change (Soule 1980, Simberloff 1988, Lande and Shannon 1996, Thomas et al. 2004). Similarly, plant restoration projects depend on a species' ability to adapt to the environmental conditions of the restoration site (Fenster and Dudash 1994, Knapp and Dyer 1998). If low genetic diversity and/or opposing indirect selection from correlated vegetative traits prevent restoration populations from responding to selection on floral traits that maximize fertilization success, they may have low establishment or persistence rates.

Unfortunately, few studies have compared selective forces among species with differing mating systems. The rarity of such studies could be due to the stringent conditions required to compare outcrossing and selfing species while avoiding confounding factors. The two species must be closely related to avoid differences created by disparate histories, such as differing floral constraints or ancestral traits. They must also grow sympatrically, with simultaneous bloom periods, so that they experience similar regimes of environmental selection. To compare evolutionary responses to selection gradients between breeding systems, the species must also be widespread to ensure independence of comparisons and lack of gene flow among populations.

Lupinus nanus and its selfing relative, *L. bicolor*, grow sympatrically in patches throughout California and are ideal study species for these questions as they meet all of the above criteria. Additionally, they exhibit high levels of floral morphological differentiation among populations. These differences are maintained when plants are grown in a common garden (Dunn 1956, Elliot et al. 1974, Riggins et al. 1977), suggesting that this variation among populations is due to genetic differences that was caused by random drift or differences in selection history.

This study combines phenotypic selection analysis, multigenerational measurements of phenotypes, and analysis of microsatellite loci from both species at five sites to address the following questions: (1) Are *L. nanus* and *L. bicolor* experiencing direct or indirect selection on floral traits? (2) Are *L. nanus* and *L. bicolor* able to respond to selection on floral traits?

We predict that *L. nanus* should be able to respond to selection, but that *L. bicolor* may not possess sufficient genetic diversity to be able to do so. Populations in which evolution of floral characters is primarily due to neutral processes should be characterized by low within-population genetic diversity and trait values that do not consistently shift in the direction predicted by selection from one generation to the next. Populations in which evolution of floral characters is primarily adaptive are likely to exhibit mid to high levels of genetic variation and a consistent response to selection (Fig. 1). If floral traits in *L. bicolor* are influenced by selection, we predict that they will be correlated with leaf traits and that indirect selection on floral traits due to direct selection on correlated vegetative traits will result in high levels of vegetative and floral colinearity and weak floral selection gradients when vegetative selection is taken into account. We predict that *L. nanus*, on the other hand, experiences and is able to respond to direct selection on floral traits, that selection has also favored breakdown of genetic correlations between leaf and floral traits. Thus, we expect weak or no correlation between vegetative and floral suites of traits and significant direct selection on floral traits, even after accounting for selection on leaf traits.

2.2 METHODS

Study System

Lupinus nanus and *L. bicolor* are annual legumes that grow in western North America. *Lupinus nanus* grows in north coastal scrub, coastal sage scrub, foothill woodland, coastal prairie, and valley grassland communities in California, Nevada, and Oregon. *Lupinus bicolor* has a wider geographic range than *L. nanus*, occurring from Arizona to British Columbia in habitats that also include closed-cone pine, Douglas fir, yellow pine, red fir, lodge pole pine, and mixed evergreen woodlands(Hickman 1993). Although both species can grow independently, they often co-occur within local plant communities.

Both plants have palmately compound leaves with whorls of pea-shaped flowers on erect spikes. The zygomorphic flowers are composed of five modified petals: the upper banner, two lateral wings, and two lower petals that are fused to form the keel(Baldwin et al. 2012). The flowers of *L. nanus* and *L. bicolor* have similar coloration, but those of *L. nanus* are larger (2-20cm) than those of *L. bicolor* (4-10mm). Genetic research on these species suggests that *L. bicolor* has a high rate of selfing, while *L. nanus* is predominantly outcrossing (Elliot et al. 1974, Harding et al. 1974, Shade 2012).

We selected five populations to represent much of the longitudinal and latitudinal range of both species in California. The two inland sites, Dye Creek Reserve (DC) and Tejon Ranch (TR) have higher temperatures and less cloud cover than the three coastal populations, Pepperwood Preserve (PW), Sibley Volcanic Regional Park (SV), and Fort Ord (FO) (NOAA 1994). Precipitation among our sites varies on a North to South gradient, with DC receiving the most rainfall and TR the least (Perica et al. 2011). To ensure the independence of our populations, we selected five locations that are 665km to 118km apart at which *L. nanus* and *L. bicolor* co-occur (Fig. 2).

Trait measurements

Within each site, 100 *L. nanus* and 100 *L. bicolor* plants were selected at random between March and April of 2009. We selected plants by generating two sets of random numbers between zero and 100: one set of x coordinates and one set of y coordinates. We laid out two 100m measuring tapes orthogonally along the population, and sampled one individual at each of the x and y location pairs from our list. Three vegetative traits (leaflet length, leaflet width, and sepal length) and five floral traits (pedicel length, banner length, wing length, lower sepal length, and wing height) were measured using digital calipers. Floral traits were averaged from measurements on two flowers from the lower flower whorl on a plant's first date of anthesis, and vegetative traits were averaged from measurements on the middle leaflet of two mature leaves located three nodes down from the apical bud (Fig. 3). Because plant traits differed in variance, we standardized our floral and vegetative data using z-scores.

We recorded five fitness components from each plant: survival to pod formation, survival to seed set, number of seeds per plant, number of fruits per plant, and number of flower scars per plant. Once all flowers were pollinated and pods were forming, we placed Polypropylene Microperforated Bread Bags (Elkay Plastics, Commerce, CA) over the fruits to catch the explosively dispersed seeds. We collected seeds between June and August of 2009, after all pods had dehisced.

Selection Analysis

To visualize vegetative and floral selection in three dimensional space, we reduced the dimensionality of our vegetative and floral trait measurements, while preserving the relationships between these two suites of traits, by performing separate PCA's on each trait group (Farris and Lechowicz 1990). We calculated the trait loadings and proportion of variance explained by each axis using the R stats package (R Development Core Team 2011). If the first axis of either the floral or vegetative PCA accounted for more than 70% of the variance, we used these first axes to describe floral and vegetative traits.

To determine if vegetative and floral first PC axis values differed between species and among sites, we performed a MANOVA using individuals values along the first vegetative and floral PC axes as dependent variables, and site and species as fixed effects. We also looked for site by species interactions to determine if the effects of sites and species on principle component values were additive. We used the Pillai-Bartlett V criterion as a multivariate test statistic for our MANOVAs, because it has been found to be more robust than other multivariate test statistics (Olson 1976, Stevens 1980). We then performed Tukey comparison tests to determine which specific population means differed from the rest. We used Bonferroni corrections to adjust for multiple testing.

To estimate the site and species-specific phenotypic selection on floral and vegetative traits we used Aster modeling, developed by Geyer and Shaw (Geyer et al. 2007, Shaw and Geyer 2010). Aster modeling is a form of multivariate regression that improves on the traditional use of Ordinary Least Squares (OLS) in selection analyses by providing accurate interpretations of data curvature and allowing multiple fitness values to be analyzed without transformation (Lande and Arnold 1983).

Lande and Arnold (1983) used OLS multiple regression to identify selection gradients (β), which describe the strength and direction of selection on each trait, and the squares and cross-products between traits (γ), which describe the curvature of the best fit quadratic selective landscape. These analyses require normally distributed fitness traits. However, because overall fitness has a compound distribution resulting from the product of multiple distinct fitness components, each with its own distribution, it is uncommon for overall fitness to have a normal distribution (Mitchell-Olds and Shaw 1987). The curvature of OLS multiple regression can also not be reliably interpreted as stabilizing or disruptive, as the maximum and minimum fitness values will not necessarily be located within the population trait range (Mitchell-Olds and Shaw 1987, Schluter 1988).

Aster modeling does not have the same limitations as OLS. It allows multiple fitness components to be included from several life-stages of a study organism, using maximum likelihood functions to model overall fitness by conditioning late-life fitness on earlier life-stage fitness components (Geyer et al. 2007, Shaw and Geyer 2010). This means that rather than using a single measurement of overall fitness, which often has a distribution pattern that does not conform to well-known parametric distributions, aster models incorporate distinct components of fitness with predictable standard probability distributions such as Poisson (in the case of offspring number, for instance) and Bernoulli (in the case of survival). Because it does not assume normality, aster analysis facilitates hypothesis testing among models, making it an ideal method to test for differences in selective surfaces between species and among sites. Additionally, because aster analysis approximates the true fitness landscape more closely than traditional OLS techniques, the curvature of the function can be accurately visualized using graphs that display the range of trait values found in nature.

We used the R package 'aster' to perform multivariate regressions on each species at each site independently, using the first axis obtained by PCA of either the floral or vegetative characters as the explanatory variables and survival and fitness components as the dependent variables (R Development Core Team 2011). Because our models only use two predictor variables and sample from a small proportion of the total lupine population at each site, we used the corrected Akaike information criterion to determine which, if any, quadratic and interaction parameters should be included in each model (Akaike 1973, Burnham and Anderson 2002). To determine whether populations differed in the selection they were experiencing we performed analyses of covariance (ANCOVA) to compare selection between species and among sites, using Bonferroni corrections to adjust for multiple testing. We visualized selection surfaces estimated by our aster analysis using the 'lattice' package in R (Sarkar 2010).

Floral and Vegetative Multicolinearity

To examine whether floral traits covary with vegetative traits, and thus may experience indirect selective responses, we calculated the Variance Inflation Factors (VIF) among the first PC axes of the vegetative and floral PCA's. Variance Inflation Factors determine the severity of within-species multicolinearity, with a VIF of one indicates no correlation between the variables, while a VIF of four or higher indicates moderate to severe multicolinearity.

Interannual Phenotypic Change

Interannual shifts in population phenotypes are due to a combination of genetic changes (due to random processes and evolutionary response to selection) and plastic responses to between-year climatic differences. Although the *L. nanus* and *L. bicolor* population-specific plastic responses to environmental factors are unknown for our sites, we use interannual phenotypic change as a coarse measure of a population's minimum ability to respond to selection.

Between March and April of 2010 we returned to the populations visited in 2009 and re-collected vegetative and floral measurements to determine whether average flower size and leaf shapes had shifted in the direction favored by selection at each site in the previous year. For this census, we measured traits on 100 *L. nanus* and *L. bicolor* individuals from FO, PW, and SV and 50 individuals from DC and TR. Because the PC axes are complex variables comprising multiple traits that we could directly measure, we transformed measurements taken in 2010 with the PCA functions calculated from the 2009 data. To test whether the 2010 floral and vegetative values had changed significantly from the 2009 values in the direction of selection, we performed one-tailed t-tests comparing the 2009 and 2010 vegetative and floral values, separately, using the null hypothesis that there was no difference between the 2009 and 2010 means, and the alternate hypothesis that the means were different (in the direction predicted by the 2009 selection gradients).

Microsatellite Analysis

Because our estimate of response to selection may be confounded by plasticity, we also examined genetic diversity levels to determine whether populations possessed sufficient genetic variance for selection to act on. We collected three young leaves from each of thirty individuals from each species (*L. nanus* and *L. bicolor*) at each site (DC, FO, PW, SV, and TR) for microsatellite analysis using the random sampling method described in the selection analysis subheading. DNA from each individual's three leaves was extracted using Qiagen's DNeasy Plant Mini kit, and we amplified Luna1-Luna6, Luna8- Luna9, Luna12- Luna13, Luna15- Luna18, and Luna20 (Molecular Ecology Resources Primer Development et al. 2012) using the protocol described in Molecular Ecology Resources Primer Development *et al.* (2012).

We used GENEPOP to estimate Nei's Gene Diversity rate to examine expected heterozygosity (Raymond and Rousset 1995), and HIERFSTAT to calculate allelic richness (the number of alleles per locus) (Goudet 2005, R_Development_Core_Team 2011). We calculated these values for each species at each site individually, resulting in site and species specific values.

2.3 RESULTS

Aster Analysis

The PCA of vegetative traits showed leaflet length loading positively, leaflet width loading negatively, and sepal length loading only slightly on the first PC axis, which accounted for 85.6% of the data variance (Table 1). This means that as the first vegetative axis increases, leaflet width decreases and leaflet length increases while sepal length does not change, resulting in longer, narrower leaflets, but relatively constant sepal length. We termed this axis the "leaflet shape axis".

The PCA of floral traits showed all traits loaded negatively on the first PC axis, so floral traits become smaller as the first floral PC axis increases (Table 1). We termed this axis, which accounted for 88.4% of the data variance, the "floral size axis".

The MANOVA performed using leaf shape and floral size as dependent variables showed that the fixed effects (site, species, and site by species interaction) were all significant (Table 2). Both leaf shape and floral size contributed significantly to these effects (Table 3). When we performed Tukey comparisons of individual population pairs for *L. nanus*, all comparisons except TR vs. DC showed significant differentiation in floral size means, and among comparisons of leaf shapes, only PW and FO were not significantly different (Fig. 4a and 4b). On the other hand, population means in *L. bicolor* were less differentiated in both leaf shape and floral size. *Lupinus bicolor* leaf shape means did not differ between PW and FO or DC, SV, and TR (Fig. 4c). Floral shapes did not differ between SV and TR, TR and DC, and DC, PW, and FO (Fig. 4d)

Selection Analysis and Multicolinearity

The aster multiple regression analyses for individual populations of *L. bicolor* found a different selection regime at each site. Figure 5 illustrates these selection regimes. Only DC exhibited no curvature for floral or vegetative traits. This site was marked by selection for shorter, wider leaflets in combination with larger flowers. This means that within the phenotypic range of the DC population plants with short, wide leaflets and large flowers were more fit at this site than plants with long, narrow leaflets and small flowers (Table 4). Three of the five sites (SV, TR, and FO) had curvature in their pattern of selection on floral traits, but no curvature in their pattern of selection on vegetative traits. At SV, for instance, there was directional selection for short, wide leaflets, but stabilizing selection that favored intermediate flower sizes. This selection gradient has a ridge of high fitness at intermediate floral sizes, which rises as leaflets become shorter and wider. Fitness decreases at an accelerating rate as floral size deviates from the ridge. At TR the floral curvature, while significant, is only minimally visible in the floral size range present at the TR site. Within this size range, selection for larger flowers appears almost linear, meaning that fitness increases steadily as flower size increases. The selection gradient also includes slight directional selection favoring leaf shapes with wider, shorter leaflets. This means that individuals at TR have the highest fitness when they have large flowers with short, wide leaflets. There is also curvature in the pattern of selection at FO, but, unlike SV and TR, this site does not impose significant direct selection on leaflet shape. Here, selection against large flowers increases as flower size increases, but the height of the fitness ridge at relatively low optimal floral values is not affected by leaf shape. PW is the only site with a fitness landscape that exhibits curvature in both the floral size and leaflet shape axes, which reflects selection on the correlation between the floral and leaflet trait suites. A fitness valley runs diagonally across the population, such that high fitness is associated with two combinations: small flowers and long, thin leaflets as well as large flowers and short, wide leaflets. The lowest fitness for this population is found in plants with long, thin leaflets and large flowers.

Each site also imposed a different pattern of selection on *L. nanus* populations (Fig. 6). Direct selection favored shorter, wider leaflets at all sites except TR, where direct selection favored longer, thinner leaflets. Direct selection at TR favored smaller flowers, whereas at SV and DC, larger flowers were favored. The latter two sites differed from each other in that SV had stronger selection on vegetative traits and weaker selection on floral traits than did DC. The only site with direct curvilinear selection on flower size was FO, at which fitness was highest for individuals with intermediate-sized flowers (Table 5).

The ANCOVAs to test the null hypothesis that populations of *L. nanus* and *L. bicolor* sharing a site have equal selective gradients showed that the fitness landscapes for the two species were significantly different ($p > 0.05$) at every site except PW ($p=0.317$). Within species, all intersite selection landscapes were significantly different ($p > 0.001$).

The Variance Inflation Factors (VIF) values for floral size and leaflet shape were below four in each site for each species (Table 5), indicating low levels of colinearity among traits.

Interannual Phenotypic Change

Most 2010 population mean phenotypes were significantly different from 2009 mean phenotypes, except *L. nanus* populations at DC and FO and the TR *L. bicolor* population, which did not have significantly different leaflet shapes between years, and the SV *L. bicolor* population, which did not have significantly different floral sizes between years (Table 6). However, only two out of five *L. bicolor* populations shifted a composite trait mean in the direction predicted by the selection analysis: at PW, mean leaflet shape was shorter and wider in 2010 than in 2009, as predicted by selection on leaflet shape in 2009, and at FO, mean flower size increased from 2009 to 2010, as predicted by the 2009 selection gradient.

In contrast, mean flower size shifted in the direction predicted by selection in all five *L. nanus* populations (Table 6), with smaller flowers at PW, and larger flowers at DC, FO, SV, and TR in 2010 than 2009. At Fort Ord, the only *L. nanus* population experiencing significant curvature in floral selection, the increase in mean flower size surpassed the estimated population optimum (Fig. 7).

Only one *L. nanus* population changed mean leaflet shape in the direction predicted by selection. PW, in which the 2009 selection gradient favored short, wide leaflets, had longer, thinner leaflets in 2009 than 2010 leaflet shape. Selection in 2009 at DC, FO, and SV also favored short, wide leaflets, but mean leaflet shape at DC and FO did not change significantly between years, and leaflets were longer and narrower in 2010 than 2009 at SV. TR, the only population with selection for long, narrow leaflets, had shorter, wider leaflets in 2010 than 2009.

Microsatellite Analysis

Lupinus bicolor populations consistently exhibited lower within-population gene diversity than the co-occurring *L. nanus* population. Populations of *L. bicolor* also had lower levels of allelic richness than their co-occurring *L. nanus* populations, indicating that *L. bicolor* had fewer alleles per locus than *L. nanus* (Table 8).

2.4 DISCUSSION

Neutral vs. Natural Selection

Because selfing populations experience a reduction in genetic diversity due to inbreeding, multiple extinction events, recolonization, and bottlenecks, it has been hypothesized that their responses to selection are constrained and their morphological evolution is dominated by neutral evolutionary processes (Lande and Schemske 1985, Charlesworth and Charlesworth 1998, Kirkpatrick and Jarne 2000, Figueroa-Castro and Holtsford 2010). Our findings are consistent with this hypothesis. *Lupinus bicolor* exhibits extremely low within-site genetic variation and changes in both floral and vegetative population means from 2009 to 2010 did not consistently follow selective predictions.

In contrast, *L. nanus* had high within-population genetic variation, and all mean 2010 sizes had shifted in the direction predicted by 2009 selection gradients (Table 6), indicating a floral response to selection. In FO, however, the 2010 *L. nanus* floral mean was higher than the optimal flower size described by 2009 selection (Fig. 7). This means that selection alone cannot account for floral changes, because even with completely heritable variation responses to selection should never exceed the selection differentials.

Other factors that might affect floral mean changes are migrants from adjacent populations with differing floral means and plastic responses to annual environmental changes. Because *L. nanus* flowers are primarily pollinated by bumblebees and honey bees (Dunn 1956, Horovitz and Harding 1972), they may be experiencing gene flow from populations located over 2km away (Danka et al. 1993, Walther-Hellwig and Frankl 2000). The generational changes in trait means would then be a function of home population means, source population means, and selection differentials. Plastic responses to annual environmental differences will also cause population means to shift. The expressed phenotype of a given genotype can change as a function of the environment, so if annual plant populations experience yearly environmental variation their phenotypes may change not only due to genetic shifts, but also due to the interaction of the environment with their developmental processes (Schlichting 1986, Scheiner 1993). Because plasticity is a heritable trait that can affect plant fitness, we suggest that future studies examine the relationship between selection on phenotypes and selection toward phenotypic plasticity in lupines, as plasticity may obscure the patterns of responses to selection observed in this study.

Migration and annual environmental fluctuation could also explain why leaflet shape means in *L. nanus* populations did not consistently shift along selection differentials. Only the PW population leaflet shape mean changed in the direction predicted by selection, while SV and TR shifted in the opposite direction from selection predictions, and DC and FO did not have annual leaflet shape mean changes.

It is also possible, however, that *L. nanus* foliage is experiencing selection on life history traits not measured by this study. Because we only measured post-flowering phenotypes we missed early-life selection events such as seedling survival and survival to flowering. Ideally, these measurements would be included in studies on vegetative selection but, because we cannot predict floral trait measurements without survival to flowering, they are not included in this study.

While we cannot make assumptions about the part that early-life selection played on vegetative mean changes, it is safe to say that *L. nanus* floral shifts are largely directed by selection and vegetative generational changes are not responding solely to post-floral selection. Unlike *L. nanus*, *L. bicolor* does not appear to have enough genetic diversity to respond to selection on any of the traits we measured. Not only do *L. bicolor* population traits shift independently of selection differentials, they also have extremely low intrasite diversity and many microsatellite loci are fixed for a population, indicating that drift is the primary source of between-population variation.

Direct vs. Indirect Selection

Patterns of trait correlations also suggest that neutral evolution is responsible for *L. bicolor* morphology. Selfing *L. bicolor* populations have lower intrafloral correlations than outcrossing *L. nanus*, but they also have smaller overall correlations, including correlations between vegetative traits and floral traits. This suggests that not only are flower traits varying independently of one another due to the removal of pollinator-imposed selection for specific floral trait relationships, but all traits are varying independently. While this could indicate independent selection on all traits, when combined with our data showing low genetic variation and inability to respond to selection over time, it is likely that traits in *L. bicolor* are decoupled due to genetic drift.

These findings do not fully support Berg's correlation-pleiades concept, which predicts that generalist plants such as *L. bicolor* will have both lower correlation among floral traits and less decoupling between floral and vegetative traits than flowers with specialized pollination systems, such as *L. nanus* (Berg 1959, 1960). Specifically, Berg's correlation hypothesis predicts that pollinator selection on floral traits of plants with specialized mating systems will result in floral trait correlation pleiades that maximize attractive flower trait size and shape relationships. If this selection pressure from pollinators is removed there will no longer be selection for floral traits to covary, so flower traits will become decoupled and vary independently from one another. In addition, however, Berg's hypotheses explains that, in plants with specialized pollination systems, floral trait correlation pleiades are decoupled from vegetative traits to allow vegetative traits to vary with environmental resources while flowers maintain a more constant size and shape that is attractive to pollinators. In plants without specialized pollination systems, such as *L. bicolor*, there is no need for flowers to be decoupled from vegetative traits, so Berg predicts that these traits will revert to ancestral correlations. Because flowers are derived from modified leaves, in the absence of opposing selection, floral-vegetative correlations should be high. While this hypothesis may hold in populations with enough trait heritability to respond to selection toward ancestral correlations, because *L. bicolor* population traits are dominated by neutral evolutionary processes, all their traits are varying independently from one another.

Lupinus nanus populations, on the other hand, exhibit the floral correlation pleiades predicted by Berg's hypothesis, but also have intermediate levels of correlation between floral and vegetative traits. However, it does not appear that floral or vegetative traits in *L. nanus* are experiencing significant indirect selection due to their correlations. Leaflet shape and floral size both have significant selection differentials in all five populations, but VIF values are low enough that colinearity between variables is negligible. This means that, while the correlations between floral and vegetative traits are higher in *L. nanus* than *L. bicolor*, they are low enough that floral and vegetative traits can respond independently to selection.

Selective Trends

Divergent selection among conspecific populations has been documented for many species, and is responsible for local adaptation and can cause species radiations (Benkman 2003, Hall and Willis 2006, Sambatti and Rice 2006, Byars et al. 2007, Verhoeven et al. 2008, Ramirez-Valiente et al. 2010). This study provides further evidence that selective surfaces within species can be vastly different among sites, as selection on the size of vegetative traits varied among sites for both species.

The variation in *L. nanus* floral selective trends could be partially due to pollinator size, with flowers growing in areas populated by small pollinators experiencing selection toward smaller flower sizes and populations inhabited by larger pollinators experiencing selection toward larger flower sizes.

However, pollinator selective pressures have been removed from the self-pollinating *L. bicolor*, so floral selective trends in this species are likely due to other factors. Because self-pollinating plants are able to set seed in areas of low mate density or reduced pollinator abundance there may be selection toward increased autonomous selfing to assure their reproduction. Under the reproductive assurance hypothesis plants should experience selection toward an optimal floral shape and size that maximizes anther-stigma contact and minimizes resource expenditure on floral displays (Dole 1992, Jarne and Charlesworth 1993, Schoen et al. 1996, Elle and Carney 2003, Moeller and Geber 2005, Davis and Turner-Jones 2008, Fishman and Willis 2008, Kennedy and Elle 2008, Vaughton et al. 2008).This means that as plants shift from an outcrossing mating system to a self-pollinating mating system they are assumed to experience selection for smaller flower sizes until pollen-stigma contact is optimized. However, only the PW *L. bicolor* population in our study was experiencing selection for smaller flowers. In fact, TR, FO, and DC were under selection for larger flower sizes, meaning that the flowers had attained a smaller size than the population optima, despite opposing selection.

Small *L. bicolor* flower sizes in the face of selection toward larger flowers could support our hypothesis that *L. bicolor* is unable to respond to selection due to lack of genetic variability. When selfing populations first split from outcrossing populations they respond to selection toward traits that increase their fitness, such as small flower sizes (Stam 1977). Over time, however, inbreeding can cause a reduction in within-site variation and an increase in allele fixation such that there may not be sufficient additive genetic variation for traits to respond to selection (Kelly and Arathi 2003, Bartkowska and Johnston 2009, Hensen et al. 2010).

Floral sizes below the optima could also be a result of past fluctuation in selection gradients due to temporal environmental variation (Caruso et al. 2003, Reynolds et al. 2010, Warner et al. 2010). Temperature, light levels, and water availability can all affect flower size, so annual fluctuations in these factors could influence floral selection (Karlsson et al. 1989, Pearson et al. 1995, Galen 1999). With yearly changes in size optima, after an annual bout of selection *L. bicolor* floral means could end up being larger or smaller than the new optimum. However, because we found low genetic variation and no consistent patterns of interannual mean changes in the direction predicted by selection in *L. bicolor* populations, we believe this hypothesis explains our results more accurately than fluctuating yearly floral optima or strong selection on fast life-cycle completion.

In addition to intraspecies variation in selective surfaces, we also found interspecies differences in selective surfaces within a site. Differing floral selection gradients can be explained by mating system differences between the two species. Vegetative selection differences, on the other hand, cannot be explained by differences in selective agents. Because both species co-occur, they are experiencing identical environmental conditions. Despite sharing environmental features, *L. nanus* and *L. bicolor* displayed differing selection gradients. At TR, for example, while the *L. nanus* population experienced selection for longer, narrower leaflets, *L. bicolor* fitness was almost entirely dependent on flower size, and selection on vegetative traits was weak. *Lupinus nanus* and *L. bicolor* have overlapping leaflet shapes at TR, and the TR *L. nanus* leaflet shape mean is not significantly different from the TR *L. bicolor* mean, so if environmental forces were affecting both species identically selection on vegetative characters should be similar. Because sympatric *L. nanus* and *L. bicolor* are experiencing different selective pressures there is likely a species by environment effect resulting in differential selective responses to identical environmental pressures.

Genotype by environment interactions are common among plants, so it comes as no surprise that *L. nanus* and *L. bicolor* have differential responses to identical environments (Mazer and Schick 1991, Cooper and Delacy 1994, Stratton 1994, Hopkins et al. 1995, Alia et al. 1997, Zewdie and Bosland 2000, Campbell and Waser 2001). Our study highlights the fact that environmental forces can act independently on individual species, and generalizations about how other plants will respond to environmental stimuli cannot be made from single species studies, because each species may have a unique reaction to the environment.

Conclusions and further exploration

We found that both *L. bicolor* and *L. nanus* have unique selection surfaces at each of the five populations in this study. However, while *L. nanus* seems to be able to respond to floral selection, *L. bicolor* lacks the genetic diversity to respond to selective pressures. This has both theoretical as well as practical implications. It supports the theory that interpopulational variation in selfing plant morphology is primarily due to neutral evolutionary forces rather than selection, but the mechanism for this outcome must be further explored. While neutral evolutionary forces may be more important than the selection regimes in the observed population mean phenotypic shifts of selfing plant traits, this does not preclude the importance of selection in the early stages of evolution from selfing to outcrossing. In future explorations of the selective pressures exerted on selfing plants, studies should focus on populations experiencing recent evolution of selfing, before genetic degradation makes response to selection impossible.

These findings also suggest that outcrossing plant populations may be able to adapt to new environments more quickly than selfing populations. Without enough genetic diversity, trait heritability may be too low to allow response to selection. In the face of climate change, this means that selfing species may be more vulnerable to rapid environmental shifts than outcrossing plants. *Lupinus nanus* and *L. bicolor* are also commonly used in restoration. If *L. bicolor* cannot respond to selection in degraded habitats, it may be more beneficial to use an outcrossing relative, such as *L. nanus*, that can adapt to the new environmental stresses and succeed in degraded restoration sites. It is also possible, however, that the need for high flower densities and insects for pollination will prevent *L. nanus* from thriving in novel habitats, despite the ability for quick adaptation. Future studies should examine local adaptation of these species to determine the potential consequence of environmental change to their populations and their restoration value.

2.5 FIGURES and TABLES

Figure 1: Distinguishing characteristics of populations experiencing different types of evolutionary processes responsible for change in floral traits over time. If floral traits are experiencing natural selection they should be characterized by higher levels of genetic diversity and greater ability to respond predictably to the direction of selection than populations dominated by neutral evolutionary processes. If they are experiencing natural selection, they could be changing due to direct selection (characterized by low correlations between vegetative and floral suites of traits and constant floral selection when vegetative selection is controlled) or indirect selection (characterized by high correlations between vegetative and floral suites of traits and low floral selection when vegetative selection is controlled).

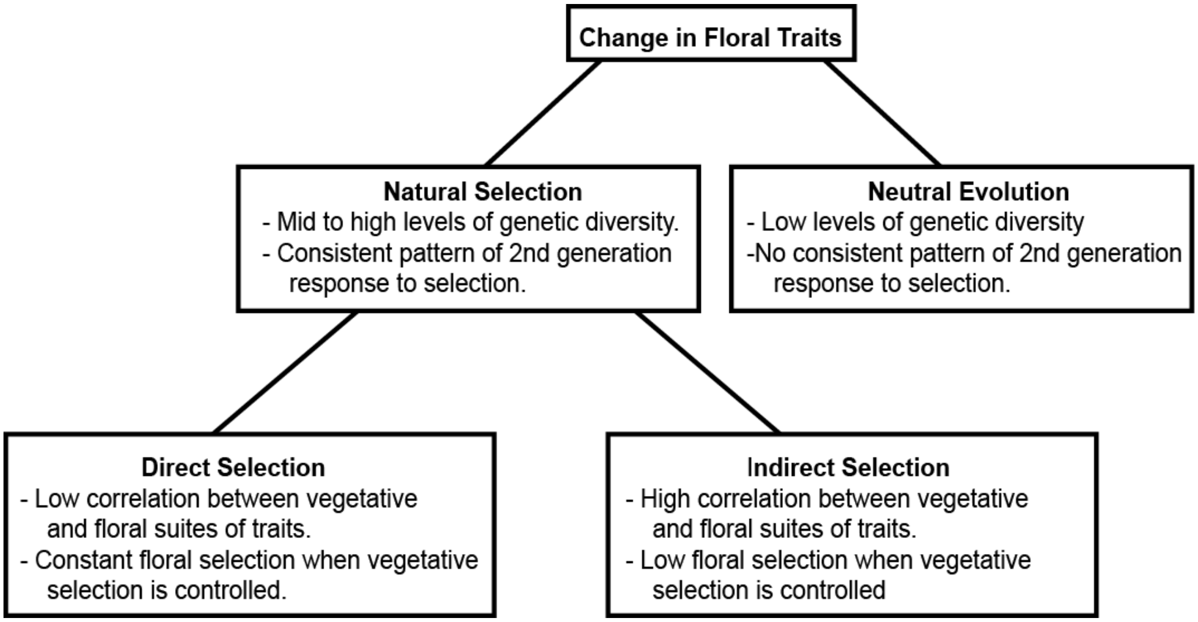
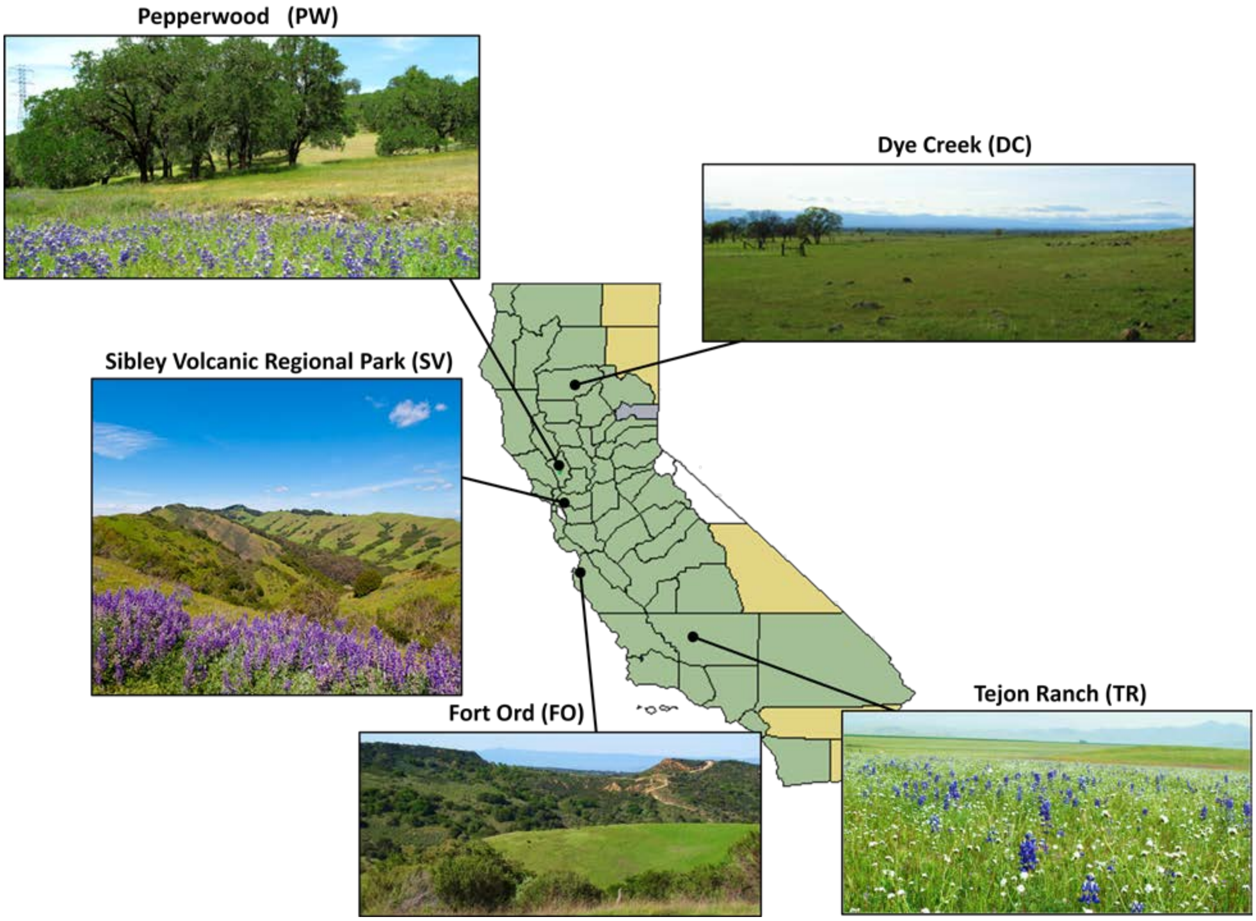


Figure 2: Locations of the five field sites. a) Map of field site locations. Counties where only *L. bicolor* grows are in beige and the county where *L. nanus* grows alone is in blue. The counties with where both species grow sympatrically are in green. b) The coordinates of the sites and their abbreviations are listed in the table from north to south.

a)



b)

Site Name	Abbreviation	Coordinates
Dye Creek	DC	40° 06' 16"N 122° 02' 38"W
Pepperwood	PW	38° 36' 08"N 122° 44' 07"W
Sibley Volcanic Regional Park	SV	37° 51' 09"N 122° 11' 26"W
Fort Ord	FO	36° 36' 14"N 121° 43' 58"W
Tejon Ranch	TR	35° 12' 34"N 118° 44' 55" W

Figure 3: We measured the following plant traits: A) leaflet width, B) leaflet length, C) sepal length, D) pedicel length, E) banner height, F) wing length, G) wing depth, H) lower sepal length.

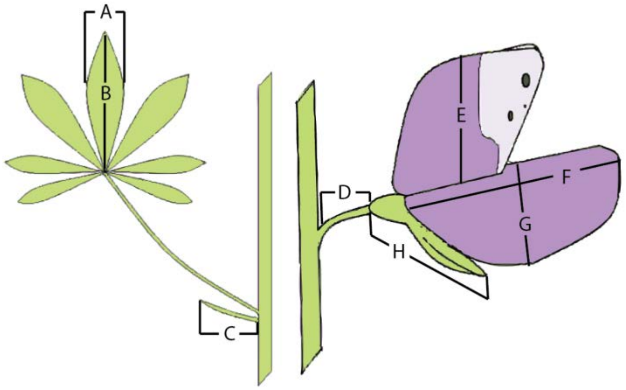


Table 1: Principal Component Analyses of vegetative traits (leaflet length, leaflet width, and sepal length) and floral traits (pedicel length, sepal length, and wing height) across all individuals, with sites and species lumped together. Vegetative axes are labeled "Veg PC" and floral axes are labeled "Floral PC". The table shows the trait loadings of raw trait values on the vegetative and floral PC axes and the total cumulative proportion of variance explained by each axis (cumulative proportion).

Vegetative Trait PCA	**Trait**	**Veg PC 1**	**Veg PC 2**			
	Leaflet Length	0.705	-0.280	0.960		
	Leaflet Width	-0.709	-0.681	-0.184		
	Sepal Length	0.020	-0.677	-0.212		
	Cumulative Proportion	0.856	0.966	1.00		
Floral Trait PCA	**Trait**	**Floral PC1**	**Floral PC2**	**Floral PC3**	**Floral PC4**	**Floral PC5**
	Pedicel Length	-0.453	-0.010	0.887	0.003	0.012
	Banner Length	-0.468	-0.002	-0.284	0.390	0.738
	Wing Length	-0.467	-0.128	-0.266	0.496	-0.671
	Sepal Length	-0.402	0.848	-0.125	-0.315	0.031
	Wing Height	-0.443	-0.507	-0.214	-0.707	0.002
	Cumulative Proportion	0.884	0.962	0.985	0.996	1.00

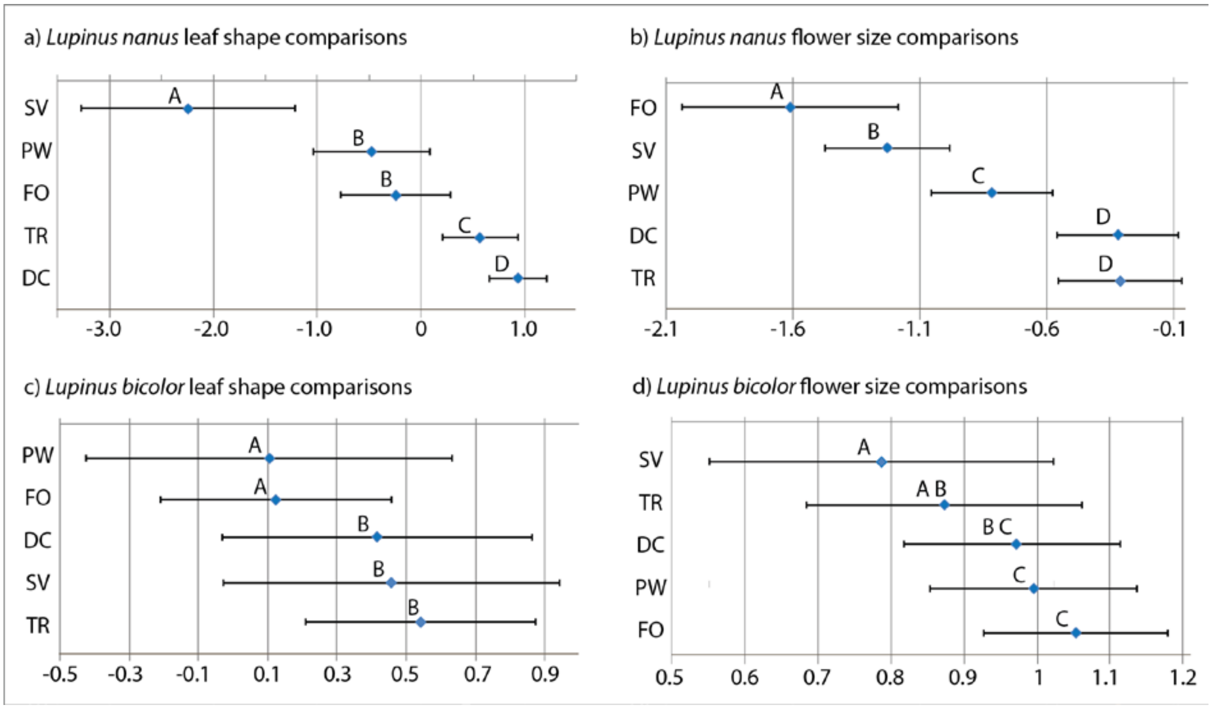
Table 2: Multivariate Analysis of Variance (MANOVA) table showing the degrees of freedom, Pillai's statistic, F-value, and p-value for the MANOVA testing differences among sites and species for leaflet shape and floral size. Site effect, species effect, and the interaction between site and species were all tested for.
* - p<0.05 ** - p<0.01 *** - p<0.001

	Degrees of freedom	Pillai statistic	Approximate F-value	P-value
Site	4	0.85	82.30	<0.001*
Species	1	1.03	3372.92	<0.001*
SitexSpecies	4	1.24	153.57	<0.001*
Residuals	435			

Table 3: Significance values of leaflet shape and floral size contribution to the Multivariate Analysis of Variance (MANOVA). Each cell shows the F statistic and p-value showing whether that trait contributed significantly to the site, species, or site by species interaction effects listed in Table 2.
* - p<0.05 ** - p<0.01 *** - p<0.001

	Leaflet shape		Floral Size	
	F statistic	p-value	F statistic	p-value
Site	165.2	<0.001***	528.8	<0.001***
Species	6595.0	<0.001***	18576.4	<0.001***
SitexSpecies	152.7	<0.001***	549.8	<0.001***

Figure 4: Multiple comparisons of differences in mean leaflet shape and floral size across sites within species. Sites are listed along the y-axis, and the x-axis describes the trait values, with means being depicted by blue dots, and lines indicating one standard deviation. The letters listed above lines show significant mean differences to the $p<0.05$ level after Bonferroni corrections, with different letters indicating significantly different means. Abbreviations as described for Figure 2.



a) *Lupinus nanus* leaf shape comparisons

b) *Lupinus nanus* flower size comparisons

c) *Lupinus bicolor* leaf shape comparisons

d) *Lupinus bicolor* flower size comparisons

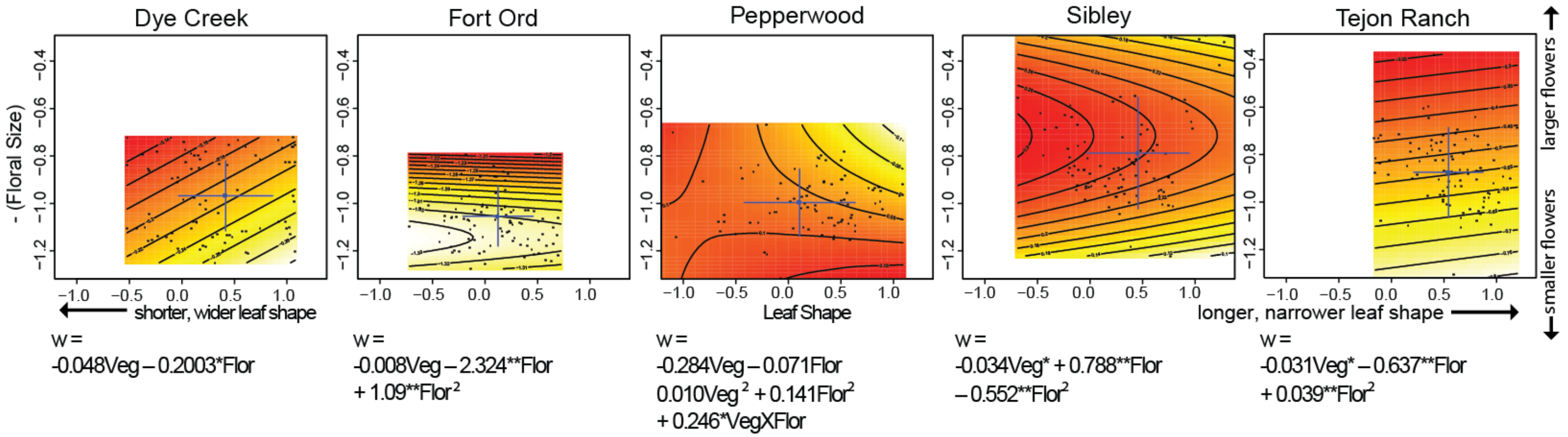


Figure 5: *L. bicolor* fitness landscapes for each site. The x-axis describes leaflet shape, with low values indicating short, wide leaflets and high values indicating long, narrow leaflets. The y-axis describes the flower size values from the first PC axis of the PCA conducted on floral traits. Because traits all loaded negatively on this axis, we transformed the flower size axis by multiplying it by -1 to avoid confusion, so higher flower size axis values indicate larger flowers. The z-axis is represented by contour lines, and describes plant fitness, with dark, red areas representing floral and vegetative combinations with high fitness, while light, yellow areas show low fitness contour areas. Graphs are presented in boxes showing the full range of species floral and vegetative values for all *L. bicolor* individuals at all sites. Blue dots and corresponding lines represent the site average and one standard deviation. Equations describe the best approximation of the selective landscape (based on AIC criterion). "w" is used to describe plant fitness, while "Veg" describes leaf shape and "Flor" describes floral size.

* $p < 0.05$ ** $p < 0.01$

Table 4: Descriptions of the optimal leaflet shape and flower size for each species at each site. Asterisks indicate that the trait combination responsible for peak fitness lies within the population floral size and leaflet shape range.

	Dye Creek	Fort Ord	Pepperwood	Sibley	Tejon Ranch
L. bicolor	short, wide leaflets and large flowers	large flowers	short, wide leaflets* with small flowers	short, wide leaflets with intermediate flower sizes	short, wide leaflets with large flowers
L. nanus	short, wide leaflets and small flowers	short, wide leaflets with intermediate flower sizes	short, wide leaflets with large flowers	short, wide leaflets with small flowers	long, thin leaflets with small flowers

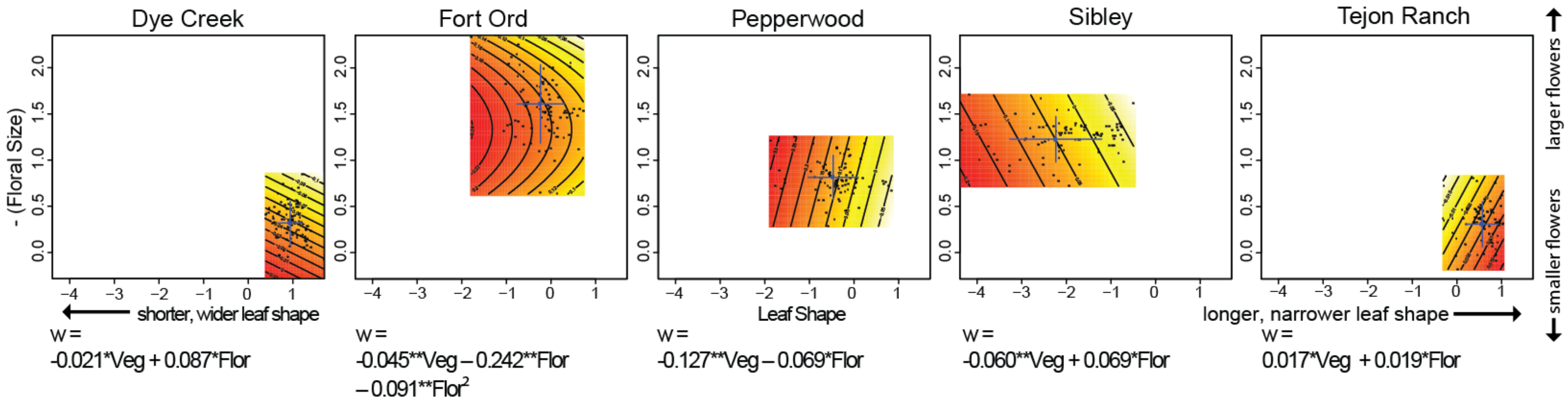


Figure 6: *L. nanus* fitness landscapes for each site. The x-axis describes leaflet shape, with low values indicating short, wide leaflets and high values indicating long, narrow leaflets. The y-axis describes the flower size values from the first PC axis of the PCA conducted on floral traits. Because traits all loaded negatively on this axis, we transformed the flower size axis by multiplying it by -1 to avoid confusion, so higher flower size axis values indicate larger flowers. The z-axis is represented by contour lines, and describes plant fitness, with dark, red areas represent floral and vegetative combinations with high fitness, while light, yellow areas show low fitness contour areas. Graphs are presented in boxes showing the full range of species floral and vegetative values. Blue dots and corresponding lines represent the site average and one standard deviation. Equations describe the best approximation of the selective landscape (based on AIC criterion). "w" is used to describe plant fitness, while "Veg" describes leaf shape and "Flor" describes floral size.

* - $p < 0.05$ ** - $p < 0.01$ *** - $p < 0.001$

Table 5: Variance Inflation Factors (VIF) values examining colinearity between leaflet shape and floral size for both species at each site.

VIF Values		
Site	*L. bicolor*	*L. nanus*
Dye Creek	1.041	1.015
Fort Ord	1.058	1.433
Pepperwood	1.010	1.071
Sibley	1.529	1.012
Tejon Ranch	1.073	1.023

Table 6: Population means and standard deviations of floral size and leaflet shape for 2009 and 2010. The change in phenotype predicted by the direction of selection is listed next to the actual directional change in phenotype from 2009 to 2010. The red arrows indicate instances where the actual direction of phenotype change agrees with the expected change, and asterisks indicate significant differences in trait means between years. Dashes indicate either minimal or non-significant selection gradients under the Expected Change column or non-significant differences in means between years in the Actual Change column. Abbreviations as described for Figure 2, with the addition that "Lb" indicates *L. bicolor* and "Ln" indicates *L. nanus.*
$^{*}p<0.05$ ** $p<0.01$ *** $p<0.001$

Species Abbreviations
Lb – L. bicolor
Ln – L. nanus

	Vegetative				Expected change	Actual change	Floral				Expected change	Actual change
	2009		2010				2009		2010			
Site	x̄	SD	x̄	SD			x̄	SD	x̄	SD		
DC*Lb*	0.415	0.432	0.476	0.401	↓	↑**	0.971	0.143	1.182	0.176	↓	↑ ***
FO*Lb*	0.137	0.321	0.317	0.423	-	↑ ***	1.061	0.115	0.885	0.316	↓	↓ ***
PW*Lb*	0.103	0.518	-0.273	0.555	↓	↓ ***	1.003	0.138	0.306	0.332	↑	↓ ***
SV*Lb*	0.462	0.454	1.310	0.261	↓	↑ ***	0.793	0.228	0.781	0.221	↓	-
TR*Lb*	0.532	0.328	0.528	0.227	-	-	0.876	0.177	1.041	0.209	↓	↑ ***
DC*Ln*	0.967	0.287	1.042	0.257	↓	-	-0.328	0.225	-0.088	0.260	↑	↑ ***
FO*Ln*	-0.236	0.502	-0.108	0.549	↓	-	-1.613	0.408	-0.829	0.416	↑	↑ ***
PW*Ln*	-0.462	0.535	-0.781	0.777	↓	↓ ***	-0.818	0.233	-1.137	0.409	↓	↓ ***
SV*Ln*	-2.228	1.028	-1.235	1.042	↓	↑ ***	-1.230	0.239	-0.861	0.345	↑	↑ ***
TR*Ln*	0.573	0.348	0.337	0.374	↑	↓ ***	-0.311	0.235	-0.198	0.274	↑	↑ ***

Table 8: Nei's gene diversity (H_e) and allelic richness within *L. nanus* and *L. bicolor* populations.

site	Dye Creek	Pepperwood	Sibley	Fort Ord	Tejon Ranch
L. bicolor					
H_e	0.07	0.30	0.10	0.01	0.12
Allelic Richness	1.25	2.26	1.33	1.06	1.36
L. nanus					
H_e	0.20	0.77	0.33	0.76	0.73
Allelic Richness	1.55	5.95	2.50	6.32	6.12

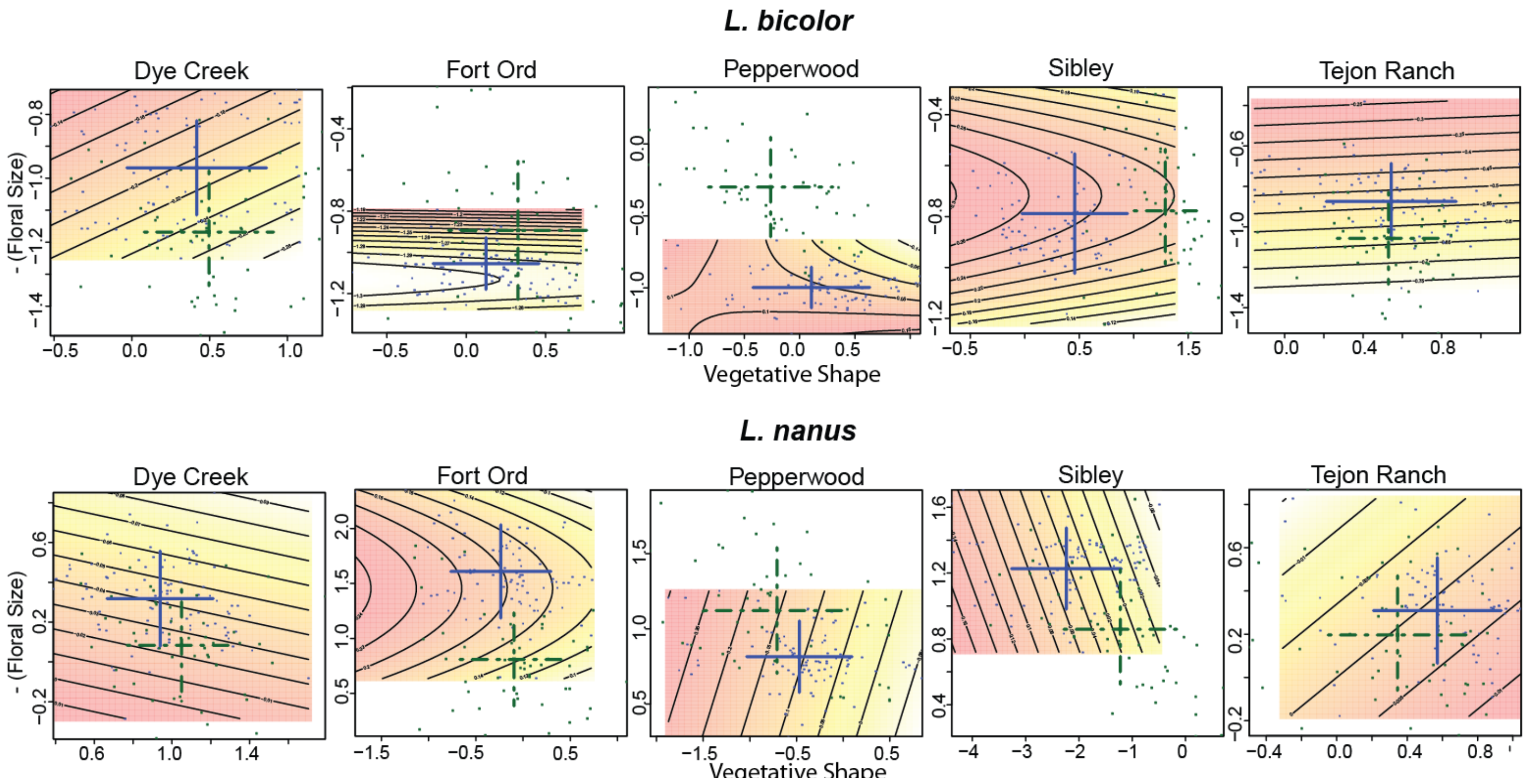


Figure 7: Phenotypes of *L. bicolor* and *L. nanus* from 2009 (plotted in blue) and 2010 (plotted in green). The x-axis describes leaflet shape, with low values indicating short, wide leaflets and high values indicating long, narrow leaflets. The y-axis describes the flower size values from the first PC axis of the PCA conducted on floral traits. Because traits all loaded negatively on this axis, we transformed the flower size axis by multiplying it by -1 to avoid confusion, so higher flower size axis values indicate larger flowers. The z-axis is represented by contour lines, and describes plant fitness. Means are plotted with standard error bars (solid lines for 2009 and dashed lines for 2010). Points are plotted in the full phenotypic range of the specific site and species for both 2009 and 2010, and selective landscapes from 2009 are included in the 2009 phenotypic range of the site and species.

Chapter 3. Local adaptation in two lupines with differing mating systems

3.1 INTRODUCTION

Most plant species are comprised of many individual populations, each experiencing selective pressures from their respective environments. If populations can respond to these spatially varying selection pressures they may become adapted to their local habitats (Schmidt and Levin 1985, van Tienderen and van der Toorn 1991, Kawecki and Ebert 2004, Geber and Eckhart 2005, Abdala-Roberts and Marquis 2007, Anderson and Geber 2010). Locally adapted genotypes will outperform non-local genotypes when grown sympatrically (Fig. 1a&c), because they have evolved morphological and physiological characteristics that maximize fitness at their home sites, even if these same characters may be detrimental to fitness in other habitats.

Local adaptation is affected by the strength of natural selection in each locale, levels of within-population genetic variation, and rate of gene flow among populations (Antonovics 1976, Schluter and Grant 1984, Linhart and Grant 1996, Stanton and Galen 1997, Hendry and Taylor 2004). If a local environment imposes strong selection on a population, that population will have a greater potential for local adaptation than populations with weaker selection differentials (Schluter and Grant 1984). Likewise, high levels of within-population genetic variation can increase a population's ability to respond to selection, because response to selection is proportional to genetic variation (Linhart and Grant 1996). Gene flow rates, on the other hand, can have a negative effect on local selection. In non-sink populations, high gene flow may prevent populations from adapting to their specific environments because it can introduce non-adaptive genes (Stanton and Galen 1997, Hendry and Taylor 2004). Such gene flow promotes cohesion among populations and thus inhibits local population specialization (Hamrick and Godt 1996).

While breeding systems can affect levels of population genetic diversity and isolation (Viard et al. 1997, Lavigne et al. 2001, Szarowska et al. 2003), the effect of pollination system on patterns of local adaptation is still not well understood (Lenormand 2002, Hereford 2010). Local adaptation has been documented in plants pollinated by a wide variety of vectors (Schmidt and Levin 1985, van Tienderen and van der Toorn 1991, Geber and Eckhart 2005, Anderson and Geber 2010), but the extent to which pollination systems play a role in the presence or absence of local adaptation is debated (Hereford 2010).

Outcrossing mating systems versus selfing mating systems could be particularly pertinent to local adaptation, because these pollination methods have differential levels of within-site genetic variation and population isolation which can have conflicting effects on local adaptation. Outcrossing plants, for instance, tend to be outcrossing or have mixed mating systems, either of which can maintain high levels of intra-site genetic variation, thus allowing for local adaptation (Jain and Bradshaw 1966, Antonovics and Bradshaw 1970, Brady et al. 2005). However, outcrossing can also increase gene flow among populations (Hamrick and Godt 1996) and prevent them from adapting to local selective pressures.

Self-pollination, on the other hand, often constrains gene flow to within sites or even family lines, which prevents local adaptation in self-pollinating species from being swamped out by gene flow. For instance, Linhart and Grant (1996) reviewed a broad array of natural plant population studies and concluded that species with high levels of selfing can show adaptive differences over small spatial scales while gene flow in outcrossing species can lead to poorly adapted progeny. Although reduced gene flow can help maintain the genetic integrity of a population, high selfing rates also affect genetic diversity. Self pollination reduces effective population size, which increases the rate of neutral evolutionary processes such as genetic drift (Wright 1931a, Charlesworth et al. 1993, Barrett 1998, Takebayashi and Morrell 2001, Hensen et al. 2010). In combination with the high rates of extinction and recolonization common in selfing species, genetic drift can reduce within-site genetic diversity (Charlesworth and Charlesworth 1995). Low levels of genetic variation will limit local adaptation in self-pollinating species, especially in small populations where genetic drift can override natural selection.

The varied effects each of these pollination systems can have on local adaptation raise the question: How does mating system affect local adaptation? Do high levels of genetic variation in outcrossing plants allow them to have higher levels of local adaptation than selfing plants despite high outcrossing rates, or does the genetic isolation among populations of selfing plants permit them to maintain higher local adaptation, regardless of the low genetic variation found within those populations?

While several studies have examined local adaptation in plants with a single pollination system (Platenkamp 1990, van Tienderen and van der Toorn 1991, Leiss and Muller-Scharer 2001, Geber and Eckhart 2005, Abdala-Roberts and Marquis 2007), few have directly compared selfing and outcrossing species (Hereford 2010). One reason these studies are rare could be that it is difficult to control for the strength of selection at a given site. Because evolutionary responses are correlated with the strength of selection, selection differentials that vary between species at different sites could confound local adaptation comparisons. To effectively compare local adaptation among selfing and outcrossing plants, the populations under consideration must be experiencing similar selection differentials. Hereford (2010) addresses this issue in his meta analysis comparing literature documenting local adaptation levels of selfing and outcrossing plants by including a variable that estimates the differences in strength of selection between source populations' native sites. However, because selection can be non-linear, it can differ among habitats in complex ways not easily described by a single variable. Thus, the only way to truly control for environmental differences in selection among locations is to compare outcrossing and selfing species that co-occur in the same locations.

In addition to environmental differences, phylogenetic differences could also confound measurements of local adaptation. If species do not share a recent common ancestor, phylogenetic differences could result in differential reactions to similar environments (Lynch and Walsh 1998). Comparing closely related species can reduce the phylogenetic effects on genotype by environment interactions.

Lupinus nanus and *L. bicolor* are ideal species to study differences in local adaptation between selfing and outcrossing plants, because not only are they closely related species with differing pollination syndromes, they also grow sympatrically, allowing for comparisons to be made under identical environmental conditions. Both species exhibit similar vegetative and reproductive phenologies, but *L. bicolor* exhibits high levels of self-pollination whereas *L. nanus* is pollinated primarily by bees and syrphid flies (Dunn 1956, Horovitz and Harding 1972, Barbour et al. 1973, Karoly 1992, Cordoba and Cocucci 2011).

Additionally, *L. nanus* and *L. bicolor* are frequently used in restoration projects (Caltrans 2003, Ashly 2005, Caltrans 2008, Balzer and Robinson 2011). Patterns of local adaptation in these species could therefore strongly affect restoration success. For example, if a species exhibits strong local adaptation, it would be important to use local seed sources for restoration attempts, because introducing maladapted plants could reduce project success (Mortlock 2000, Hamilton 2001, Krauss and Koch 2004). Introducing non-native seeds to locally adapted populations could also reduce biodiversity through hybridization with evolutionarily distinct local lineages (Baldwin 2000).

In this study we compare local adaptation at three California sites to answer the question: Do *L. nanus* or *L. bicolor* populations exhibit higher levels of local adaptation? We hypothesized that, although *L. nanus* experiences high outcrossing rates, which can limit local adaptation, the extremely low levels of genetic variation in *L. bicolor* populations will cause them to exhibit lower levels of local adaptation than *L. nanus*.

We also differentiate between local adaptation and home-site advantage (Kawecki and Ebert 2004). Local adaptation is the fitness advantage of a local population at its home site, as compared to sympatric non-local individuals (Fig. 1a&c). In contrast, home-site advantage happens when a genotype experiences higher fitness when grown at its home-site than when grown in away habitats (Fig. 1b&c). These home versus away comparisons run the risk of mistaking responses to selection with habitat quality. For instance, if individuals with genotypes adapted to low-nutrient soil (Population A) were to be grown in high-nutrient soil they may experience a higher overall mean fitness than they would in their home sites, but would not be able to reach a mean fitness level as high as individuals with genotypes that were adapted to the high-nutrient soils (Population B). In this case, if one were to only compare the mean fitness of Population A genotypes across sites, this population's adaptation to its native habitat would be masked by the differences in soil nutrient levels. In contrast, if mean fitness between population genotypes were to be compared within sites (local vs. foreign comparisons) the local genotype, Population A, would perform better in its native habitat than the foreign genotype, Population B, thus illustrating local adaptation (Fig 1a). This example describes a case where local adaptation is present without home site advantage, thus demonstrating the need to compare mean fitness between genotypes within sites rather than within a genotype between sites to determine whether a population is locally adapted to its environment.

It is also possible for demes to experience a home site advantage without local adaptation (Fig 1b). This would be the case, for example, if the Population A genotype performed better in its home habitat than in the away habitat, but was out-performed in comparison to the Population B genotype in both habitats. This could occur if a beneficial mutation were spreading through the population, but had not yet reached population A, or if Population A had lower fitness due to genetic factors such as inbreeding depression. It is also common for both local adaptation and home-site advantage to be present in a species (Fig 1c) (Gandon 1998, Thrall et al. 2002, Belotte et al. 2003). Therefore, in addition to examining local adaptation levels in *L. bicolor* and *L. nanus*, we also determine whether our study populations are experiencing home-site advantage.

3.2 METHODS

Study System

Lupinus bicolor and *L. nanus* are annual legumes that have palmately compound leaves and erect spicate inflorescences of pea-shaped flowers. These zygomorphic flowers are composed of five modified petals: the upper banner, two lateral wings, and two lower petals that are fused to form the keel (Baldwin et al. 2012). Petal coloration is usually blue with white banner spots, although rare pink, lavender, and white flowered individuals have been documented (Baldwin et al. 2012).

Lupinus bicolor flowers are 4-10mm long; pedicels are generally less than 3mm long. *Lupinus nanus* has larger inflorescences than *L. bicolor*, with flowers that can range up to 15mm long and pedicels up to 7mm long (Baldwin et al. 2012). *Lupinus bicolor* has higher selfing rates than *L. nanus*, but both species are self-compatible (Elliot et al. 1974, Harding et al. 1974).

Lupinus nanus and *L. bicolor* grow in open or disturbed areas, and often occur sympatrically. The range of *L. nanus* stretches through California, Nevada and Oregon, with common occurrences in north coastal scrub, coastal sage scrub, foothill woodland, coastal prairie, and valley grassland communities. *Lupinus bicolor* is found throughout the range of *L. nanus*, and also in Arizona, Washington, and British Columbia (Baldwin et al. 2012).

For this study we selected three sympatric populations of *L. nanus* and *L. bicolor* that were between 96km and 239km apart to ensure that populations were genetically distinct. The northernmost site, Pepperwood Preserve (38° 36′ 08″N, 122° 44′ 07″W), is located along Martin Creek in Sonoma County and has moist soil until late in the growing season. Lupines at this location grow densely in the loamy soil around the creek and have a longer flowering season than other populations due to the late season water availability (USDA 1978). The site at Sibley Volcanic Regional Park (37° 51′ 09″N, 122° 11′ 26″W), in Alameda County, is drier than Pepperwood, but the coastal fog keeps temperatures cool in the summer. Sibley has low soil acidity due to the basaltic rocks that are common at this site (Sloan 2006). Fort Ord (36° 36′ 14″N, 121° 43′ 58″W), in Monterey County, is our southernmost site, and has loamy sand soils, a maritime climate, and moderate summer fog (Cook 1978).

Seed Collection

Seeds used in this study were collected from 100 plants of each species at each location between June and August on 2009. Plants were selected for seed collection by generating two sets of random numbers between zero and 100: one set of x coordinates and one set of y coordinates. We laid out two 100m measuring tapes orthogonally along the population edge, and collected seeds from one individual at each of the x and y location pairs from our random number list. To collect the explosively dispersed seeds we placed Polypropylene Micro-perforated Bread Bags (Elkay Plastics, Commerce, CA) over the fruits of pollinated pods, which we gathered after dehiscence. Seeds were stored in coin envelopes at 23 °C.

The sites from which seeds were collected are referred to as seed sources and the sites into which plants from each seed source were planted are referred to as planting sites.

Germination Study

To examine seed source and planting site effects on germination rates, we planted out 100 seeds of each species from each site at Pepperwood, Sibley, and Fort Ord. We selected 50 maternal families of each species at random and planted two seeds from each maternal family at each planting site.

We used the planting technique described by Moore (2009) to distinguish study seeds from naturally occurring plants. Briefly, seeds were glued to plastic toothpicks of different colors using water-soluble glue (2 seeds per toothpick), with each toothpick color denoting a specific seed source site and species. In October, 2009, these toothpicks were sown in 2 × 2 × 4 mm holes along a 100m transect, spaced evenly every 2m. Six toothpicks, one for each species from each site, were sewn 5cm apart in a line perpendicular to the transect. Planting sites were monitored bi-monthly for survival through August, 2010.

Seed Scarification

Because the seed coats of some lupines are extremely hard, dormancy can be difficult to break (Rolston 1978). We therefore expected our seed plantings to yield low germination rates. To examine the effects of selection on later fitness stages such as seedling survival, survival to flowering, number of flowers per plant, and number of seeds produced, we also performed a reciprocal transplant experiment with 3-week old plants.

To induce seed germination, past studies have used concentrated sulfuric acid to erode pits in the testa, thus allowing water entry into the seeds, so we tested acid soaking treatments as a way to increase germination rates (Burns 1959). We set up three trials using ten *L. nanus* and *L. bicolor* seeds to determine the amount of time the seeds should be soaked in concentrated sulfuric acid to erode the testa without damaging the seeds (20 min, 30 min, and 40 min soak times). Rupturing of the hilum has also been documented as a dormancy breaking technique, so we added a fourth trial that included a 40 min sulfuric acid soak followed by scratching along the hilum with a razor blade (Hyde 1954).

Using the acid scarification techniques described by Simms *et al.* (2006), we soaked the seeds in sulfuric acid until they had soaked for a total of 20min, 30min, or 40min, depending on the trial soak time. Seeds that were knicked, in addition to acid scarification, were then scratched with a razor blade along the hilum. After scarification seeds were transferred into sterile 96-well plates with 200ul of sterile water. These plates were covered and left to germinate at 15°C in the dark. We checked the seeds after 3 days for signs of imbibitions.

Because the combined hilum scratching after 40min acid scarification yielded the highest germination rates, this is how we treated the seeds used in our reciprocal transplant (Appendix 1).

Reciprocal Transplant

We treated ten seeds (using the protocol listed above) from each of 50 maternal plant families of *L. nanus* and *L. bicolor* collected from each of the three sites (500 seeds per species per site). Maternal plant families were chosen at random for inclusion in this study, with the requirement that there were at least ten seeds per family. Treated seeds were left to imbibe for a week at 15°C in sterile 96-well plates in the dark.

We filled 66ml Ray Leach Cone-tainer cells (Stuewe and Sons, Tangent, Oregon) with soil from each intended planting site and transferred seeds that had imbibed into these cells. We placed two seeds from each maternal family in soil from each site. We allowed plants to grow for three weeks in the greenhouse before transplanting them to their respective field sites. During this time we replaced any seedlings that died to make sure we had a total of 100 plants (2 plants from 50 maternal families) of each species from each seed source site for transplant to each planting site.

Seedlings were planted along a 100m transect in February, 2010. At every meter mark one *L. bicolor* and one *L. nanus* seedling from each seed source site were planted 5cm apart in a line perpendicular to the transect. To avoid confusing planted seedlings with natural recruits, we weeded naturally occurring seedlings around the planted seedlings in a diameter of 50cm, but we did not remove other vegetation around the seedlings before planting. All seedlings were watered by hand immediately after transplantation. When pods began to form on the plants we collected seeds by placing Micro-perforated Polypropylene Bread Bags (Elkay Plastics, Commerce, CA) over the fruits of pollinated pods.

Planting sites were censused bi-monthly for three components of fitness: first month survival, seedling survival to flowering, and number of flowers per plant. We quantified total fitness as the total number of seeds produced per seedling planted.

Statistical Analyses

We used ANOVAs to examine the effects of seed source and planting site on total fitness. Our data violated assumptions of homoscedacity, so we square-root transformed total fitness (seed number) before performing ANOVAs.

When the seed source by planting site interaction was statistically significant we used Tukey comparison tests to examine independent contrasts. To test for local adaptation we compared fitness of local populations with non-local populations within planting sites. To test for home-site advantage we compared fitness of a population genotype at its home planting site with that at away planting sites. We used Bonferroni corrections to adjust for multiple testing.

If local adaptation or home-site advantage was detected we then examined individual fitness components separately (first month survival, seedling survival to flowering, and number of flowers per plant) using ANOVAs to determine their role in the observed patterns. Before performing these analyses we square-root transformed number of flowers per plant, and arcsine-square-root transformed first month survival and seedling survival to flowering. When a fitness component showed a significant seed source by planting site interaction we graphed the fitness component by planting site and seed source and performed independent contrasts using Tukey comparisons (and Bonferroni corrections) to determine whether it played an important role in the patterns of local adaptation and home-site advantage we observed with total fitness. To visualize fitness comparisons (component and total fitness) we used the "ggplot2" package in R (Wickham 2009) to graph the untransformed data.

Because using transformed fitness variables in ANOVA analyses may compromise biological interpretations of ecological patterns, we also used the R package '*aster*' to confirm patterns of planting site and seed source effects on overall fitness identified by the ANOVAs (Geyer et al. 2007, R Development Core Team 2011).

Aster modeling allows multiple fitness values with different probability distributions to be analyzed jointly without requiring fitness transformations. Because overall fitness has a compound distribution resulting from the product of multiple distinct fitness components, each with its own distribution, it is uncommon for multiplicative fitness to be normally distributed (Mitchell-Olds and Shaw 1987). Aster models do not require multiplicative fitness values to be normally distributed, because they use maximum likelihood functions to model fitness by conditioning late-life fitness on earlier life-stage fitness components (Geyer et al. 2007, Shaw and Geyer 2010). Thus, rather than using a single multiplicative fitness value, which often has a distribution pattern that does not conform to well-known parametric distributions, aster models incorporate distinct components of fitness with predictable standard probability distributions such as Poisson (in the case of offspring number, for instance) and Bernoulli (in the case of survival). Because it does not violate the assumptions of normality, aster analysis also facilitates hypothesis testing among models.

To examine how seed source site and planting site influenced overall fitness we fit an aster model that incorporated the following fitness components from different lupine life stages: first month survival, survival to flowering, number of flowers, and number of seeds produced. Because germination of field-site sewn seeds was extremely low (less than 2%, on average) it was not possible to include field germination data in our analyses. We used seed source site, planting site, and the interaction between seed source site and planting site as fixed effects. We then compared this full model to reduced models that sequentially dropped terms, starting with the interaction effect. In total we compared four models: A full model including all the fixed effects listed above, a restricted model including both seed source and planting site (which was compared to the full model), and two partial models including either seed source or planting site, only. The partial models were compared to the restricted model to examine whether term additions improved the analysis in a step-wise fashion.

3.3 RESULTS

Field-Sewn Seed Germination

Germination of seeds sewn directly at field sites was extremely low (Fig. 2). None of the seeds planted at Pepperwood grew. At the Sibley planting site two *L. bicolor* seeds collected from Pepperwood, one *L. nanus* seed from Sibley, and one *L. bicolor* seed from Fort Ord germinated. At the Fort Ord planting site one *L. bicolor* seed from Sibley, one *L. bicolor* seed from Fort Ord, and two *L. nanus* seeds from Fort Ord germinated. Because these germination levels were so low, they were not included in further analyses (See Appendix 2 for more information).

Overall Fitness

Both the *L. bicolor* and *L. nanus* ANOVAs for total fitness showed significant interactions between seed source and planting site (Table 1). Aster analyses were in accordance with the ANOVA results, as the inclusion of all three terms (seed source, planting site, and the seed source by planting site interaction) significantly improved the model (Table 2).

All *L. nanus* seed sources exhibited home-site advantage (Fig. 3b). Seeds from all three seed source sites had significantly higher fitness when planted at their home site than at either away site (Table 3b).

Two of the three *Lupinus nanus* populations exhibited local adaptation (Fig. 3b). Pepperwood genotypes were significantly more fit at the Pepperwood planting site than were seeds from the other two sources. Sibley seeds were also significantly more fit at the Sibley planting site than were seeds from Pepperwood or Fort Ord. At the Fort Ord site, however, Pepperwood seeds were significantly more fit than the local Fort Ord seeds (Table 4b).

In contrast, *L. bicolor* exhibited neither local adaptation nor home site advantage (Fig. 3a). At the Fort Ord planting site, seedlings sourced from Pepperwood did significantly better than the local Fort Ord seedlings. At the Pepperwood planting site, there were no significant differences in fitness among seed sources. Finally, at the Sibley planting site Pepperwood seeds were significantly more fit than the local Sibley seeds (Table 4a). Fitness of Fort Ord seeds at their home site did not differ significantly from their fitness at the Pepperwood planting site, although their fitness at both of these sites was significantly higher than at the Sibley planting site. Pepperwood seeds were least fit at their home planting site, and did significantly better at the Fort Ord planting site. Sibley seeds were also least fit at their home planting site, and had significantly higher fitness at the Fort Ord planting site (Table 3a).

Fitness Components

Because all three *L. nanus* populations exhibited home-site advantage and the Pepperwood and Sibley populations were locally adapted, we examined the *L. nanus* fitness components separately to determine whether these patterns could be observed at multiple life stages.

All measured *L. nanus* fitness components showed significant interactions between planting site and seed source (Table 5). Survival to flowering did not exhibit a significant planting site effect but, because the interaction between planting site and seed source was significant, we left this term in the model.

All three fitness components contributed to local adaptation of the Pepperwood and Sibley populations to their home sites (Fig. 4, Table 6), whereas at the Fort Ord planting site Pepperwood seeds had higher first month survival, survival to flowering, and number of flowers per plants than did the local Fort Ord seeds.

All three fitness components contributed to home-site advantage in the Sibley population (Fig. 4, Table 7). In the Pepperwood population, survival to flowering and number of flowers per plant both contributed to home-site advantage. However, first-month survival of Pepperwood seeds was not significantly different between the Pepperwood and Fort Ord sites, which indicates that the *L. nanus* Pepperwood population did not show a pattern of home site advantage for this fitness component. Finally, in the Fort Ord population none of the fitness components showed a pattern of home-site advantage.

3.4 DISCUSSION

We hypothesized that low levels of genetic variation in the selfing *L. bicolor* populations would prevent this species from exhibiting local adaptation across sites. Our data partially supported this hypothesis, because, while we did find a significant interaction between planting site and seed source for *L. bicolor*, this interaction was not indicative of local adaptation. In fact, we found several instances where plants from local seed sources did worse than plants from either of the non-local seed sources. There was also no pattern of home-site advantage in any of the seed source genotypes.

The low fitness of local seeds growing in their native habitats when compared to non-local seeds suggests that some *L. bicolor* populations may not be well adapted to their native locations. We can think of two explanations for this observation. First, selection favoring local adaptation happens at earlier life stages than we were able to observe. For example, seed germination is particularly important in grassland species, because native species are often seed limited, and germination rates can have a large effect on total population fitness (Turnbull et al. 2000, Foster and Tilman 2003, Clark et al. 2007, Stanley et al. 2011). Date of emergence is also important for total fitness (Kalisz 1986). Early emergence allows plants to achieve a larger size early in the growing season, thus increasing their competitive ability (Black and Wilkinson 1963, Ross and Harper 1972, Harper and White 1974, Cook 1980, Fowler 1984). This is especially important for small annuals, such as *L. bicolor*, which rarely exceeds 4dm in height (Baldwin et al. 2012) and can quickly be shaded out by invasive grasses. It has been hypothesized that selfing species employ a strategy of early emergence and swift maturation to ensure reproduction through rapid seed-set (Snell and Aarssen 2005, Mazer et al. 2010). If so, then emergence date could be an especially important fitness component for *L. bicolor*, because early emergence would allow *L. bicolor* populations to complete their life-cycles before environmental stresses can decrease population fitness (Primack and Kang 1989).

However, because *L. bicolor* is a selfing species with low intrapopulation genetic diversity (Shade, Chapter 1), it is more likely that local adaptation is not present in *L. bicolor* populations because these populations lack the necessary genetic component of phenotypic diversity for selection to act on. High self-pollination rates can lead to low levels of within-site genetic diversity (Charlesworth and Charlesworth 1995), which reduces the heritability of traits in selfing populations (Nyquist 1991). Because response to selection is proportional to the heritability of the traits on which selection is acting, selfing species may be unable to respond to selection (Lande and Schemske 1985, Charlesworth and Charlesworth 1998, Kirkpatrick and Jarne 2000, Figueroa-Castro and Holtsford 2010). In this case, *L. bicolor* traits will change over time due to neutral evolutionary processes such as genetic drift without regard to the direction of the selection gradient. If these trait changes happen against the direction of the local selection differential it could result in a population that is poorly adapted to its local environment. Previous studies have found extremely low levels of genetic diversity within *L. bicolor* populations (Shade, Chapter 1), so we hypothesize that the lack of local adaptation or home-site advantage in this species is largely due to the absence of intrapopulation genetic diversity.

While the selfing species *L. bicolor* did not show evidence of local adaptation or home-site advantage, all three outcrossing *L. nanus* populations exhibited home site advantage and two populations exhibited local adaptation. This partially supports our hypothesis that *L. nanus* is more locally adapted to its environments than *L. bicolor*. This finding suggests that while *L. nanus* gene flow levels are high enough to maintain sufficient genetic variation for selection to work on, the amount of gene flow is restricted enough to prevent genetic homogenization among populations.

Although *L. nanus* exhibited home site advantage at all sites and local adaptation at more sites than *L. bicolor*, *L. nanus* was not locally adapted at all sites. The *L. nanus* Fort Ord population was not locally adapted, despite the fact that it exhibited home site advantage. Biologically this means that while seeds from Fort Ord had higher fitness when grown at their home site than away sites, the high fitness level they exhibited at their home site was not as high as the fitness levels of non-local seeds grown at the same site. There are four possible explanations for this pattern: a) the *L. nanus* Fort Ord population is the only *L. nanus* population experiencing inbreeding depression, b) a beneficial mutation is spreading through *L. nanus* populations but has not reached the Fort Ord population, c) Fort Ord experienced a temporal shift of climate, or d) different populations have evolved toward different adaptive peaks of varying heights.

It is possible that the Fort Ord *L. nanus* population is experiencing inbreeding depression, while the other two populations have sufficient outcrossing to prevent inbreeding depression. Seeds from different *L. nanus* populations could have varying levels of inbreeding because *L. nanus* is self-compatible and variable outcrossing rates have been documented for this species (Dunn 1956, Harding 1970, Horovitz and Harding 1972, Harding et al. 1974, Karoly 1992). Self-fertilization in *L. nanus* is prevented by protandry and a collar of peristigmatic hairs that prevent internal pollen from reaching the stigma. These hairs wilt with time, thus allowing self-pollination in flowers that have not been pollinated (Juncosa and Webster 1989). Therefore, *L. nanus* populations with fewer pollinators may have higher inbreeding levels. Pollinator species composition may also affect *L. nanus* inbreeding levels. For example, Horovitz and Thorp (1970) found higher inbreeding rates in *L. nanus* populations that were visited by honey bees than in those visited primarily by bumble bees. If the *L. nanus* population growing at Fort Ord has higher inbreeding levels than the Sibley or Pepperwood populations then inbreeding depression could cause the fitness of plants grown from Fort Ord seeds to be lower than individuals from non-local seeds with higher outcrossing (Husband and Schemske 1996). This would not alter the pattern of home-site advantage, because all Fort Ord seeds would have similar levels of fitness reduction due to inbreeding, regardless of their planting site. While Fort Ord plants all experienced reduced fitness those at the native planting site would still have a home site advantage if they were better adapted to that location than the other two planting sites.

Local adaptation at Fort Ord could also be obscured by a beneficial mutation spreading through the populations. If the beneficial alleles have been fixed at Sibley and Pepperwood but not Fort Ord, Sibley and Pepperwood populations would have a higher fitness potential than Fort Ord. In this case, even if the Fort Ord population was well adapted to its local environment the advantages associated with the new mutation could result in Pepperwood and Sibley plants exhibiting higher fitness than Fort Ord plants, even when grown at Fort Ord's home site. The Pepperwood site is closer to the Sibley site (96.1km) than either site is to Fort Ord, which lies 144.8km south of Sibley and 239.4km south of Pepperwood, so it is possible that a beneficial mutation is spreading along a north to south gradient and has not yet reached Fort Ord. However, several studies have shown that beneficial mutations should spread rapidly across populations even when selection strength is moderate and levels of migration are low (Slatkin 1976, Rieseberg et al. 2003, Whitlock 2003). Beneficial mutations that result in significant fitness increases are likely to be strongly selected for, so if this was the case the spread rate would be especially rapid. Additionally, Fort Ord *L. nanus* populations have low levels of genetic isolation in comparison with Sibley populations (Shade, Chapter 1), so if a beneficial mutation was to become fixed in the Sibley populations it is unlikely the mutation would not occur in the Fort Ord population.

The lack of local adaptation at Fort Ord could also be due to a local shift in climate. Rapid climate changes have been documented in several habitats (Franks et al. 2007), and are especially common in California, where short-term climate stability is lower than most other Mediterranean-type ecosystems (Cowling et al. 2004, Cowling et al. 2005). If the climactic conditions at Fort Ord shifted during our planting season to be more similar to the environmental conditions that Pepperwood plants had become adapted to, then Pepperwood plants would thrive. In this case, even if Fort Ord plants had become locally adapted to the environment they had experienced in the past, this local adaptation would not be apparent in our study due to the shift in climate away from previous climactic conditions.

Finally, evolution toward differing adaptive peaks could also explain the lack of local adaptation at the Fort Ord site. Adaptive landscapes are used to describe genetic combinations of traits that are associated with varying fitness levels. Certain combinations of traits produce high fitness, resulting in high-fitness peaks, while other traits are less advantageous, and result in low-fitness valleys (Wright 1931b, 1932). Adaptive landscapes are often rugged, with several fitness peaks of varying heights (Whitlock et al. 1995). If the founders of a population, or remaining individuals after a population bottleneck possess traits that are located on a fitness slope, natural selection will cause them to adapt toward the local equilibrium of that fitness peak. Unless populations experience genetic drift mediated peak shifts or environmental fluctuation they could be stuck at a local fitness peak even if higher peaks exist (Wright 1931b, Dodson and Hallam 1977, Kirkpatrick 1982, Milligan 1986, Whitlock 1997). This could be the case for the *L. nanus* population at Fort Ord. If the Fort Ord population has adapted toward a fitness peak that is lower than the peaks Sibley and Pepperwood populations evolved toward, then Fort Ord plants may not have the potential to reach a fitness level as high as Sibley or

Pepperwood plants. The trait combinations resulting in the Fort Ord fitness peak could still be more beneficial at the Fort Ord planting site than the other planting sites, thus explaining the pattern of home-site advantage, but the higher fitness potential of Sibley and Pepperwood trait combinations could cause these plants to outperform local Fort Ord plants at their home site. It should be pointed out, however, that this scenario is contingent upon extremely limited gene flow between Fort Ord and other *L. nanus* populations. Even low levels of gene flow could bring new genetic combinations into the Fort Ord population, thus shifting this population away from its suboptimal peak (Lenormand 2002). Because *L. nanus* is an outcrossing species with mid- to high levels of gene flow, we suggest that this scenario is an unlikely explanation for the lack of local adaptation in the Fort Ord population.

While differential inbreeding, beneficial mutation spread, climatic shifts, and varying adaptive peaks could all explain the combination of home-site advantage presence, but lack of local adaptation at Fort Ord, these patterns should be re-examined with data that include early life-stage fitness measurements. As with *L. bicolor*, selection on germination date and survival could be important factors influencing total *L. nanus* fitness.

However, our analysis of *L. nanus* fitness components indicated that while all fitness components are indicative of total-fitness local-adaptation patterns, early life stage fitness may be less accurate at predicting patterns of home-site advantage for some populations. All *L. nanus* fitness components showed the same configurations of local adaptation as total fitness. This is consistent with several studies that have found similar local adaptation patterns across fitness components (Jordan 1992, Nagy and Rice 1997, Galloway and Fenster 2000). Interestingly, this is not the case for patterns of home-site advantage. While Fort Ord did exhibit home-site advantage when total fitness was examined, this population did not show home-site advantage for any of the individual fitness components. Additionally, Pepperwood did not exhibit home-site advantage during first month survival, as local seeds did not have significantly higher survival during the first month at the Pepperwood home-site than the Fort Ord away site.

The lack of clear home-site advantage patterns for two of the three populations during the first month survival could indicate that total fitness differences between populations planted at their home sites versus their away sites is more influenced by adult-stage life history components than juvenile survival. Several studies document instances where mortality at juvenile life stages is not strongly selective (Antonovics and Primack 1982, van Tienderen and van der Toorn 1991), so selection on first month survival may be lower than selection on late life stages for Pepperwood and Fort Ord populations. Because Fort Ord only exhibited home-site advantage when seed number differences were observed, the selective pressures on seed number could have a disproportionate effect on fitness differences among planting sites for this population.

Conclusions

This study shows that in comparison with *L. bicolor*, most *L. nanus* populations are more locally adapted to their environments and have higher home-site advantages. Previous studies have found *L. bicolor* populations to be genetically depauperate, so we hypothesize that the reduction of intrapopulation genetic variation caused by selfing is at least partially responsible for the lack of local adaptation and home-site advantage in this species.

Because *L. nanus* and *L. bicolor* are commonly used in restoration these results could have several important ecological implications. For example, the presence of local adaptation in some *L. nanus* populations means that it may be important to use locally collected *L. nanus* seed in restoration projects, because a) foreign genotypes may not perform well in new environments and b) gene flow with existing populations could reduce the fitness of future generations (McKay et al. 2005). Projects that use non-local seeds for restoration may have low success rates, because the foreign plants may be poorly adapted to the environment in which the restoration site is located (Knapp and Rice 1994). Additionally, the introduction of novel genotypes to a restoration site could negatively affect adjacent native populations (Lesica and Allendorf 1999, Keller et al. 2000). If there is gene flow between the existing populations and the introduced populations the resulting progeny could have reduced fitness due to outbreeding depression caused by genetic introgression of foreign provenances that are not adapted to the local environment (Edmands and Timmerman 2003, Hufford and Mazer 2003, McKay et al. 2005).

Although *L. bicolor* does not exhibit local adaptation or home-site advantage, the mechanisms behind this finding should be investigated further, because they may influence restoration success. For instance, if a low level of genetic diversity is responsible for the lack of local adaptation in *L. bicolor* it may not do well in restoration areas because it lacks the heritability needed to respond to the novel selection pressures of the degraded habitat. To ensure the long-term survival of a restoration population there must be sufficient genetic diversity to grant the population the ability to adapt to novel environmental conditions (Booy et al. 2000), so low levels of intrapopulation genetic diversity could lead to poor population establishment in restoration areas (Falk and Holsinger 1991, Hufford and Mazer 2003).

3.5 FIGURES and TABLES

Figure 1: Three possible types of genotype by environment interactions. Panel a) depicts an instance where both populations are locally adapted, but Population A does not experience home site advantage. Panel b) depicts an instance of home site advantage for both populations, but Population A is not locally adapted to its native habitat. Panel c) depicts an example where populations are experiencing both local adaptation and home site advantage.

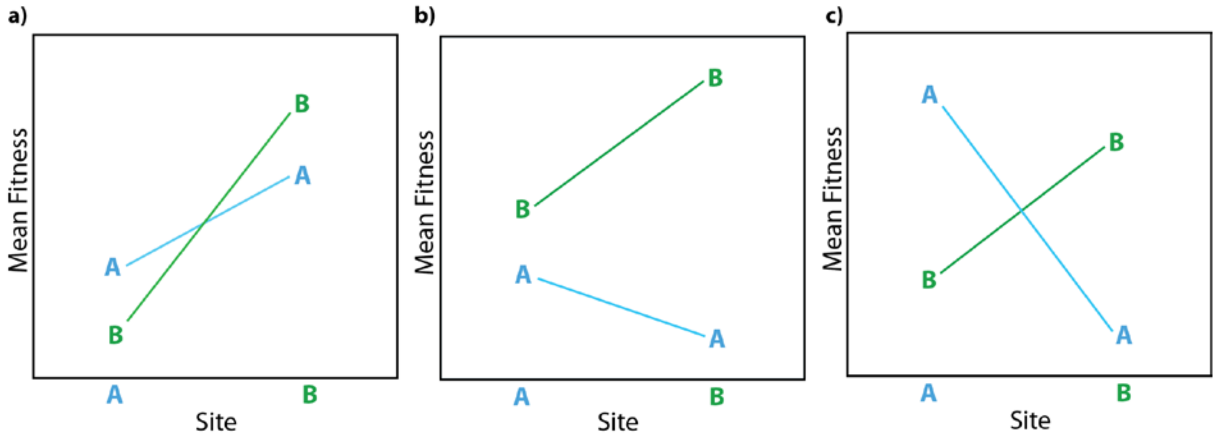


Figure 2: Seed germination in the field at Sibley and Fort Ord. Pepperwood is not depicted because no seeds planted at that location germinated. Site abbreviations are as follows: PW – Pepperwood; SV – Sibley; FO – Fort Ord.

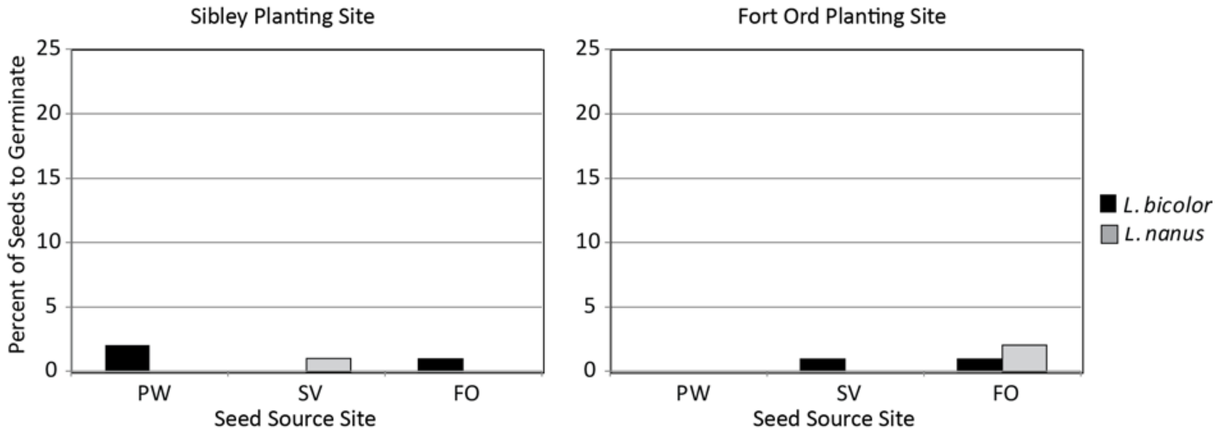


Table 1: Analysis of Variance (ANOVA) in total seed count for a) *L. bicolor* and b) *L. nanus*. Degrees of freedom (d.f.), F-value, and p-value for the ANOVA examining planting site and seed source effects on total seed counts.
* - p<0.05 ** - p<0.01 *** - p<0.001

a) *L. bicolor*

ANOVA Factor	d.f.	F value	P (>F)
Planting Site	2	19.67	<0.001***
Seed Source	2	13.15	<0.001***
Planting Site x Seed Source	4	3.31	0.01*

b) *L. nanus*

ANOVA Factor	d.f.	F value	P (>F)
Planting Site	2	3.88	0.02*
Seed Source	2	57.24	<0.001***
Planting Site x Seed Source	4	82.85	<0.001***

Table 2: Aster model comparisons for a) *L. bicolor* and b) *L. nanus* between the full model and the reduced models with sequentially dropped terms. The full model included fixed effects for seed source (Seed), planting site (Planting), and seed source by planting site interaction (Seed x Planting), with lifetime fitness, consisting of multiple life history stages (first month survival, seedling survival to flowering, number of flowers, and number of seeds produced), as the response. The restricted model only included seed source and planting site effects (no interaction effect), and the partial models only included one fixed effect (seed source or planting site). The restricted model was tested against the full model, while the partial models were tested against the restricted model. The test degrees of freedom (d.f.), test analysis of deviance (-2 log likelihood), and chi-squared p-values refer to comparisons between the model listed in that column and the more complete model it was tested against.

a) *L. bicolor*

Model	Fixed Effects	Model d.f.	Model deviance	Test d.f.	Test deviance	p-value
Full	Seed x Planting	11	650	-	-	-
Restricted	Seed + Planting	7	717	4	67	<0.001
Partial	Seed	5	752	2	35	<0.001
Partial	Planting	5	799	2	82	<0.001

b) *L. nanus*

Model	Fixed Effects	Model d.f.	Model deviance	Test d.f.	Test deviance	p-value
Full	Seed x Soil	11	-48,864	-	-	-
Restricted	Seed + Soil	7	-45,308	4	3,556	<0.001
Partial	Seed	5	-45,281	2	27	<0.001
Partial	Soil	5	-44,745	2	563	<0.001

Figure 3: Graphs showing total fitness (number of seeds produced per seedling planted) for a) *L. bicolor* and b) *L. nanus*. Planting sites are listed along the x-axis, and total fitness (Seed #) is listed along the y-axis. Fort Ord seed source is represented by blue filled squares and solid lines, Pepperwood seed source is represented by red empty squares and dashed lines, and Sibley seed source is represented by green empty diamonds and dotted lines. For clarity, error bars were omitted, but site averages and standard deviations are listed in Appendix 3. Site abbreviations are described in Figure 2.

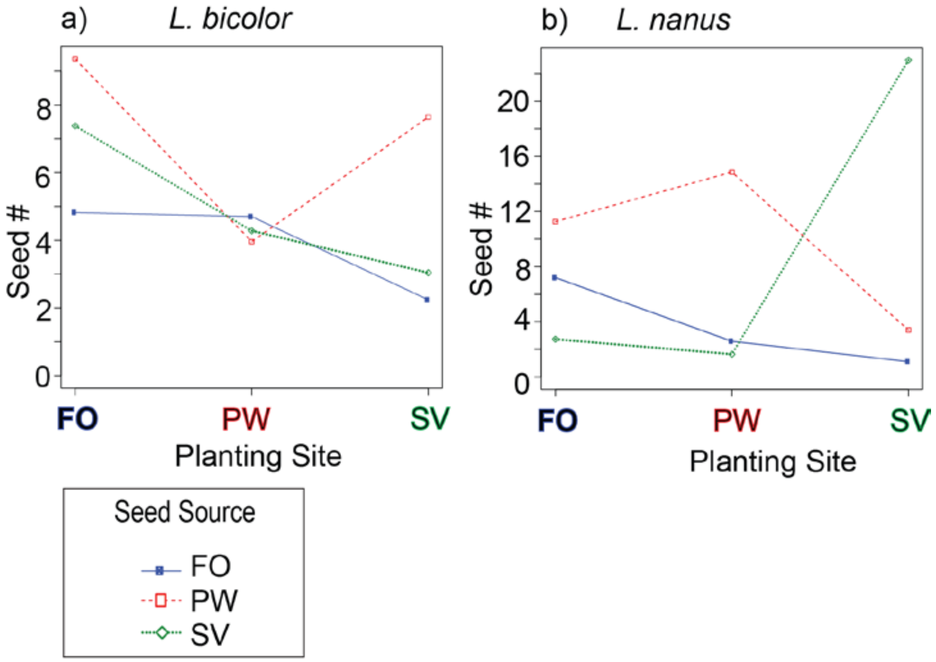


Table 3: Home site advantage tables for a) *L. bicolor* and b) *L. nanus* showing differences between planting site pairs, when compared within seed sources. Planting site pair comparisons are listed in the left hand column as two planting sites with a dash between them. The seed sources that the comparisons were made within are listed in the upper column under the label Seed Sources. The seed source of populations experiencing home site advantage are listed in bold. Site abbreviations are described in Figure 2.
* - $p<0.05$ ** - $p<0.01$ *** - $p<0.001$

a) *L. bicolor*

	Seed Sources:		
	FO	PW	SV
Planting Site Comparisons:	p-values		
PW-SV	0.024*	1.8	0.324
SV-FO	0.001**	0.081	<0.001***
FO-PW	1.404	<0.001***	0.009**

b) *L. nanus*

	Seed Sources:		
	FO	**PW**	**SV**
Planting Site Comparisons:	p-values		
PW-SV	0.342	<0.001***	<0.001***
SV-FO	<0.001***	<0.001***	<0.001***
FO-PW	0.012*	0.024*	0.378

Table 4: Local adaptation tables for a) *L. bicolor* and b) *L. nanus* showing differences between seed source pairs, when compared within planting sites. The seed source pairs being compared in each row are listed in the left hand column as with a dash between them. The local planting sites of populations experiencing local adaptation are listed in bold. Site abbreviations are described in Figure 2.
* - $p<0.05$ ** - $p<0.01$ *** - $p<0.001$

a) *L. bicolor*

	Planting Sites:		
	FO	PW	SV
Seed Source Comparisons	p-values		
PW-SV	0.384	2.7	0.003**
SV-FO	0.069	2.76	0.63
FO-PW	<0.001***	2.493	<0.001***

b) *L. nanus*

	Planting Sites:		
	FO	PW	SV
Seed Source Comparisons	p-values		
PW-SV	<0.001***	<0.001***	<0.001***
SV-FO	0.078	1.749	<0.001***
FO-PW	<0.001***	<0.001***	<0.001***

Table 5: Analysis of Variance (ANOVA) tables for *L. nanus* fitness components, including a) first month survival, b) survival to flowering, and c) flowers per plant. Degrees of freedom (df), F-value, and p-value for the ANOVA are included.
* - $p<0.01$ ** - $p<0.001$

a) First Month Survival

	df	F value	p (>F)
Planting Site	2	7.27	<0.001**
Seed Source	2	25.71	<0.001**
Planting Site x Seed Source	4	25.2	<0.001**

b) Survival to Flowering

	df	F value	p (>F)
Planting Site	2	4.54	0.856
Seed Source	2	30.84	<0.001**
Planting Site x Seed Source	4	32.94	<0.001**

c) Flowers per Plant

	df	F value	p (>F)
Planting Site	2	10.15	0.008*
Seed Source	2	33.12	<0.001**
Planting Site x Seed Source	4	12.16	<0.001**

Figure 4: Graphs showing *L. nanus* fitness components at each planting site for each seed source. Planting sites are listed along the x-axis, and the fitness component is listed along the y-axis. Fitness components include a) first month survival, b) survival to flowering, and c) flowers per plant. Fort Ord seed source is represented by blue filled squares and solid lines, Pepperwood seed source is represented by red empty squares and dashed lines, and Sibley seed source is represented by green dotted lines. For clarity error bars were omitted, but site averages and standard deviations are listed in Appendix 3. Site abbreviations are described in Figure 2.

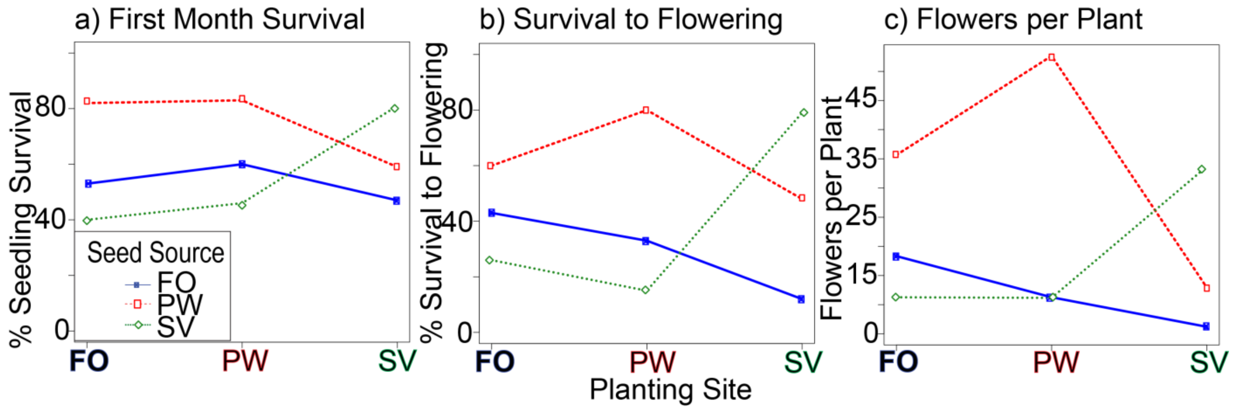


Table 6: Local adaptation tables for *L. nanus* fitness components showing differences between seed source pairs, when compared within planting sites. Fitness components include a) first month survival, b) survival to flowering, and c) flowers per plant. Seed source pair comparisons are listed in the left hand column as two seed source sites with a dash between them. The planting sites that the comparisons were made within are listed in the upper column under the label Planting Sites. The local planting site of populations experiencing local adaptation are listed in bold. Site abbreviations are described in Figure 2.

* - p<0.05 ** - p<0.01 *** - p<0.001

a) First Month Survival

	Planting Sites:		
	FO	**PW**	**SV**
Seed Source Comparisons	p-values		
PW-SV	<0.001***	<0.001***	<0.001***
SV-FO	0.195	0.141	<0.001***
FO-PW	<0.001***	<0.001***	0.267

b) Survival to Flowering

	Planting Sites:		
	FO	**PW**	**SV**
Seed Source Comparisons	p-values		
PW-SV	<0.001***	<0.001***	<0.001***
SV-FO	0.033*	0.006**	<0.001***
FO-PW	0.048*	<0.001***	<0.001***

c) Flowers per Plant

	Planting Sites:		
	FO	**PW**	**SV**
Seed Source Comparisons	p-values		
PW-SV	<0.001***	<0.001***	<0.001***
SV-FO	0.243	0.549	<0.001***
FO-PW	<0.001***	<0.001***	<0.001***

Table 7: Home site advantage tables for *L. nanus* fitness components showing differences between planting site pairs, when compared within seed sources. Fitness components include a) first month survival, b) survival to flowering, and c) flowers per plant. Planting site pair comparisons are listed in the left hand column as two planting sites with a dash between them. The seed sources that the comparisons were made within are listed in the upper column under the label Seed Sources. The seed source of populations experiencing home site advantage are listed in bold. Site abbreviations are described in Figure 2.

* - $p<0.05$ ** - $p<0.01$ *** - $p<0.001$

a) First Month Survival

	Seed Sources:		
	FO	PW	**SV**
Planting Site Comparisons	p-values		
PW-SV	0.195	<0.001***	<0.001***
SV-FO	1.194	<0.001***	<0.001***
FO-PW	0.96	2.559	1.182

b) Survival to Flowering

	Seed Sources:		
	FO	**PW**	**SV**
Planting Site Comparisons	p-values		
PW-SV	<0.001***	<0.001***	<0.001***
SV-FO	<0.001***	0.267	<0.001***
FO-PW	0.438	0.006**	0.162

c) Flowers per Plant

	Seed Sources:		
	FO	**PW**	**SV**
Planting Site Comparisons	p-values		
PW-SV	<0.001***	<0.001***	<0.001***
SV-FO	<0.001***	<0.001***	<0.001***
FO-PW	0.333	<0.001***	0.774

3.6 APPENDICES

Appendix 1: **Seed Treatments**

Table depicting the seed treatments, including concentrated sulfuric acid soak time (Acid Soak Time) and razor blade rupturing of the hilum (Razor Blade Knicking), and percent seed imbibitions for *L. bicolor* and *L. nanus*. We used ten seeds from each species for each trial. The fourth trial, which incorporated razor blade scratching with a 40 minute concentrated sulfuric acid soak time, resulted in the highest percentage of imbibed seeds after 3 days, so we used this method to germinate our reciprocal transplant seeds.

Trial #	Acid Soak Time	Razor Blade Knicking	*L. bicolor* % Imbibition	*L. nanus* % Imbibition
1	20 min	none	10%	0%
2	30min	none	30%	20%
3	40 min	none	20%	20%
4	40 min	hilum scratch	90%	70%

Appendix 2: **Seed Germination**

The low seed germination we observed at our sites is not unusual for seeds with hard seed coats that have not been scarified (Grime et al. 1981, Bewley and Black 1994). The family Fabaceae, in particular, is known to need specific germination clues including temperature fluctuations (Roberts and Boddrell 1985, Van Assche et al. 2003), heat shock (Keeley 1991, Probert 2000, Maret and Wilson 2005), cold stratification, and physical scarification (Baskin and Baskin 2001, Kaye and Kuykendall 2001).

Because we stored *Lupinus* seeds at a constant temperature with low exposure to elements that could wear down the seed coat it is likely that they were not exposed to the necessary germination cues that are present in the natural environment. However, the specific germination cues required by plants in the genus *Lupinus* remain poorly understood (Elliott et al. 2011).

Future studies should examine mechanisms that break *Lupinus* seed germination, because identifying factors that improve the germination of ecologically important native plants can improve the efficacy of restoration attempts (Primack 1996, Seabloom et al. 2003, Corbin and D'Antonio 2004, MacDougall and Turkington 2006). One tactic that may increase germination of untreated seeds is planting in the mid to late summer, when *L. nanus* and *L. bicolor* naturally set seed. This would allow seeds to experience natural temperature fluctuations and seed coat abrasion. However, post-dispersal seed predators may reduce seed numbers as a function of time. Few studies, if any, have examined the effects of seed sew timing on germination rates versus seed decrease due to natural predators, so we recommend future investigations into this area.

Appendix 3: **Fitness Averages and Standard Deviations**

Table showing *L. nanus* and *L. bicolor* number of seeds produced per seedling planted (Seed #), first month survival (1^{st} Mo. Survival), survival to flowering (Flower Survival), and flowers per plant (Flowers/Plant) averages and standard deviations for each seed source population (Seed) at each planting site (Soil). Site abbreviations are described in Figure 2.

Soil	Seed	Species	Seed # Average	Seed # SD	% 1^{st} Month Survival Average	% 1^{st} Month Survival SD	% Flower Survival Average	% Flower Survival SD	Flowers/Plant Average	Flowers/Plant SD
FO	FO	*L. bicolor*	4.82	8.44	66	47.61	53	50.16	4.25	5.45
FO	FO	*L. nanus*	7.20	19.82	51	48.24	41	50.16	13.46	45.47
FO	PW	*L. bicolor*	9.36	9.10	84	36.85	72	45.13	7.22	5.98
FO	PW	*L. nanus*	11.26	13.64	81	35.89	60	44.62	30.50	31.03
FO	SV	*L. bicolor*	7.38	10.47	80	40.20	61	49.02	5.96	6.17
FO	SV	*L. nanus*	2.73	8.66	40	49.24	23	44.08	6.27	13.07
PW	FO	*L. bicolor*	4.70	9.37	64	48.24	48	50.21	4.83	6.62
PW	FO	*L. nanus*	2.58	11.88	59	46.06	36	49.60	7.34	13.38
PW	PW	*L. bicolor*	3.96	7.01	80	40.20	54	50.09	5.07	6.20
PW	PW	*L. nanus*	14.86	15.18	81	43.80	80	30.15	48.08	48.06
PW	SV	*L. bicolor*	4.28	8.48	76	42.92	49	50.24	5.48	8.20
PW	SV	*L. nanus*	1.64	6.34	42	49.89	18	39.43	6.88	18.02
SV	FO	*L. bicolor*	2.24	10.12	72	45.13	32	46.88	3.23	5.70
SV	FO	*L. nanus*	1.10	6.64	48	49.76	17	32.66	4.45	4.43
SV	PW	*L. bicolor*	7.64	13.95	81	39.43	58	49.60	8.99	13.28
SV	PW	*L. nanus*	3.40	5.71	61	46.48	51	50.21	10.03	11.21
SV	SV	*L. bicolor*	3.04	8.66	41	49.43	29	45.60	3.74	7.37
SV	SV	*L. nanus*	22.97	22.12	80	25.64	80	34.87	29.79	22.84

Chapter 4. Effect of mating system on phenotypic variance-covariance matrix similarities across sites in two species of *Lupinus*

4.1 INTRODUCTION

Plant mating systems are one of the key factors affecting the evolutionary trajectory of plant floral and vegetative structures (Fenster et al. 2004, Hargreaves et al. 2004). In particular, the split between selective forces on groups of plants with specialized pollinator relationships versus those with generalized pollination strategies has been cited as being responsible for current patterns of floral and vegetative traits (Berg 1960, Waser et al. 1996, Fenster et al. 2004, Hargreaves et al. 2004). Because plants with specialized pollination syndromes have flowers that need to be visited by a specific group of animal vectors for effective fertilization, many of these plants have zygomorphic flowers with floral structures that protect floral rewards such as nectar and pollen from being accessed by pollinators that carry pollen from other species or deposit pollen ineffectively. Plants with generalized pollination syndromes, on the other hand, are self-pollinated, pollinated by abiotic vectors such as wind or water, or pollinated by a diverse range of animals and thus do not require a precise match between floral and pollinator architecture.

The selective pressures associated with each of these mating systems can result in mean trait differences among species (Armbruster et al. 1999). However, because selection does not act uni-dimensionally, the covariance structure of plant traits can also be affected (Berg 1960, Antonovics 1976, Waitt and Levin 1993, Campbell et al. 1994, Bennington and McGraw 1995, Nicotra et al. 1997, Murren 2002, Caruso et al. 2003, Gong and Huang 2009, Ramirez-Valiente et al. 2010, Reynolds et al. 2010). Berg (1959, 1960) first pointed out the differences in phenotypic variance-covariance matrices, or P-matrices, between plants with specialized versus generalized pollination systems, hypothesizing that each group experienced distinct selective pressures that would ultimately cause phenotypic correlations among traits to be modified. She noted that plants experiencing specialized pollination are under selection by their pollinators for stable floral structures, which should result in high correlations among functionally related floral traits (Stebbins 1950, Conner and Via 1993, Waitt and Levin 1998, Bissell and Diggle 2010). Vegetative traits, on the other hand, often exhibit large phenotypic variation and are under selection from environmental factors that are less directly associated with floral evolution, such as precipitation and cloud cover. Because maximum plant fitness is achieved by responding to vegetative and floral selection independently, there should be selection for the decoupling of floral traits from vegetative traits. This would allow floral structures to retain a constant architecture that optimizes stigma contact with pollen deposits, while vegetative traits vary with environmental factors.

While plants with specialized pollination are predicted to experience selection for two distinct correlation pleiades (a vegetative trait pleiad composed of loosely correlated vegetative traits and a floral pleiad composed of tightly correlated floral traits), the selection responsible for this decoupling should be absent in plants with generalized pollination syndromes. The latter species do not experience selection for a precise correspondence between pollinator and flower morphology, and should thus have lower integration within floral trait suites and less decoupling between vegetative and floral trait suites (Berg 1960, Stebbins 1974).

Armbruster *et al.* (1999) expanded Berg's Correlation-Pleiades Concept to address specific expected differences between groups of plants with specialized pollination syndromes and those with generalized pollination syndromes. Specifically, they hypothesized that plants with specialized pollinators should have highly integrated floral traits that are decoupled and buffered from vegetative traits. Plants with a generalist pollination system should exhibit the opposite characteristics, with floral traits that are not decoupled from and/or not buffered from vegetative traits, a less tightly correlated floral suite, and similar correlations within vegetative trait suites as floral trait suites.

While these hypotheses have been tested using multiple traits on a variety of species (Meng et al. 2008, Pelabon et al. 2011, Rosas-Guerrero et al. 2011), there have been few studies that extend Berg's theory to multiple populations of a single species (Nattero et al. 2010b, Ceolin and Miotto 2011) . Adding independent populations to these analyses is important, because it allows us to clarify how mating systems affect patterns of phenotypic responses to local selection pressures and test for parallel or divergent evolution.

Examining patterns of floral architecture among populations would allow system specific evolution to be examined. Plants pollinated by specialists, for example, should experience parallel floral evolution across populations, because, if the same groups of specialized animal vectors are present and depended upon for pollination across populations, there should be similar selection on floral architecture among sites (Wolfe and Krstolic 1999). Several studies have documented this pattern of constant floral selection gradients across sites in species pollinated by specialized pollinators (Hansen and Totland 2006, Nattero et al. 2010a). Because selection can affect the architecture of variance-covariance matrices, parallel selection on relationships among floral traits should result in floral phenotype matrix similarities among populations of plants with specialized pollination systems (Roff and Mousseau 2005).

Vegetative traits of plants with specialized pollination systems, however, should differ among populations because they are subject to selective pressures from site-specific environmental factors such as precipitation amount, temperature, and herbivore presence, and thus should experience selection gradients that vary among sites with their habitat. Because vegetative structures are predicted to be decoupled from floral traits in plants pollinated by specialists, they should be able to adapt to the local environment independently from floral traits, resulting in differing vegetative phenotype relationships and values among sites (Berg 1960).

Plants with a generalized pollination system, on the other hand, would not experience floral selection from identical pollinators across sites. This is especially true with self-pollinating species, as these plants are freed entirely from the selective pressures imposed by pollinators. While plants with specialized pollinators are under selection for highly integrated floral characters, the selective pressures imposed by pollinators are absent in selfing plants. Therefore, selfing populations would not exhibit the consistent high within-site floral integration that outcrossing plants experience. This removal of pollinator selective pressures maintaining patterns of high floral integration could result in variable levels of intrapopulation integration in selfing populations, depending on population-specific costs to integration (Anderson and Busch 2006). Therefore, selfing plants could exhibit greater population differences in both floral trait values and covariance matrices among selfing populations.

In addition to these mating-system-specific hypotheses about interpopulation evolution within species, intrapopulation differences and similarities between plants with opposing mating systems should be tested. For example, if selfing and specialized outcrossing species co-occur, the vegetative structures of both species could experience similar intra-site environmental conditions, which may result in similar vegetative trait variance-covariance matrices between species within a site.

Floral structures, on the other hand, would experience different selective forces between species within a site, since specific pollinators would be exerting selection on the outcrossing species but not the selfing species. This would lead to different floral relationships between species within sites.

To compare trait covariance structures among species, the study organisms must be closely related to avoid differences in responses to selection due to phylogeny. If the two species under comparison have differing ancestral states the dissimilarities of vegetative or floral architecture could be due to those ancestral differences rather than divergent evolution. Similarly, genotype by environment interaction is common within species, and could be amplified among distant relatives, resulting in different evolutionary responses to shared selection pressures rather than parallel evolution (Lynch and Walsh 1998).

Lupinus nanus and its selfing congener, *L. bicolor*, are ideal species in which to examine spatial relationships among vegetative and floral trait matrices. Not only are these co-occurring species closely related, but they also occur sympatrically throughout California, allowing for comparisons to be made between species within sites, in addition to within species among sites. Both species have zygomorphic flowers, but, while *L. bicolor* exhibits high levels of self-pollination, *L. nanus* has a specialized pollination syndrome, as it is pollinated primarily by bees and syrphid flies with sufficient flight control and mass to access the floral rewards (Dunn 1956, Horovitz and Harding 1972, Barbour et al. 1973, Karoly 1992, Cordoba and Cocucci 2011).

This study uses six *L. nanus* and *L. bicolor* populations to investigate the following four hypotheses: Because both species share the same environments at each site and therefore experience the same patterns of selection pressure on their foliage, the two species should 1) share vegetative covariance structures within a site (Fig. 1a) and 2) exhibit between-site differences in those characters (Fig1b). Additionally, because *L. bicolor* and *L. nanus* experience different floral selection pressures, their floral trait covariance matrices should 3) differ between species within sites (Fig 1c). Outcrossing *L. nanus* depends on specific pollinators at all sites, so 4) it should share floral trait structures across sites (Fig 1d). In contrast, because *L. bicolor* experiences no selection pressures from pollinators, it should be released from parallel selection, and therefore its floral architecture might vary between sites either due to correlated responses to selection on vegetative characters or to neutral evolution. We compare variance-covariance matrices so that we can examine differences among sites and between species on the relationships among these traits. We also examine the correlation matrices between species to see if *L. nanus* and *L. bicolor* adhere to Berg's theory of correlation plieades, which predicts that *L. nanus*, as a specialized outcrosser, has higher floral integration and lower correlation among floral and vegetative traits than *L. bicolor*. Additionally, we compare individual trait differences and similarities among sites and between species to examine how mating system affects mean trait values.

4.2 METHODS

Study System and Measurements

L. nanus is an outcrossing annual legume that grows in north coastal scrub, coastal sage scrub, foothill woodland, coastal prairie, and valley grassland communities in California, Nevada, and Oregon. *L. bicolor* is closely related to *L. nanus*, but is highly selfing (Elliot et al. 1974, Harding et al. 1974, Shade 2012), and has a wider range than *L. nanus*, occurring from Arizona to British Columbia with habitats that also include closed-cone pine, Douglas fir, yellow pine, red fir, lodgepole pine, and mixed evergreen woodlands (Baldwin et al. 2012). Bees have been documented as the main pollinators of *L. nanus*, but *L. bicolor* flowers are rarely visited by insects (Dunn 1956).

L. nanus and *L. bicolor* have palmately compound leaves with stipules and whorls of pea-shaped flowers on erect spikes. The zygomorphic flowers are composed of 5 modified petals: the upper banner, two lateral wings, and two lower petals that are fused to form the keel (Baldwin et al. 2012). The flowers of *L. nanus* and *L. bicolor* have similar structure and coloration, but those of *L. nanus* are larger than those of *L. bicolor* (Baldwin et al. 2012).

We selected six sympatric populations of *L. nanus* and *L. bicolor* growing between 4km and 665km apart (Fig. 2). The three inland sites, Dye Creek Reserve (DC), Canyon Head (CH), and Tejon Ranch (TR), have higher temperatures and less cloud cover than the coastal populations, Pepperwood Preserve (PW), Sibley Volcanic Regional Park (SV), and Fort Ord (FO).

One hundred *L. nanus* and *L. bicolor* plants were selected at random in each site between March and April of 2009. We selected plants by generating two sets of random numbers between zero and 100: one set of x coordinates and one set of y coordinates. We laid out two 100m measuring tapes orthogonally along the population, and sampled individuals at the x and y locations from our list. Vegetative traits (leaflet length, leaflet width, and stipule length) and floral traits (pedicel length, banner height, wing length, lower sepal length, and wing height) were measured and averaged on two flowers from each plant from the lower whorl on their first date of anthesis (Fig. 3).

Trait Analyses and Correlations

We examined which traits played a significant role in population differentiation by performing Pillai's Multiple Analysis of Variance (MANOVA) on all eight morphological traits with a fixed effects model that included site, species, and the site by species interaction as independent variables (Scheiner and Gurevitch 2001). We used the Pillai-Bartlett V criterion as a multivariate test statistic for our MANOVAs, because it has been found to be more robust than other multivariate test statistics (Olson 1976, Stevens 1980).

We then performed Tukey comparison tests to examine differences in interspecies vegetative and floral trait means among sites and between species. We used Bonferroni corrections to adjust for multiple testing (Dunn and Massey 1965). Because floral and vegetative traits have different size scales (the vegetative traits we measured were generally larger than floral traits), we standardized our measurements across sites using z-scores for analyses that used both floral and vegetative variables.

To examine integration within floral and vegetative suites of traits and levels of decoupling between vegetative and floral trait suites, we examined 1) species-specific correlation matrices, 2) relative eigenvalue standard deviation, and 3) canonical correlations between vegetative and floral trait groups.

First, we used species-specific correlation matrices to examine overall patterns of integration within floral and vegetative trait suites and decoupling between these two functional groups. We calculated Pearson's correlations for each site and averaged correlations by speices, using Steiger's Z-test to calculate the significance of the correlation values. This statistic determines how unlikely it is that the given correlation coefficient would occur between the two variables given that the variables are unrelated.

Second, we used relative eigenvalue standard deviations to examine site and species-specific morphological integration of floral and vegetative traits to test Berg's hypothesis that a) specialized outcrossers have greater floral integration than generalists, and b) floral traits of specialized outcrossers are more integrated than their vegetative traits, while generalist plants have floral traits that are integrated at a similar level as their vegetative traits (Pavlicev et al. 2009). Eigenvalue variability is used to examine the integration of trait suites because when traits are highly correlated the independent variance is concentrated in the first few eigenvalues rather than spread evenly through all the eigenvalues (Wagner 1984, Cheverud et al. 1989). This means that if phenotypic units are highly integrated they will have high eigenvalue variance, while traits with low integration will have low eigenvalue variance. However, because eigenvalue variance depends on the number of eigenvalues, in addition to the integration of traits, the variance must be standardized so that comparisons among phenotypic units comprised of different numbers of traits can be made. Unfortunately, eigenvalue variance does not scale linearly with the mean correlation of a matrix, but rather the square of the mean correlation. The standard deviation of the eigenvalues, on the other hand, does scale linearly with the correlation. Therefore, using the standardized eigenvalue standard deviation is a good estimation of overall morphological integration.

Third, we performed a site and species-specific canonical correlation analysis (CCA) to examine the levels of decoupling between floral and vegetative trait suites and thus determine whether our data supported Berg's hypothesis that species with specialized pollination systems would have more decoupling of vegetative and floral traits from each other and a more highly integrated floral trait suite than plants with generalist pollination strategies. CCA is a way of examining relationships between two pre-defined groups of multidimensional variables while maximizing the inter-group correlation. In this case, we defined a vegetative group consisting of leaflet length, leaflet width, and stipule length and a floral group consisting of pedicel length, banner height, wing length, sepal length, and wing height. We calculated the correlation between the first major axes for the two groups as well as the proportion of variance accounted for by the set of floral and vegetative axes used for the correlation calculation. We also used the F statistic based on Rao's approximation to test the statistical significance of the canonical dimensions (Bartlett 1941). All CCA calculations were performed in R, using the R package 'calibrate' (Graffelman 2010).

Principal Component Analyses and Comparisons

To compare site- and species-specific variance-covariance matrices we used the hierarchy method of Principal Component Comparisons (PCC) described by Phillips and Arnold (1999). This allowed us to test our hypotheses that 1) interspecies vegetative covariance structure would be similar within sites (Fig. 1a), 2) intraspecies vegetative covariance structure would differ among sites (Fig 1b), 3) interspecies floral covariance structure would differ within sites (Fig 1c), and 4) intraspecies floral covariance structure would be similar among sites for *L. nanus* but not *L. bicolor* (Fig 1d). This approach uses hierarchical comparisons developed by Flury (1988) to test a variety of hypotheses addressing different levels of matrix similarities. Flurry introduced the idea that there are complex relationships between matrices ranging from a) Equality, where matrices share eigenvectors and eigenvalues, b) Proportionality, where matrices share identical eigenvectors, but have eigenvalues that differ by a proportional constant, c) Complete Common Principal Components (CPC), where all eigenvectors are shared but eigenvalues differ, d) Partial Principal Components (PCPC), where any number of eigenvalues are shared up to p-2 (where matrices have dimensions of pxp), to e) Unrelated structures (Flury 1988). The reason PCPC matrices can only reach p-2 shared principal components is because, since principal component axes are orthogonal in nature, once p-1 axes have been defined the last axis is already determined.

Flury hierarchical PCC is a process that compares matrices, starting with the null hypothesis that matrices are unrelated. If that hypothesis is rejected, matrices are again compared to inspect similarities at the next hierarchical level, continuing to equality (Phillips and Arnold 1999). We used the model building approach to matrix comparisons, using Akaike information criterion (AIC) to determine which hierarchical level best fits the model. Because this model does not provide a statistical test, we compared it to other model testing approaches to search for inconsistencies, such as the step-up approach, in which relationships between matrices are tested in a stepwise fashion, and the jump-up approach, in which matrix relationships are tested directly against unrelated structure.

We performed PCC's individually on floral and vegetative traits to test the hypotheses that *L. bicolor* should have fewer similarities among sites in floral traits than *L. nanus*, and vegetative traits should be more similar between species than among sites. We measured fewer vegetative traits than floral traits, so we only report equality, proportionality, CPCP, and unrelated structure rather than also reporting the exact number of shared principal components between matrices. Because size scales within functional groups of traits were similar, all PPC's were performed on covariance matrices rather than correlation matrices.

4.3 RESULTS

Trait Comparisons

Pillai's MANOVA using vegetative and floral traits as dependent variables showed significant differences among sites, species, and a site by species interaction (Table 1). All eight traits contributed significantly to the interaction term. Leaflet length did not contribute to the between-species differences (p=0.144), but because this trait did contribute significantly to the interaction terms we must conclude that it is an important factor in the model (Table 2). Floral trait means for *L. bicolor* populations were consistently smaller than those in *L. nanus* populations and all species and site-specific floral trait means differed significantly from one another. However, as described by the significant site by species interactions, the level of differentiation between species varied among sites (Fig. 4). Vegetative trait means had more overlap between species than floral trait means, but all intraspecies trait means differed among sites and all intrasite trait means differed significantly between species, except leaf length at Sibley (p=0.090), and leaf length and leaf width at Tejon Ranch (p=0.132).

Correlations

The correlation matrix averaged across sites for *L. bicolor* showed a decoupling between vegetative traits and floral traits, as no vegetative traits were significantly correlated with floral traits (Table 3). Decoupling of vegetative and floral suites of traits was also evident in *L. nanus*, but this species exhibited fewer significant correlations within functional groups (Table 4). Additionally, floral integration was not higher than vegetative integration for either species, as leaflet length and leaflet width had consistently high significant correlations.

The relative eigenvalue standard deviations showed that all *L. nanus* and *L. bicolor* populations had similar overall levels of integration, except at the Sibley and Fort Ord sites (Fig. 5a). At Sibley *L. bicolor* had more overall integration than *L. nanus*, and at Fort Ord *L. bicolor* had much less overall integration than *L. nanus*. These patterns seem to be driven by floral and vegetative integration levels as well as inter-group associations, because higher *L. bicolor* than *L. nanus* integration at Sibley and lower *L. bicolor* than *L. nanus* integration at Fort Ord was consistent in both the vegetative and floral analyses. Floral integration tended to be higher in *L. nanus* populations than *L. bicolor* populations except at Pepperwood and Sibley (Fig. 5b). Additionally, *L. bicolor* showed more variation in floral integration levels than *L. nanus*. The ranges of relative eigenvalue standard deviations examining vegetative integration, on the other hand, were nearly equal for *L. nanus* and *L. bicolor* (Fig. 5c). Most sites had similar levels of vegetative integration between species, except Sibley and Fort Ord, which followed the patterns described above in the overall integration analysis. Floral integration was not higher for *L. bicolor* than *L. nanus*. Additionally, floral integration was not consistently higher than vegetative integration for either species (Fig. 6).

Canonical correlations (Table 5) did not support the hypothesis that *L. bicolor* would have higher correlations, and thus less decoupling, between vegetative and floral suites than *L. nanus*. Individual population comparisons showed slightly higher intra-group correlations for *L. bicolor* than *L. nanus* for four of the six populations, but neither of these patterns were significant (p=0.556).

Principal Component Comparisons

Principal Component Comparisons (PCC) on floral traits showed more similarities among sites in *L. nanus* than *L. bicolor* (Fig. 7). For *L. bicolor* there were five pairs of sites with PCA similarities: FO-CH, DC-PW, DC-TR, SV-PW, and DC-CH. The DC-CH pair of sites had proportional PCAs, while the other pairs had shared principal components. There were nine site pairs with PCA similarities within *L. nanus*. SV-DC had proportional PCAs, and SV-FO, FO-DC, SV-PW, DC-CH, FO-CH, DC-PW, SV-CH, and PW-CH shared principal components. There are also more inter-species similarities in the floral trait PCAs than the PCAs calculated with both vegetative and floral traits. All sites showed PCA similarities between species except TR. *L. nanus* and *L. bicolor* had proportional PCAs at DC and CH, and shared principal components at FO, SV, and PW.

Comparisons among sites and species for PCAs performed on vegetative traits show few similarities among sites (Fig 8). *L. bicolor* had five site pairs with PCA similarities. FO-DC and CH-TR shared principal components, SV-FO and SV-DC were proportional, and PW-SV had identical PCAs. *L. nanus* only showed PCA similarities between PW-FO, which had identical PCAs. Half of the species comparisons showed common principal components in between-species comparisons for vegetative traits. *L. nanus* and *L. bicolor* had proportional vegetative PCAs at TR, and shared principal components at FO and PW.

The model building approach to Principal Component Comparisons that we used to determine relationships among variance-covariance matrices chose models that were in agreement with the significant models chosen by the step-up and jump-up approach, so these results are based on PCC relationships with the lowest AIC values.

4.4 DISCUSSION

Trait Pleiades

Berg hypothesized that a) plants with specialized pollination syndromes should have higher floral integration than plants with general pollination syndromes and b) generalist plants should have similar levels of within group floral and vegetative integration, while specialized outcrossers have higher levels of floral integration than vegetative integration (Berg 1960). Because outcrossing plants with zygomorphic flowers are considered to have specialized pollination syndromes while self-pollinating plants are considered to have generalized pollination syndromes (Berg 1960, Wolfe and Krstolic 1999, Ushimaru et al. 2006) we predicted that the outcrossing *L. nanus* would have higher floral integration than the selfing species *L. bicolor*, and that floral integration would be higher than vegetative integration within *L. nanus*, while *L. bicolor* floral and vegetative integration would be equal.

Our *L. nanus* and *L. bicolor* populations did not support these hypotheses, as we did not find consistently higher floral integration in the outcrosser, *L. nanus* than the selfer, *L. bicolor*. While floral integration was relatively constant in *L. nanus*, *L. bicolor* showed high variation in integration levels among populations. This could suggest that while *L. nanus* has consistent selection toward a common level of floral integration among all sites these selection pressures have been removed from *L. bicolor*, allowing floral integration levels to vary without fitness consequences. This could only occur, however, if the maintenance of floral integration was not associated with a trade-off cost, because if maintaining integrated floral characters was costly then integration should decrease once selection relaxed. It is also possible that the large variation in *L. bicolor* floral integration levels was caused by neutral evolutionary processes, such as genetic drift. Because *L. bicolor* populations are selfing they have low effective population sizes, and are therefore at risk for random fixation of genes (Shade 2012). Thus, high interpopulation variation of floral integration could be the result of genetic drift rather than selective forces.

While across-site variation in floral integration varied between species, high correlation between wing length and banner height was consistent for both *L. nanus* and *L. bicolor*. The fact that these two characters are tightly correlated in both species suggests that this relationship may be maintained more by genetic constraints than selection. While a strong correlation between banner height and wing length may be selectively important for *L. nanus* if pollinators are more attracted to a certain banner to wing ratio, this selection pressure should be absent from *L. bicolor*, so maintenance of this relationship could be indicative of linked traits or genetic fixation of trait correlations.

In addition to not finding higher floral integration for *L. nanus* than *L. bicolor*, we also did not find a higher floral to vegetative integration ratio in *L. nanus* than *L. bicolor*. Overall correlations among vegetative traits were as high as those among floral traits, and correlations among leaflet length and leaflet width were especially high for both species. Within-site integration levels also did not support this part of Berg's hypothesis, as relationships between floral and vegetative integration levels were inconsistent for both species.

Another tenet of Berg's hypothesis is that plants with generalized pollination systems, such as *L. bicolor*, should have less decoupling between floral and vegetative suites of traits than plants with specialized pollination systems, such as *L. nanus*. However, we found decoupling in both *L. nanus* and *L. bicolor*. The similarities in decoupling between *L. nanus* and *L. bicolor* could be due to several factors. For instance, it is possible that because *L. nanus* and *L. bicolor* are closely related *L. bicolor* retains some of the genetic architecture of *L. nanus*. If selection against decoupling is weak or there are genetic constraints preventing re-integration among the two suites of traits *L. bicolor* would maintain similar correlations among floral and vegetative traits as *L. nanus* (Dorn and Mitchell-Olds 1991, Ashman and Majectic 2006, Bissell and Diggle 2008).

It is also possible, however, that selfing species do not fit the generalized pollination strategy model. Although self-pollinating species do not experience sexual selection from pollinators they may still experience selection for a flower architecture that facilitates fertilization. For instance, if a particular flower shape and size minimizes floral expenditures while maximizing the likelihood of stigma-anther contact then there should be strong selection toward this optimal flower shape and size. This idea has recently been examined by Perez *et al.* (2007) and Rosas-Guerrero *et al.* (2011) who found that self-compatible flowers often have floral integration that is as high as or higher than their self-incompatible relatives. This would mean that selfing species should not be categorized as typical generalist pollination system plants, because they experience floral selection for specific flower shapes to maximize pollination success, rather than reduced selection for precise floral architecture.

Vegetative Traits within Sites

Because the vegetative traits of *L. nanus* and *L. bicolor* are exposed to similar environmental selective pressures we hypothesized that species would be similar within sites (Fig 1a). However, our data did not support this hypothesis, as we found within-site species differentiation in vegetative covariance structures for half of the sites we observed.

The lack of support we found for our hypothesis that species would have similar within-site covariance matrix structures could be due to environment by species effects. If the same environmental factors affect *L. nanus* and *L. bicolor* differently then selective pressures and responses to these pressures could differ between species. These types of complex differences in responses to environmental factors among species have been documented among close relatives as well as within species, so it is possible that sites in our study not exhibiting vegetative similarities between species are experiencing environment by species interactions (Travis 1994, Sultan 2001, Eckhart et al. 2004).

It is also possible that, while *L. nanus* and *L. bicolor* experience similar selective pressures on vegetative traits from their shared environment, *L. bicolor* does not have enough genetic variation to respond to these selective pressures. Because selfing populations have a history of inbreeding and multiple extinction and recolonization events, neutral evolutionary processes might overpower responses to selection (Takebayashi and Morrell 2001, Dudash et al. 2005). In our study this would mean that, while both *L. nanus* and *L. bicolor* may be experiencing identical environmental selection gradients toward a specific leaf size or shape, only *L. nanus* has enough heritable variance in vegetative traits to respond to this selection. The changes over time in *L. bicolor* foliage would be dominated by neutral evolutionary processes such as genetic drift, leading to differences in vegetative structures between species within sites.

Vegetative Traits among Sites

Because among-site environmental selective pressures on vegetative traits differ, we hypothesized that there should be few similarities in vegetative trait covariance matrices among sites, even within species (Fig 1b). Our data partially supported this hypothesis, showing few vegetative variance-covariance matrix similarities among sites. However, we did find some evidence of matrix similarities between sites. For instance, *L. nanus* Principal Component Comparisons between PW and FO showed identical matrix structures. This means that the relationships among vegetative traits are identical at these two sites, and the variance accounted for by each of these traits is equal. *L. bicolor* sites had a greater number of similarities among sites, as SV-PW had identical principal component structure, SV-FO and SV-DC had proportional matrix relationships, and FO-DC and TR-CH had shared principal components.

It is possible that the higher number of phenotypic matrix similarities among sites in *L. bicolor* than *L. nanus* is due to ancestral variance-covariance matrix constraints. *L. bicolor* has high rates of genetic drift due to its selfing nature, so it is possible that at some point in the past the genetic architecture of the relationships between vegetative traits drifted to fixation in one of the populations of *L. bicolor*. As this population spread it would mean that while vegetative trait means were still able to vary among sites in response to evolutionary processes and plastic responses to environmental factors, the relationships among these traits would remain stable.

Floral Traits within Sites

L. bicolor and *L. nanus* experience different sexual selection pressures on floral traits, so we predicted that floral traits between these two species should differ within sites (Fig 1c). However, our data did not support this hypothesis, as five out of our six study sites had similarities in variance-covariance matrices between species. *L. nanus* and *L. bicolor* shared principal components at FO, SV, and PW, meaning that the variance on one of their floral principal component axes was explained by the same traits. At DC and CH floral matrices between species were proportional, meaning that not only do they share principal component axes, but the total amount of variation explained by each axis differed by a constant variable. Biologically this means that while the size and shapes of the flowers may differ between species the specific floral traits that are responsible for the range in intrapopulational flower differences are constant, and that the total amount of variation explained by each orthogonal combination of traits differs by a constant variable.

While floral covariance matrices were shared between species at most of our sites, we found that floral trait means between species differed at all sites. One possibility that would account for the differences in floral trait means among species but similarities in covariance structure is that floral trait relationships are genetically constrained from changing in these two closely related species. This would mean that while mean trait sizes are able to respond to selection pressures the structures of the relationships among this floral module would be genetically confined. An example of these constraints in floral modules was described in *Nicotiana* across the species *Nicotiana alata* and *N. forgetiana* (Bissell and Diggle 2008, Bissell and Diggle 2010). Like *L.* nanus and *L. bicolor* these two species have very different floral trait means, but the relationships among pollinator attraction modules were conserved. In 2010 Bissell and Diggle confirmed the genetic basis for these similarities, supporting the theory that floral modules are genetically constrained.

However, because we found few similarities in floral trait variance-covariance matrices among sites (see the following section), the genetic constraint explanation for matrix structure conservation between species has potential implications about the phylogenetic relationships of *L. nanus* and *L. bicolor* populations. Specifically, if genetic constraints are responsible for similarities between species within a site, but these genetic constraints have broken down within species among sites it would mean that *L. bicolor* and *L. nanus* populations growing sympatrically may be more closely related than within-species populations growing at different locations. This could be possible if the ancestor species to *L. nanus* and *L. bicolor* spread through California and populations subsequently split into an outcrossing variety (*L. nanus*) and selfing variety (*L. bicolor*), rather than a single ancestral split followed by multiple independent colonization events.

While both nuclear and chloroplast regions support the monophyly of *Lupinus* (Ainouche and Bayer 1999, Ainouche et al. 2004, Cronk 2006) species delineation within this genus remains unclear (Kass and Wink 1997, Drummond 2008). Drummond (2008), for instance, used two populations of *L. bicolor* and *L. nanus* in his analysis, and found one population of *L. bicolor* to be more closely related to the two populations of *L. nanus* than to the other population of *L. bicolor*. The phylogenetic relationships of *L. nanus* and *L. bicolor* populations must be further investigated to determine if genetic constraints among species within sites can explain the similarities we found in floral variance-covariance matrices structures.

Floral Traits among Sites

Because *L. nanus* experiences sexual selective pressures on floral traits from specific insects at each site, we hypothesized that *L. nanus* populations would have similar floral phenotypes across sites (Fig 1d). Because *L. bicolor* plants are not visited by pollinators (Dunn 1956) these selective pressures are absent from *L. bicolor* populations, so we predicted floral phenotypic differences across sites for *L. bicolor*.

We only found weak support for these hypotheses from our data, however. While we did find more matrix similarities among *L. nanus* populations than *L. bicolor* populations, these patterns are not strong enough to be conclusive. Among *L. bicolor* populations our PCCs found proportional similarities between DC-CH and shared principal components between SV-PW, PW-DC, DC-TR, and FO-CH. The proportional similarities between CH and DC could be due to the proximity of these sites. Because CH and DC are located only 3.8 km apart the *L. bicolor* populations at these sites may be recently split. It is possible that while the trait means were able to diverge quickly due to genetic drift or responses to environmental differences the genetic architecture of the relationships between these traits has not had time to diverge. Genetic constraints could also explain the variance-covariance similarities among the other four population pairs, if SV-PW, PW-DC, DC-TR, and FO-CH are more closely related to each other than to populations with which they do not share principal components.

These floral architecture similarities among *L. bicolor* populations could also be explained by parallel evolutionary responses to selective pressures, however. As we mentioned earlier, selfing plants may be experiencing selection on floral structure to minimize expenditures on floral displays while maximizing effective pollination. If this is the case, then *L. bicolor* individuals at the five population pairs that share variance-covariance matrix architecture may be responding to these selective pressures in a similar manner. Populations without similarities in matrix architecture could be responding to these selective pressures in a novel way, or they could have such limited within-population genetic variation that they are unable to respond to selection.

Among *L. nanus* populations, while we found floral covariance matrix similarities among nine population pairs, we did not find similarities among six population pairs. These differences in *L. nanus* floral trait matrices could be explained by differences in pollinator composition among sites. While *L. nanus* flowers are almost exclusively visited by bees, the composition of bee species may differ among sites. For instance, *L. nanus* populations growing at sites dominated by large dominated by large *Bombus* species may experience selection for different trait relationships than *L. nanus* populations growing at sites predominantly inhabited by small megachilids such as O*smia* and *Anthidium* species, because different bee species may have differing floral architectural preferences (Horovitz and Harding 1972, Barbour et al. 1973, Karoly 1992).

One noteworthy pattern in *L. nanus* variance-covariance matrix comparisons is the lack of similarities between TR floral matrices and those of other populations. The *L. nanus* population at TR is the only *L. nanus* population with no inter-population floral matrix similarities. Tejon Ranch is the most distant population, with its nearest population (FO) 390.6 km away. If these populations experience isolation by distance (Shade 2012) this could mean that TR is less genetically similar to the other populations than they are to each other. Genetic differentiation could explain the distinctive floral variance-covariance matrix architecture found in this population. Tejon Ranch also experiences the highest summer temperatures and lowest total precipitation, so it is also possible that the novel floral variance-covariance pattern at this site is due to phenotypic interactions with its unique environment.

4.5 FIGURES and TABLES

Figure 1: Diagram depicting different comparisons in covariance matrices between species and across sites. Panel a) describes intrasite comparisons of vegetative traits between species, panel b) describes intrasite comparisons of floral traits between species, panel c) describes intraspecies comparisons of vegetative traits among species, and panel d) describes intraspecies comparisons of floral traits across sites. Comparisons that we hypothesized would result in covariance matrix differences are marked with an asterisk (*).

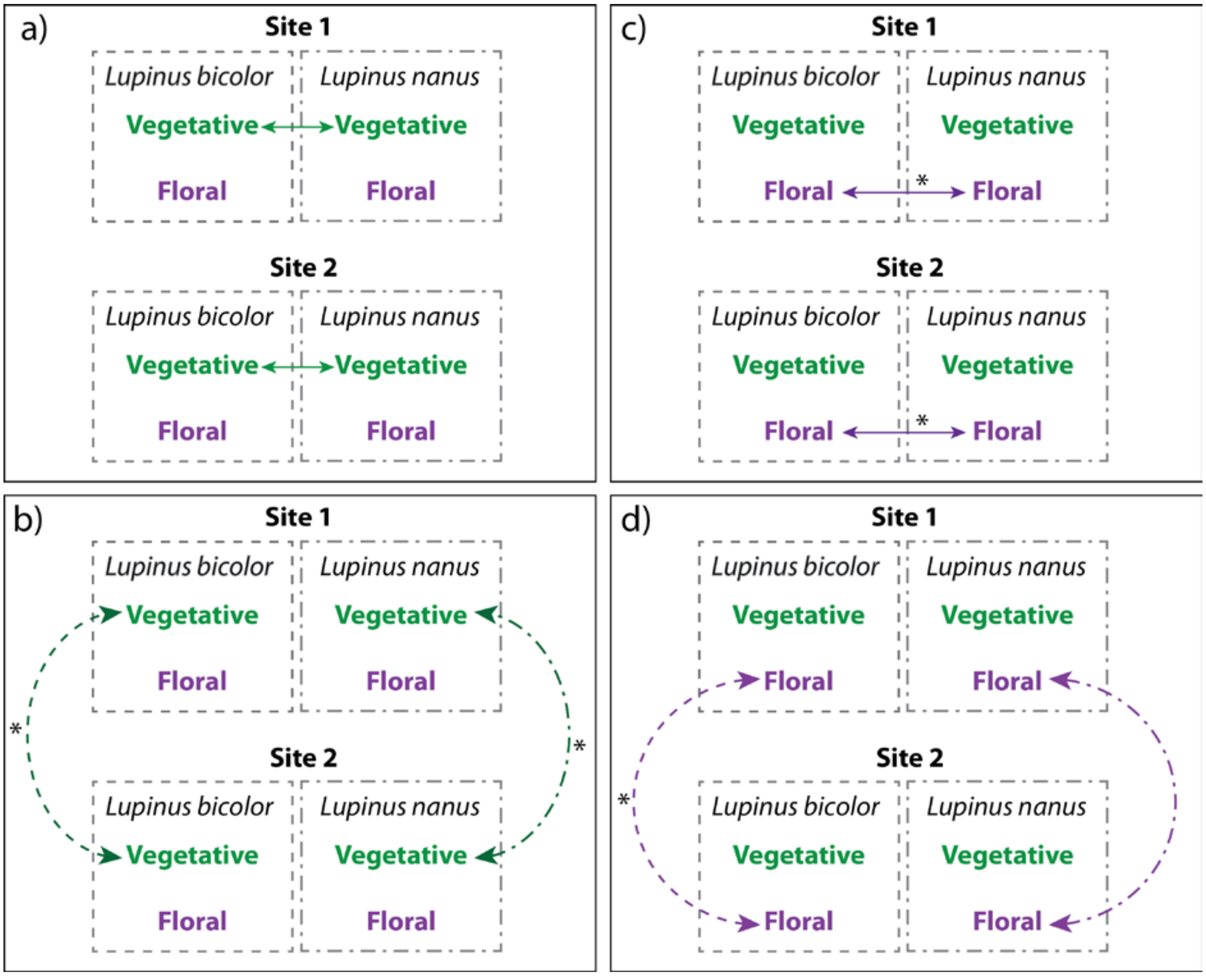
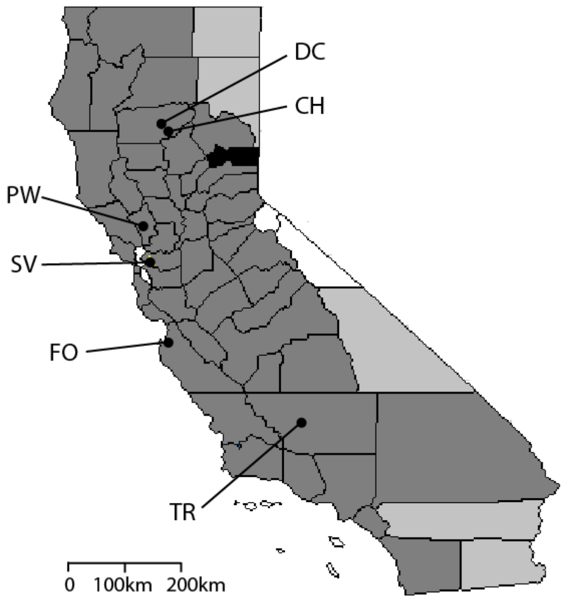


Figure 2: Locations of all ten field sites. Counties where only *L. bicolor* grows are in light gray and the county where *L. nanus* grows alone is in black. The counties in which both species grow sympatrically are in dark gray. The coordinates of the sites and their abbreviations are listed in the table from North to South.



Site Name	Abbreviation	Coordinates
Dye Creek	DC	40° 06' 16"N 122° 02' 38"W
Canyon Head	CH	39° 51' 16"N 122° 12' 01"W
Pepperwood	PW	38° 36' 08"N 122° 44' 07"W
Sibley Volcanic Regional Park	SV	37° 51' 09"N 122° 11' 26"W
Fort Ord	FO	36° 36' 14"N 121° 43' 58"W
Tejon Ranch	TR	35° 12' 34"N 118° 44' 55" W

Figure 3: We measured the following plant traits: A) leaflet width, B) leaflet length, C) stipule length, D) pedicel length, E) banner height, F) wing length, G) wing depth, H) lower sepal length.

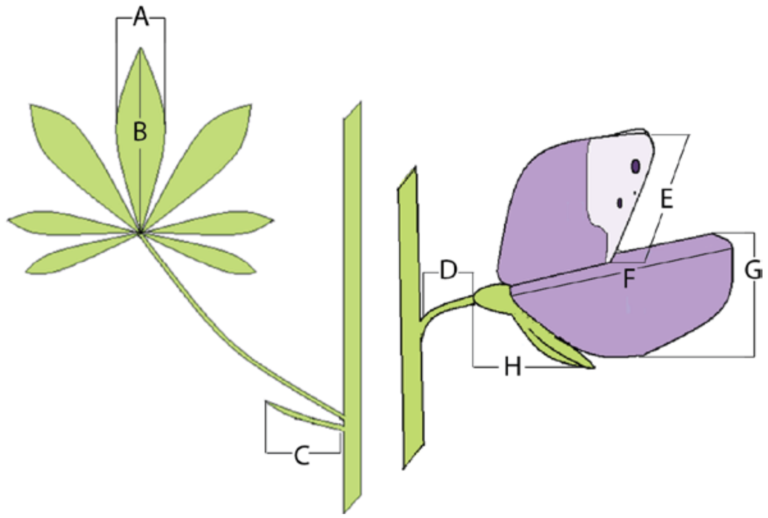


Table 1: Multivariate Analysis of Variance (MANOVA) table showing the degrees of freedom, Pillai's statistic, F-value, and p-value for the MANOVA testing differences among sites and species for vegetative traits and floral traits. Site effect, species effect, and the interaction between site and species were all tested for.
* - $p<0.05$ ** - $p<0.01$ *** - $p<0.001$

	Degrees of freedom	Pillai statistic	Approximate F-value	P-value
Site	5	2.116	125.0	<0.001*
Species	1	0.959	4019.2	<0.001*
SitexSpecies	5	2.128	126.3	<0.001*
Residuals	435			

Table 2: Significance values of the trait's contribution to the Multivariate Analysis of Variance (MANOVA). Each cell shows the p-value showing whether that trait contributed significantly to the site, species, or site by species interaction effects listed in Table 1.
* - $p<0.05$ ** - $p<0.01$ *** - $p<0.001$

	Leaflet Length	Leaflet Width	Stipule Length	Pedicel Length	Banner Height	Wing Length	Sepal Length	Wing Height
Site	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***
Species	0.144	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***
SitexSpecies	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***

Figure 4: Trait means compared among sites and between species. Species are listed on the x-axis, traits are described on the y-axis, and individual populations are indicated by different colors

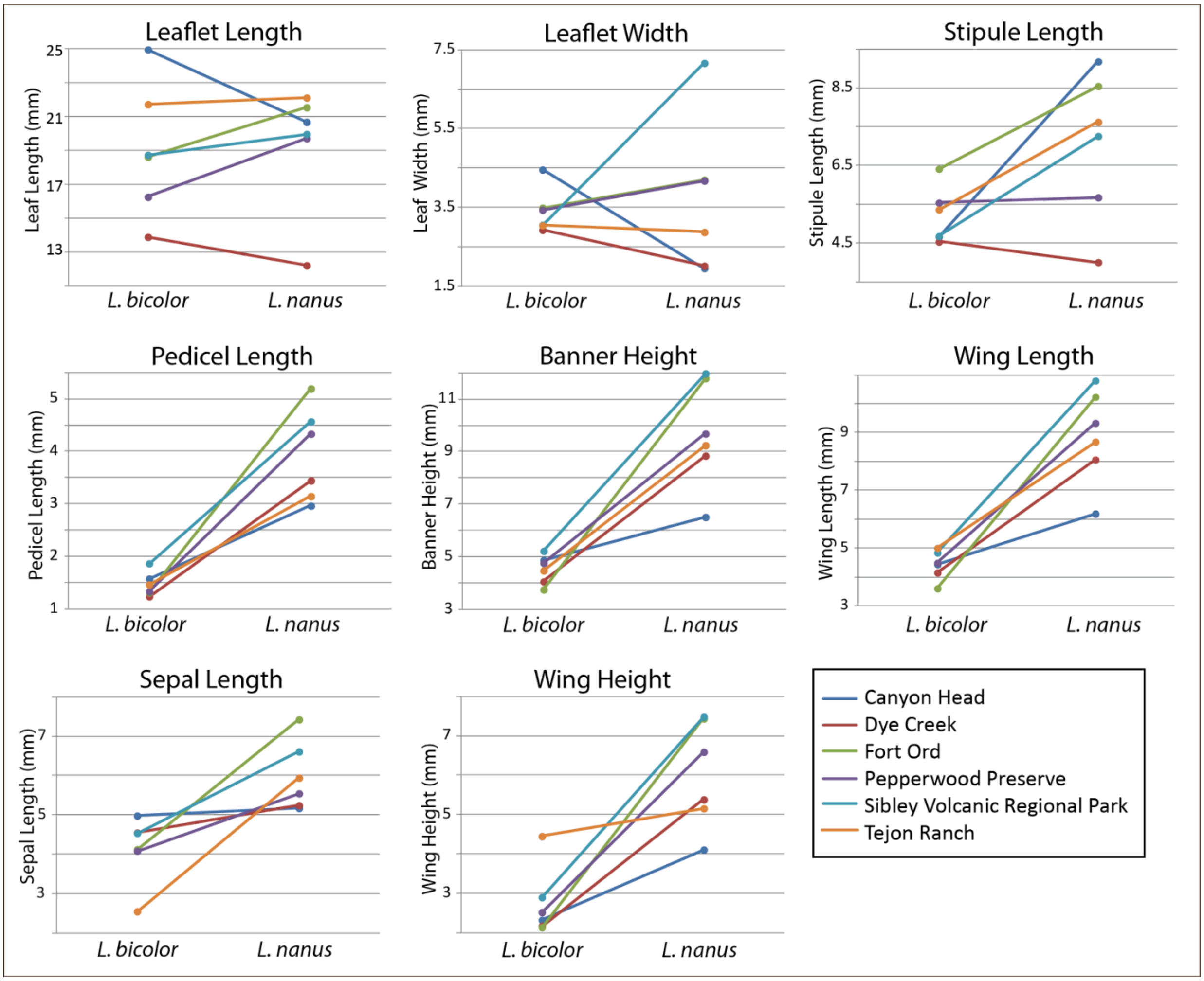


Table 3: Average trait correlation matrix when correlation matrices from individual *L. bicolor* populations are averaged. Traits that have correlations over 0.5 are bolded.
*- $p<0.05$ **- $p<0.01$ ***- $p<0.001$

	Leaflet Length	Leaflet Width	Stipule Length	Pedicel Length	Banner Height	Wing Length	Sepal Length	Wing Height
Leaflet Length	1							
Leaflet Width	**0.652****	1						
Stipule Length	0.375	0.301*	1					
Pedicel Length	0.038	0.032	0.072	1				
Banner Height	0.113	0.137	0.060	0.341**	1			
Wing Length	0.123	0.130	0.068	0.366**	**0.628****	1		
Sepal Length	0.140	0.111	0.156	0.316**	0.443*	0.462***	1	
Wing Height	0.049	0.091	0.021	0.129	0.412***	0.353*	0.190	1

Table 4: Average trait correlation matrix when correlation matrices from individual *L. nanus* populations are averaged. Traits that have correlations over 0.5 are bolded.
*- $p<0.05$ **- $p<0.01$ ***- $p<0.001$

	Leaf Length	Leaf Width	Stipule Length	Pedicel Length	Banner Height	Wing Length	Sepal Length	Wing Height
Leaf Length	1							
Leaf Width	**0.668*****	1						
Stipule Length	0.403	0.289	1					
Pedicel Length	0.061	-0.021	-0.072	1				
Banner Height	-0.014	-0.036	0.034	0.258*	1			
Wing Length	-0.008	-0.040	0.004	0.312	**0.546****	1		
Sepal Length	0.148	0.061	0.106	0.232	0.281	0.325	1	
Wing Height	-0.111	-0.012	-0.057	0.204	0.291	0.316	0.084	1

Figure 5: Relative eigenvalue standard deviations indicating a) overall integration, b) floral integration, and c) vegetative integration. Species are listed on the x-axis, relative eigenvalue standard deviation values are listed on the y-axis, and individual populations are indicated by different colors.

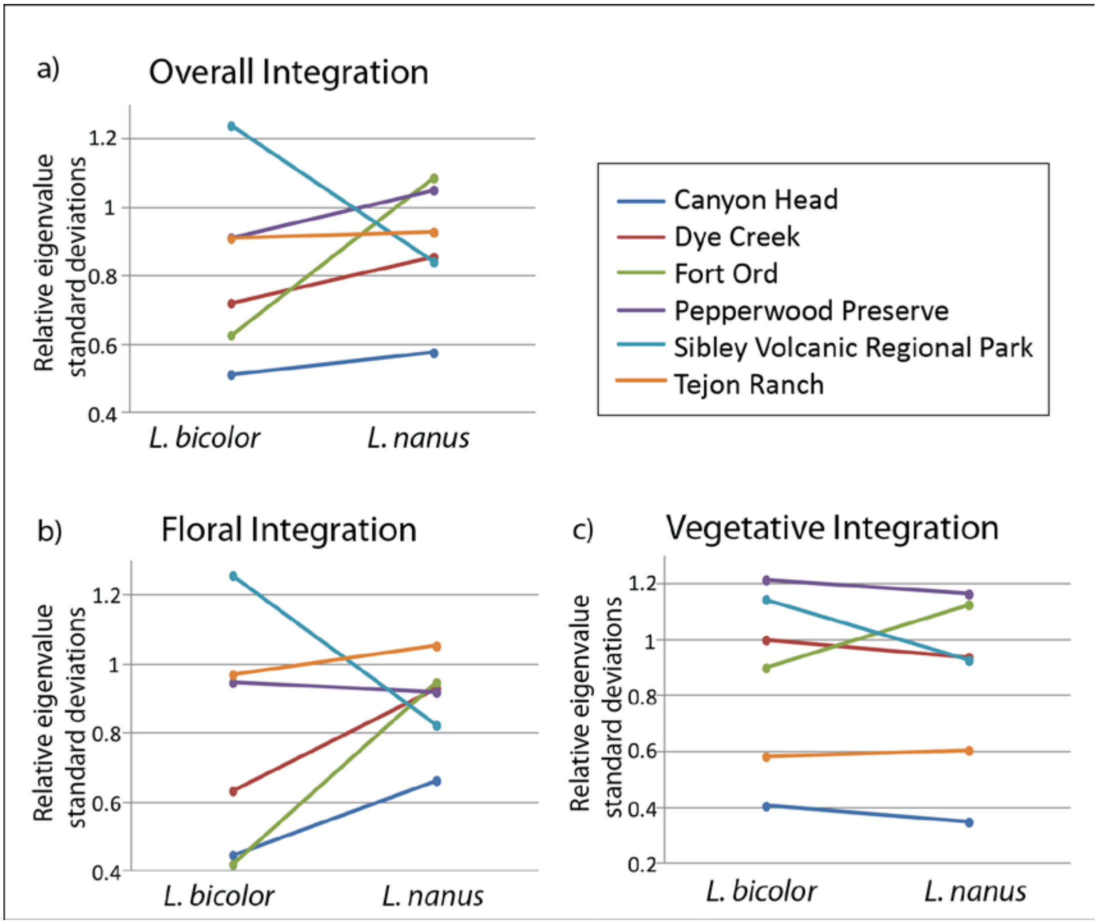


Figure 6: Relative eigenvalue standard deviations comparing floral integration to vegetative integration for *L. nanus* and *L. bicolor* at each site. Integration types (floral vs. vegetative) are listed on the x-axis, relative eigenvalue standard deviation values are listed on the y-axis, site locations are indicated by different colors, *L. nanus* populations are indicated by circle markers and black solid lines, and *L. bicolor* populations are indicated by square markers and grey dashed lines.

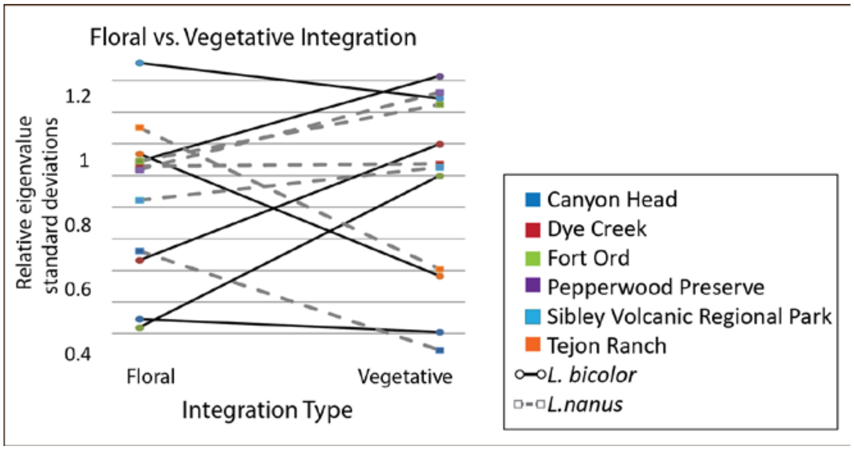


Table 5: Site-specific first-axes canonical correlations between floral and vegetative traits for *L. bicolor* and *L. nanus*. The amount of variation explained by the first floral and vegetative axes is also included. All first canonical axes were significant (p<0.001).

Site Abbreviations

CH – Canyon Head
DC – Dye Creek
FO – Fort Ord
PW – Pepperwood Preserve
SV – Sibley Volcanic Regional Park
TR – Tejon Ranch

	1st Canonical Axis			
	L. bicolor		*L. nanus*	
	Correlation	% variation	Correlation	% variation
DC	0.400	0.542	0.362	0.629
FO	0.369	0.535	0.614	0.752
PW	0.392	0.747	0.453	0.750
SV	0.491	0.527	0.313	0.563
TR	0.439	0.726	0.296	0.607
CH	0.433	0.836	0.217	0.554
Ave	0.421		0.376	
SD	0.043		0.140	

Figure 7: Floral trait PCA relationships among sites and species. There are more relationships among sites in *L. nanus* than *L. bicolor*, and there are similarities between species in all sites except TR.

Site Abbreviations
CH – Canyon Head
DC – Dye Creek
FO – Fort Ord
PW – Pepperwood Preserve
SV – Sibley Volcanic Regional Park
TR – Tejon Ranch

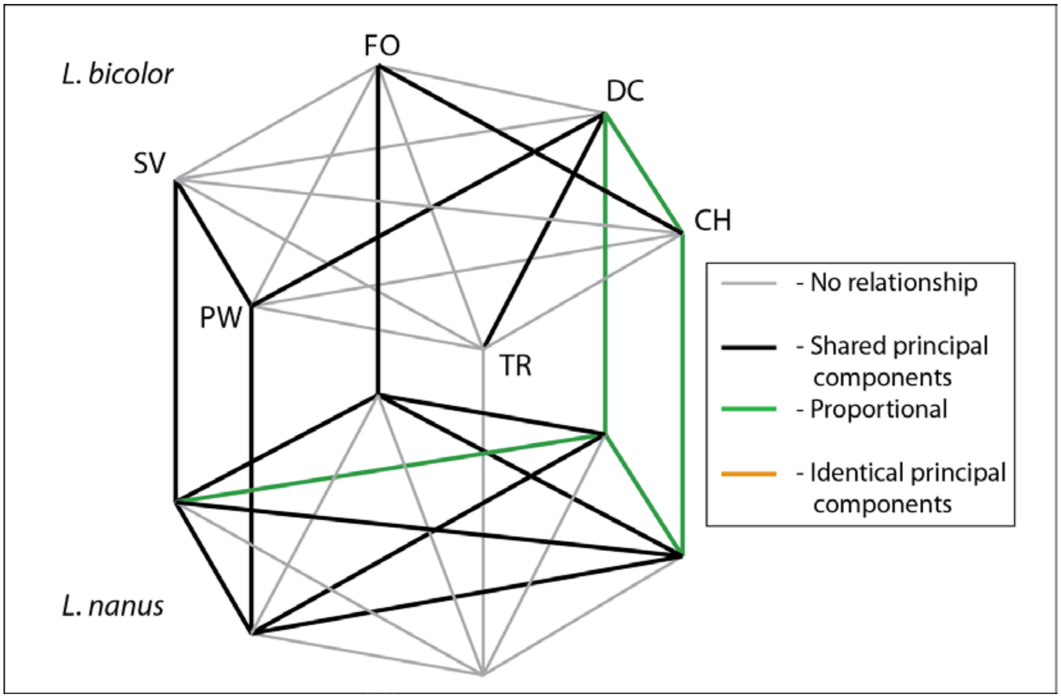
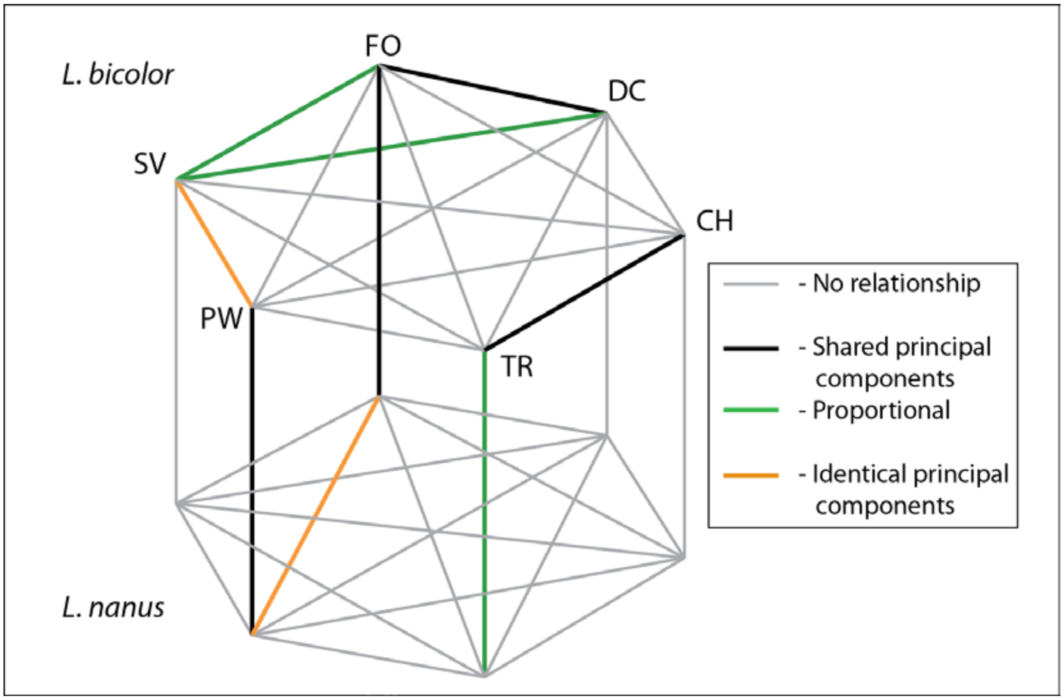


Figure 8: Vegetative trait PCA relationships among sites and species. There are very few similarities in PCAs among sites when only vegetative traits are used in the analysis, but half of the sites show shared Principal Components.

Site Abbreviations
CH – Canyon Head
DC – Dye Creek
FO – Fort Ord
PW – Pepperwood Preserve
SV – Sibley Volcanic Regional Park
TR – Tejon Ranch



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