Title
Nurture over nature: Summer germinating Lupinus nanus are a result of anthropogenic germination cues and are not an independently evolving population

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Nurture over nature: Summer germinating *Lupinus nanus* are a result of anthropogenic germination cues and are not an independently evolving population

by

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Committee in charge:

Professor Ellen Simms, Co-chair
Professor George Roderick, Co-chair
Professor Patrick O'Grady
Professor Paul Fine

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Abstract

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Folsom Lake is a dammed reservoir experiencing anthropogenic lake level fluctuations that have created a novel germination time for *Lupinus nanus*. The recently discovered summer germinating plants begin to grow in May-June, just after the typical winter germinating plants in that location die. Since flowering of summer and winter germinating plants is temporally isolated, and plants are subject to differing selective pressures, they could be two independently evolving populations. Alternatively, they could be part of one population that can respond to both natural and anthropogenic germination cues. Through a common garden experiment, a germination experiment, and microsatellite analyses of plants from these environments, these hypotheses are tested. All evidence supports the hypothesis that summer and winter germinating plants are part of the same population, which can respond to germination cues at various times of the year.

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Chapter 1: Introduction

Earth is a human-dominated environment, with one-third to one-half of its land surface altered by human actions (Vitousek et al 1997). River systems are one of the most altered environments, with an average of ten new dams built every year in the US (Gleick 2000). As of 1996, only two percent of the rivers in the United States run unimpeded (Abramovitz 1996), and current policies indicate river alterations will continue to be implemented in the near future.

Damming of rivers often creates lakes with artificially fluctuating water levels that can impact local plant populations. For example, inundation of lakeshore seeds during spring floods has been shown to hamper germination (Nishihiro et al 2004). Fluctuating water levels in man-made dams can also create novel moisture zonation (Ali 2006). The extent to which dams cause novel selection pressure and the consequences for local adaptation and population subdivision is unknown.

The Folsom Lake Study System

In this dissertation, I am studying Folsom Lake, which was created in 1955 by the damming of the American River. This lake experiences seasonal water level fluctuations ranging from an elevation of 123 meters to 136 meters. The lake level rises with winter rains and spring snowmelt, and drops as water is used for hydroelectric power and irrigation during the dry season (Figure 1.1). Folsom Lake is located in a Mediterranean climate region which entails hot dry summers, and cool wet winters. As a result, there is little or no precipitation to fill the lake

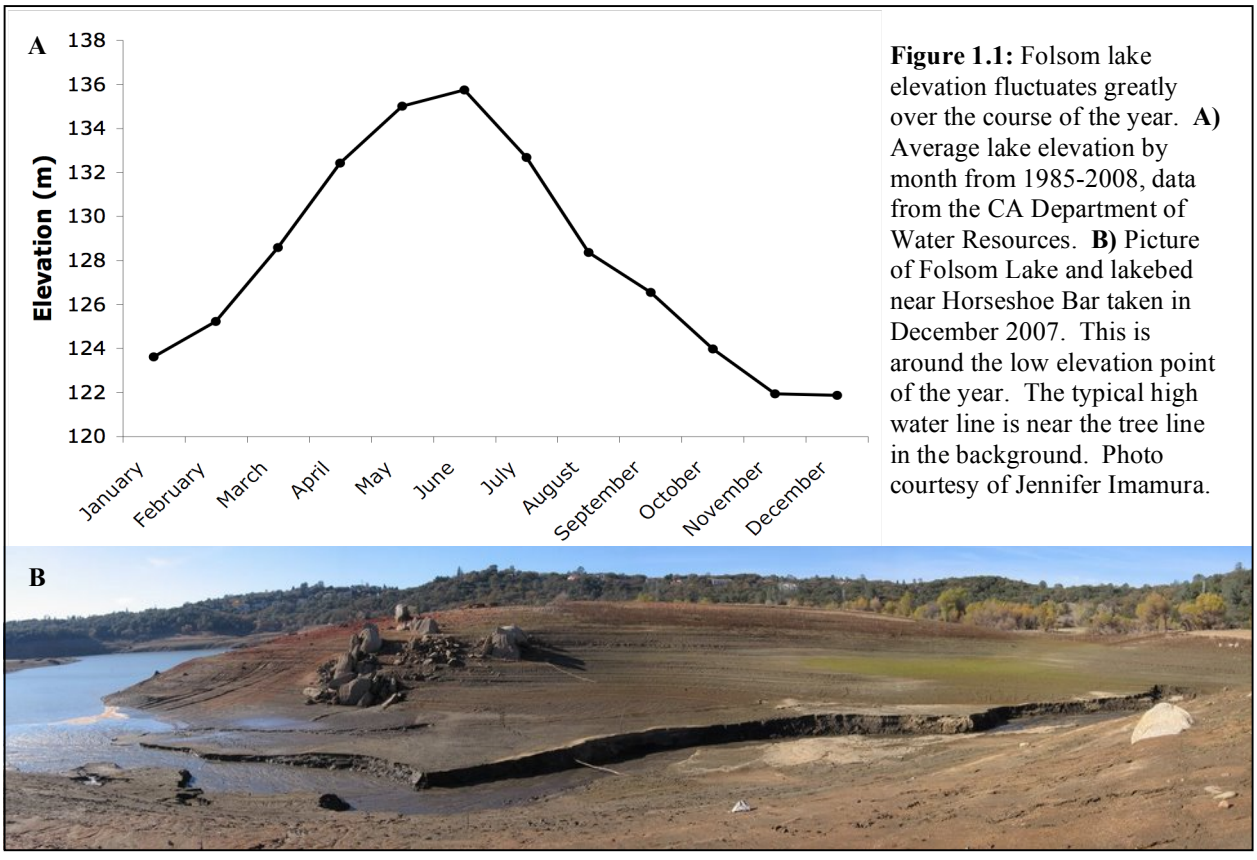

Figure 1.1: Folsom lake elevation fluctuates greatly over the course of the year. **A)** Average lake elevation by month from 1985-2008, data from the CA Department of Water Resources. **B)** Picture of Folsom Lake and lakebed near Horseshoe Bar taken in December 2007. This is around the low elevation point of the year. The typical high water line is near the tree line in the background. Photo courtesy of Jennifer Imamura.

during the dry season from June to November. Lake levels are usually highest in May-June and lowest in November-December (CA Dept of Water Res 2007).

One very common plant that lives around Folsom lake is the sky lupine, *Lupinus nanus* Benth., a native herbaceous annual in the family Fabaceae. It was first collected in the area that is now Folsom Lake in 1907 (Published record of the Consortium of California Herbaria 2007). Large numbers of *L. nanus* grow around Folsom Lake on the shoreline and further upland (CA Dept of Water Res 2007).

Lupinus nanus typically germinates in December, at the start of the rainy season, and flowers in May. However, I have discovered a novel summer germinating phenotype at Folsom Lake. These plants are present in a narrow band at the high water line, and germinate right after the lake level begins to drop in June, and flower in November and December (Figures 1.2, 1.3). Summer germinating plants grow exclusively at the lake high water line (Summer Lake site), whereas the more common and presumably ancestral winter germinating plants grow both along the shoreline (Winter Lake site) and upland, independent of any lake effect (Winter Upland site) (Figure 1.4). At the Winter Lake location, it does appear that the plants are more numerous in the area where past inundation has disturbed the soil than they are above the average high water line.

Experimental Background

Summer and Winter Lake environments represent extremely different challenges for *L. nanus*. While winter germinating seeds experience frequent rains and cool temperatures, Summer germinating seeds are inundated by lake water, then exposed in a very hot, dry environment. Selection in the Winter Lake (WL) environment is likely driven by many factors, including competition with other plants, and inundation of mature plants by the rising lake level (Figure 1.5). However in the Summer Lake (SL) environment, selective forces might include drought, high heat, and intense solar radiation.

Traits that may be advantageous for the plants living in the Summer Lake environment, such as drought tolerance, may be neutral, or even disadvantageous in the Winter Lake environment where mature plants are inundated. For example, in *Cakile edentula*, traits like

Figure 1.2: Summer Lake plants germinate in a narrow band along the high water line. The right side of the picture lacks previous year's dead plants because it was recently inundated and plant material was decomposed. Picture was taken in June 2008.

Figure 1.3: Winter and Summer Lake plants from the same vantage point. **Top:** Winter Lake plants grow in a wide swath around most of the lake. Picture taken in April 2008. **Bottom:** Summer Lake plants grow in a narrow strip at the previous year's high water line. Picture taken in November of 2007.

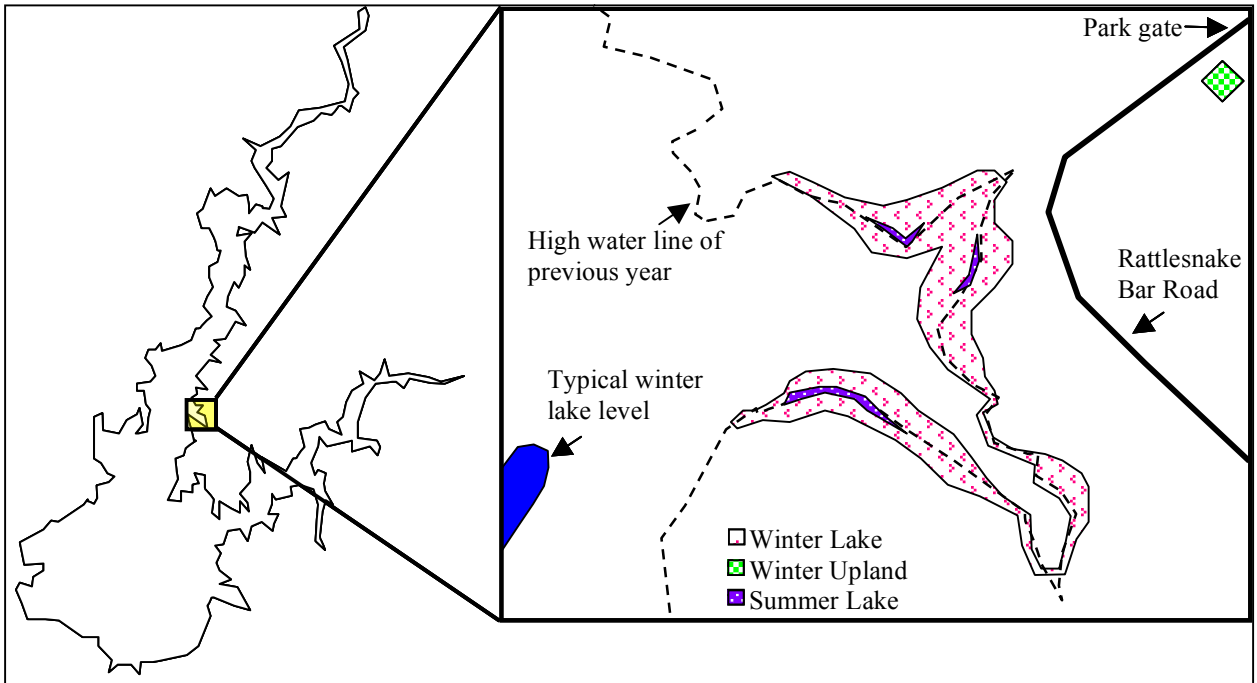

Figure 1.4: Map of Folsom Lake showing detail at main study site.

water-use efficiency and intermediate leaf size that are advantageous in a dry environment are maladaptive and lead to smaller plant biomass in a wet environment (Dudley 1996). Picotte et al (2007) also found that for *Piriqueta caroliniana* in wet environments, greater leaf area has significant effects on plant growth. However in dry environments, narrower leaves, higher water-use efficiency, and increased trichome density significantly increased plant growth (Picotte et al 2007).

Another potential selective difference between Summer Lake and Winter Lake *L. nanus* is the time during their life cycle at which they experience drought conditions. While Summer Lake germinating plants experience drought early in their growing season, Winter Lake germinating plants that are not inundated by rising lake levels experience drought near the end of their growing season. In *Impatiens capensis*, drought stress early in the growing season causes selection for lower water-use efficiency. However drought stress later in the growing season causes selection for high water-use efficiency (Heschel and Riginos 2005).

Reproductive isolation limits gene flow between groups of organisms. In habitats with differing selective pressures, reproductive isolation allows groups of organisms to evolve independently to each environment. Reproductive isolation can occur before or after fertilization, called pre- and post-zygotic reproductive isolation, respectively. In post-zygotic reproductive isolation, fertilization takes place but hybrids do not survive or reproduce. Pre-zygotic reproductive isolation is a result of barriers that prevent fertilization from occurring. A common pre-zygotic barrier for plants is a temporal barrier, where plants of two populations flower at different times and so cannot exchange gametes (Hendry et al 2007). Were it to persist over many generations, reproductive isolation could produce population differentiation, especially coupled with the differing selective pressures between Summer Lake and Winter Lake environments.

Variable selective pressures during the year, coupled with limited gene flow between temporally isolated populations, may lead to genetically differentiated seasonal populations. In the pine processionary moth, larval development normally occurs in winter; however a novel summer larval population was discovered in 1997. These winter and summer populations have differing selective pressures, and winter moths invest more in silk spinning and produce more larvae, whereas summer moths invest more in egg cold resistance (Santos et al 2007). Microsatellite data has shown significant genetic differentiation between these populations (Santos et al 2007). The Madeiran storm-petrel is a seabird with sympatric cool-season and warm-season nesting populations experiencing differing selective pressures. Microsatellite data demonstrates very little gene flow between these populations, suggesting they may be subspecies (Friesen et al 2007).

Figure 1.5: Winter Lake plants being submerged by rising water. Picture taken in April of 2008.

In the examples above, temporally varying selective pressures in combination with non-overlapping reproductive stages have allowed for independent evolution and adaptation to different seasonal environments. Were it to persist over many generations, reproductive isolation between Summer Lake and Winter Lake germinating plants at Folsom Lake could produce population differentiation, especially if coupled with the differing selective pressures between Summer and Winter environments.

At Folsom Lake, Summer Lake and Winter Lake plants have non-overlapping flowering times, hence cannot share gametes within a reproductive period. However, an extensive and long-lived seed bank might prevent population differentiation (Delcastillo 1994, McCue and Holtsford 1998, Honnay et al 2008). At other sites, *L. nanus* is known to have an extensive seed bank with 6.3 seeds per liter of soil (Moore 2009). Although viability of those seeds was not determined, a related species, *Lupinus polyphyllus*, has seeds that are estimated to remain viable at 30 degrees Celsius with 5 percent humidity for 1.86 years. Conditions underground may enable *L. polyphyllus* seeds to remain viable over 70 years (Sapra et al 2003).

Even with an extensive and long-lived seed bank, population differentiation might still occur if there were genetic variation for response to temporally specific germination cues. Temperature requirements for germination have been shown to be under genetic control in several species (Whittington et al 1970, Stratton 1991). Variation between populations in germination response to soil moisture has been seen in many species (Baskin and Baskin 1998).

Genetic variation in germination would create a genetic correlation between germination time and flowering time, and facilitate population differentiation. In contrast, if there is no genetic variance for germination response, for example if the response is phenotypically plastic and every genotype is equally likely to germinate in summer if the right cues are provided, then population differentiation would not be expected.

The possibility of phenotypic plasticity in germination response has been documented in plants with impermeable seed coats like lupines. *L. nanus* has a tough water-resistant seed coat that enforces dormancy. Daily temperature fluctuations can break dormancy and cause germination in many species with hard, impermeable seed coats (Baskin and Baskin 1998). When exposed to temperature fluctuations of 31 degrees Celsius, seeds from *Lupinus digitatus*, *L. luteus*, and related legumes with impermeable seed coats *Medicago tribulus* and *Trifolium subeterraneum,* germinated (Quinlivan 1961). A 15 degree Celsius daily temperature fluctuation regardless of the initial temperature (from 15 to 60 degrees Celsius) has been shown to cause *T. subterraneum* germination (Quinlivan 1966). Since a 15 degree fluctuation can cause germination at a variety of temperatures, it is possible that germination could occur during any season experiencing that amplitude of temperature fluctuation.

Phenotypic plasticity is a major source of genetic variation, and species with greater potential for plasticity are thought to be more likely to survive anthropogenic change (Sultan 2004). High phenotypic plasticity of many traits including germination response in the selfing grass *Bromus tectorum* has allowed nearly identical genotypes of these grasses to live in a wide range of environments, leading the grass to become invasive in Western North America (Novak et al 1991, Meyer et al 1997). Phenotypic plasticity is also thought to be the initial stepping stone towards evolutionary divergence, where phenotypic plasticity allows organisms to live in novel environments to which they can later adapt (West-Eberhard 1989).

The potential existence of genetic variation in germination response points to two alternative predictions: either Summer Lake and Winter Lake plants are differentiated or they are not. If they are differentiated (hypothesis A in Figure 1.6), Winter Lake and Summer Lake plants would comprise two separate populations reproducing independently, having undergone natural selection causing germination in response to a particular cue that is unique to that time of year, and adapted to their specific temporal environment. If they are not differentiated (hypothesis B in Figure 1.6), then all the Folsom Lake plants would belong to one population, where seeds produced in one growing season might either germinate immediately or remain in the seed bank, depending on environmental cues. There is additionally a third hypothesis, which is that they will eventually become separated, because a scenario for isolation is occurring, but that there has been insufficient time for this to occur. Given the extensive and long-lived seed bank, it could take a century or longer for this to occur.

To test these hypotheses, the following steps were completed, corresponding with Chapters 2-5 of this dissertation:

2) I outline the development of twenty-one microsatellite loci to be used for population genetic analysis of *Lupinus nanus*.

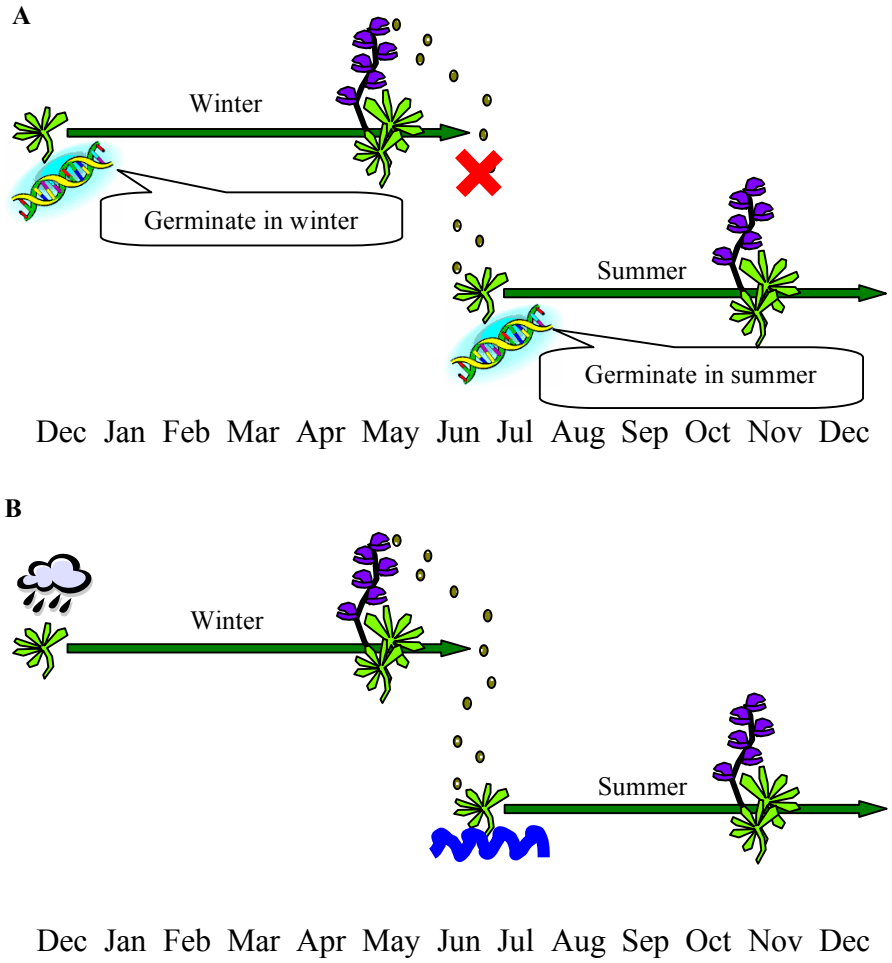

Figure 1.6: Graphical depiction of the competing hypotheses to be addressed in this dissertation. **Hypothesis A)** Winter Lake and Summer Lake plants are two independent populations, which are each adapted to their native environment and are reproductively isolated. **Hypothesis B)** Winter Lake and Summer Lake plants are part of the same population that can respond to a germination cue during multiple times of year.

3) I test Summer Lake, Winter Lake, and Winter Upland plants' responses to Summer selective pressures. If Summer and Winter plants are separate populations, we would expect plants from the Summer Lake environment to survive the harsh Summer Lake conditions better than plants from the Winter environments. I show that this is unlikely to be the case. Additionally, we would expect to see selection on natively growing Summer plants during the course of the season affecting some genotypes more than others. I find no evidence this is occurring.

4) I test for response to an environmental cue for germination in the Summer Lake environment. If Summer Lake and Winter Lake and Upland plants are part of the same population, the seeds must germinate in response to the same environmental cue. The specific water level fluctuations at Folsom Lake during Summer Lake germination may mimic weather conditions during Winter germination. By replicating these conditions, I show that Winter Lake seeds can germination in response to this potential Summer germination cue.

5) I determine the population genetic structure of plants growing around the lake. If Summer Lake, Winter Lake, and Winter Upland plants represent differentiated populations, they

should have different population genetic parameters. I show they share the same population parameters.

Chapter 2: Development of microsatellite loci for *Lithocarpus denisflorus* and *Lupinus nanus*

This chapter outlines the development of microsatellite loci from two species: *Lithocarpus densiflorus* Hook. & Arn. and *Lupinus nanus* Benth. During the course of development of the *L. nanus* microsatellites, I assisted in the creation of microsatellite loci from several other species as follows: seventeen microsatellites were developed for the planthopper *Nesosydne chambersi* (Goodman et al 2008), and eleven microsatellites were developed for *Grindelia hirsutula* Hook. and Arn. (Moore et al 2009), with three more in progress for other species of *Grindelia* (Moore, personal communication). Microsatellites for *Lupinus arboreus* Sims are still in progress, but approximately fifteen to twenty microsatellite loci are expected (Caroline Lee, personal communication).

Microsatellites are sequences of DNA that contain short tandem repeats of one to six bases. It is thought that slippage of the polymerase on the repeated bases during replication is responsible for the high polymorphism of these areas (Schlötterer and Tautz 1992). The high variability of microsatellite loci makes them suitable for individual and population level analysis. While microsatellite loci are more common in non-coding DNA, they may also be found in coding regions of the genome (Zane et al 2002). One common example is Huntington's Disease, which is caused by expansion of a trinucleotide repeat (Zuehlke et al 1993). This is somewhat at odds with the common assumption for most population genetic models that variation is neutral.

There are two well-documented models of evolution for microsatellites: the infinite alleles model (Kimura and Crow 1964), and the stepwise mutation model (Kimura and Otha 1978). In the infinite alleles model, it is assumed that each new mutation results in a unique allele, and that there are an infinite number of these unique alleles. In the stepwise mutation model, it is assumed that mutation occurs by the addition or subtraction of one or more tandem repeat units, and those different mutation events can result in alleles that appear to be the same, or are homoplastic. For example, an allele with nine repeats could lose a tandem repeat to become eight repeats long, but that same eight-repeat allele could also arise when an allele with six repeats gains two tandem repeats. The stepwise mutation model more accurately reflects how microsatellites are thought to mutate, with one or a few repeats being added or deleted and no way to detect homoplasy (reviewed in Estoup et al 2002). Therefore, we can never be sure if alleles with the same identity (length) are actually identical by descent (genetic relationship).

Microsatellites are commonly used in analyses of population genetics across a wide range of organisms. To assess this, a *Web of Science* v4.6 (www.isiknowledge.com) search was performed in October 2009 for the topic "microsatellite". It yielded 29,277 primary research articles from fields as diverse as psychology, geography, chemistry, anthropology, and dentistry, as well as from the expected areas of evolution and ecology. In the field of molecular ecology, analysis of microsatellite data is often used to determine population structure and gene flow in natural populations because they mutate relatively quickly on an evolutionary timescale.

Lithocarpus densiflorus microsatellite development (Morris and Dodd 2006)

Coastal woodlands of northern California and southern Oregon are suffering heavy mortality from infection by *Phytophthora ramorum*, which causes sudden oak death

(McPhearson et al 2005). Tanoak, *Lithocarpus densiflorus*, is a common component of these coastal woodlands and is also found in interior woodlands of the Sierra Nevada in California. This species is unusual in that *P. ramorum* infects foliage as well as the main stem, where cankers are produced. Recently, heavy losses of tanoak have become a major concern to natural resource managers in this region of the Pacific coast of North America. Tanoak is able to reproduce vegetatively as well as by seed, but no data are available on the population genetic structure of this species and how different modes of reproduction may affect spatial distribution of genotypes. Population genetic studies are important for understanding host-pathogen interactions, as some populations appear to me more resistant to pathogens than others.

Mature *L. densiflorus* leaves were obtained from Soquel State Demonstration Forest, Santa Cruz Co., California. Genomic DNA was extracted using a hexadecyl trimethyl ammonium bromide (CTAB) method (Cullings 1992). This DNA was used to build a microsatellite-enriched library by following the Glen and Schable (2005) protocol modified as described here. Four micrograms of extracted DNA were restricted with RsaI (New England Biolabs) and ligated to double-stranded SuperSNX-24 linkers (forward 5'-GTTTAAGGCCTAGCTAGCAGAATC-3', reverse 5'-GATTCTGCTAGCTAGGCCTTAAACAAAA-3'). The restriction ligation reaction was hybridized to mixtures of the following single-stranded biotinylated oligonucleotide probes: $(CA)_{13}$ $(GA)_{15}$ $(AT)_4$ $(AAT)_{12}$ $(CAA)_9$ $(AACC)_5$ $(AACG)_5$ $(AAGC)_5$ $(AAGG)_8$ $(ATCC)_5$ $(AAAC)_6$ $(AAAG)_6$ $(AATC)_6$ $(AATG)_6$ $(ACCT)_6$ $(ACAG)_6$ $(ACTC)_6$ $(ACTG)_6$ $(AAAT)_8$ $(AACT)_8$ $(AAGT)_8$ $(ACAT)_8$ $(AGAT)_8$. Magnetic beads coated with streptavidin (Dyna1) were used to capture hybridized DNA, while unhybridized DNA was discarded. The enrichment procedure was then repeated. Polymerase chain reaction (PCR) was used to recover enriched DNA, and 4 μl were cloned with a TOPO TA kit (Invitrogen). XL1-Blue (Qiagen) chemically competent *Escherichia coli* were transformed with PCR II-TOPO TA cloning vectors ligated to enriched DNA per the manufacturer's instructions. We screened for successful transformation using the β-galactosidase gene and amplified the inserts from positive colonies using M13 primers. The two hundred and twenty-four fragments found of the correct size (500-1000 bp long) were sequenced with M13 forward and reverse primers using BIG DYE v3.1 (Applied Biosystems) on an ABI 3100 automated sequencer.

We designed primers for eighty-seven sequences that were positive for microsatellite repeats and were had enough sequence data on either end available for primer design of flanking regions. Primers were designed manually and with the aid of the software FastPCR (Kalendar 2005). Synthesized primers (Operon and Invitrogen) were optimized for amplification on the source tree's DNA and were visualized on a 2.5% agarose gel. Thirty-seven primers yielded products that were both the expected size and appeared to be polymorphic across four individuals. Fluorescently-labeled primers (6-Fam and Hex, Operon) were obtained for these thirty-seven.

To screen for polymorphism and test performance in the species' range we carried out PCR on extracted DNA from 20 individuals from the Soquel State Demonstration Forest, seven individuals from populations in northern coastal California (Forestville, Cazadero, Salt Point Park and Fish Rock road) and one individual from an interior population in the Plumas National Forest, Butte Co., California. PCR conditions were as follows: 20 mM Tris-HCl (pH 8.4), 50

Table 2.1: Primer sequences designed to amplify microsatellite loci in *Lithocarpus densiflorus* and their characterization in the native range of the species. H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, fixation index; *Allele sizes and number of alleles for samples from six populations in the species range; *Denotes primer fluorescently labeled with hex; +Denotes primer fluorescently labeled with 6-fam.

Locus	GenBank accession number	Sequence 5'-3'	Repeat sequence	Allele range (bp)*	Total no. of alleles	H_O	H_E	F_{IS}
Ld1	DQ272386	F:CTGATGAAGAGGAAGCCGAAG* R:GTGGCCCTTTCTGACATGG	$(CTAT)_3$	224-228	2	0.350	0.358	0.022
Ld2	DQ272387	F:GGCACATAGAGTTAAACCC+ R:GGCCCACCAAAATGCTATCTC	$(C(A)_n)_{10}$	268-299	4	0.350	0.349	-0.004
Ld3	DQ272388	F:GCTAAAATTGGTGACTATG+ R:GGTTTACTAGAGCTCCAAAGG	$(AG)_{22}$	348-378	10	0.450	0.749	0.405
Ld4	DQ272389	F:GCCGGTCAATATAATTTGTTGC* R:GAGTAGGGTAGGGCTGATC	$(A)_{21}$	190-197	2	---	---	---
Ld5	DQ272390	F:TGCTCCGAACCCATGTA+ R:GAAATCGTTTCTTTGGGGTGTG	$(GTT)_6$	188-200	3	0.300	0.349	0.143
Ld6	DQ272391	F:CTAGAGAGTCAGGGTAAGCACC+ R:CAGAAAAGAAATGAAGATGCTG	$(A)_{21}$	452-455	2	---	---	---
Ld7	DQ272392	F:ACCACACGAATGCAGCACAATCAC+ R:GAATACCTCCTGTCCCACGTGAC	$(CT)_9$	407-415	3	0.300	0.328	0.088
Ld8	DQ272393	F:GTATCGGCGGCTTCGGTGGTC* R:CAAATAGCCACGTTGCAACAC	$(ATTT)_7$	165-177	3	0.700	0.591	-0.190
Ld9	DQ272394	F:GGCAAGAGATCCTGATGCATGTG* R:CAGAATCAATCTGCAATCTC	$(GA)_{14}$	117-121	2	0.200	0.185	-0.086
Ld10	DQ272395	F:GAGACAAGAATGAGCATCTC R:GTGATTGCATGTCTAGCTG*	$(C(T)_{3-5})_4$	195-201	2	0.700	0.508	-0.393
Ld11	DQ272396	F:CTGTTGGGTATGGTTGTCACTC+ R:CCTTAATTATGAGGAAAAAAC	$(CTTT)_8$	248-264	4	0.200	0.623	0.685
Ld12	DQ272397	F:CATCATCAAAACTACCGAC+ R:CGGTATCGATCTTGGAACAAC	$(CCAAA)_4$	118-153	5	0.650	0.545	-0.199
Ld13	DQ272398	F:GATTCGCAATACGATTCACG+ R:CGCATATGTATTTTCGTGGGAG	$(TATG)_7$	206-234	5	0.050	0.576	0.915
Ld14	DQ272399	F:GTCCAGGCTGCAGGCAATAG+ R:ATTGCCCTTGCCATTG	$((CAA)_{2-5})_4$	191-206	5	0.650	0.688	0.057
Ld15	DQ272400	F:GCAGCACACAATGCAATTTCC+ R:GTTCCATCAACTATTGACTCTG	$((GA)_{2-14})_5$	380-430	6	0.211	0.589	0.649
Ld16	DQ272401	F:CCTTCATTTCACATAATAGTGAATC+ R:GGAGTTGCCACCTGATTATAGG	$(AC)_9$	428-432	3	0.000	0.185	1
Ld17	DQ272402	F:CACAAGTTTATTCAATTTATTGG+ R:CAAGACCATTAGAGCACC	$(AT)_6(GTAT)_3$	141-145	3	0.650	0.617	-0.056
Ld18	DQ272403	F:GTTTGGCTTTGGCGCCACCTTCAC* R:GGAGTGACTTCGAGGTCGTTTGG	$(CT)_{20}$	147-181	7	0.150	0.355	0.584
Ld19	DQ272404	F:GAATTTCATTTTCAGGAGAG+ R:GATATGGCGTGGGATACACTTC	$(GA)_{11}$	158-182	6	0.450	0.704	0.367

mM KCl, 25 μg/ml BSA for all except locus Ld3 which used 12.5 μg/ml BSA, 4 mM $MgCl_2$ for all except loci Ld1 and Ld3 which each took 2 mM $MgCl_2$, 0.52 mM of each primer, 150 μM of each dNTP, 1 unit Taq DNA Polymerase (Invitrogen), and 2.5 μl of a 1:20 dilution of extracted DNA in a 25 μl reaction. PCR profile started with one activation cycle at 95°C for 10 min followed by 2 cycles of 1 min denaturing at 94°C, 1 min annealing at 60°C and 35 sec extension at 70°C. This was followed by 18 cycles of 45 sec denaturing at 93°C, 45 sec annealing at a step-down starting from 59°C and going down 0.5°C per cycle, and 45 sec extension at 70°C. Then there were twenty cycles of 30 sec denaturing at 92°C, 30 sec annealing at 50°C and 1 min extension at 70°C. This was followed by a final extension for 5 min at 72°C.

Two μl of PCR product was mixed with a solution of 8 μl of formamide and 0.5 μl of appropriate ROX size standard (ROX 350 or ROX 500, Applied Biosystems) and electrophoresed on an ABI 3100 automated sequencer. Results were analyzed with GENESCAN 3.7 and GENOTYPER 3.7 software (Applied Biosystems).

Of the 37 tested primers, 19 showed polymorphism within the species range examined and yielded reproducible and scorable bands (Table 1). The twenty individuals from a single population in Soquel were then screened for fragment size variation at these loci. Observed and expected heterozygosity in the population were obtained using the program CERVUS 2.0 (Marshall et al., 1998). Deviation from Hardy-Weinberg equilibrium and linkage disequilibrium were tested with GENEPOP 3.4 (Raymond and Rousset 1995) using the probability test with default values.

Of the 19 tested primers, 17 showed polymorphism within the Soquel population (Table 2.1). Loci Ld4 and Ld6 were fixed. Allelic richness across scored individuals ranged from 2 to 10, while allelic richness within the Soquel population ranged from 2 to 8, excluding the fixed loci. Significant linkage disequilibrium was observed between loci Ld1 and Ld2. This could suggest these loci are on the same chromosome, but might also indicate a history of admixture between two or more populations. The later hypothesis is supported by detection of significant deviation from HW equilibrium ($p<0.002$) being detected for loci Ld11, Ld13 and Ld18.

***Lupinus nanus* microsatellite development**

To address the hypotheses outlined in Chapter One of this dissertation, it was necessary to develop inexpensive, easy-to-use molecular markers with polymorphism at the population level. Although LEGCYC1A gene sequencing has been used to elucidate phylogenetic relationships within lupines (Ree et al 2004), sequencing is costly, optimization of the primers is difficult, and it is unlikely that polymorphism within populations would be sufficient for genetic analysis. While a handful of microsatellites have been developed for other lupines (Drummond and Hamilton 2005), prior to this work none had been developed and optimized specifically for *L. nanus*.

Lupinus nanus seeds haphazardly collected from plants setting seed at the Bodega Marine Reserve (BMR), Bodega Bay, in Sonoma County, CA (38°18'52"N 123°03'38"W) were surface-sterilized using a mixture of 50 ml bleach and 500 ul liquid Alconox. Seeds were mixed with one ml of the bleach solution for two minutes, vortexing once per minute. The bleach solution was pipetted off, and one ml of sterile water was immediately added; then pipetted off without vortexing. Five rinses of one ml sterile water with vortexing followed. Individual seeds were placed in wells of a 96-well microtiter plate with 200 ul sterile water, and left at 16 degrees Celsius for one to five days.

Scarification was necessary to break *L. nanus* dormancy, and in order to prevent contamination with fungus, it was carried out in a sterile laminar flow hood, using sterile equipment. Seeds were removed with forceps and placed in a Petri dish where a razor blade was used to cut the hilum end of the seed coat from the seeds. They were then placed individually into 300 ul 96-well plates with 250 ul sterile water, and left to imbibe at 16 degrees Celsius for

two to three days. Subsequently, three seeds were planted per cell in a 12-cell tray (Novosel Enterprises), using sterile pro-mix (Premier Horticulture, Quakertown, PA). Plants were watered daily for 21 days. Whole seedlings were harvested and 100 mg tissue was used immediately for DNA extraction using the DNeasy Plant Mini kit (Qiagen) according to protocol.

DNA was also extracted as described from young leaves of mature plants grown in the same fashion as described above for microsatellite analysis. This tissue was sampled from populations around California: BMR, Fort Ord Natural Reserve (FONR) in Monterey County (36°40'39"N 121°46'35"W), Kellogg (KG) in Sonoma County (38°37'53"N 122°40'29"W), and Hastings Natural History Reserve (HNHR) in Upper Carmel Valley of Monterey County (36°23'23"N 121°33'05"W) (Joshua Povich, personal communication).

DNA from a whole BMR seedling was used to build a microsatellite-enriched library by following the Glen and Schable (2005) protocol modified as described here. Four micrograms of extracted DNA were restricted with BstUI (New England Biolabs) and ligated to double-stranded SuperSNX-24 linkers as described for *Lithocarpus densiflorus*. The restriction ligation reaction was hybridized to mixtures of the following single-stranded biotinylated oligonucleotide probes: $(AG)_{12}$ $(TG)_{12}$ $(AAC)_6$ $(AAG)_8$ $(AAT)_{12}$ $(ACT)_{12}$ $(ATC)_8$ $(ATCC)_5$ $(AAAC)_6$ $(AAAG)_6$ $(AATC)_6$ $(AATG)_6$ $(ACCT)_6$ $(ACAG)_6$ $(ACTC)_6$ $(ACTG)_6$ $(AAAT)_8$ $(AACT)_8$ $(AAGT)_8$ $(ACAT)_8$ $(AGAT)_8$. Magnetic beads coated with streptavidin (Dyna1) were used to capture hybridized DNA, while unhybridized DNA was discarded. The enrichment procedure was then repeated. PCR was used to recover enriched DNA, and 4 μl were cloned with a TOPO TA kit (Invitrogen). XL1-Blue (Qiagen) chemically competent *Escherichia coli* were transformed with PCR II-TOPO TA cloning vectors ligated to enriched DNA per the manufacturer's instructions. These were screened for successful transformation using the β-galactosidase gene and inserts were amplified from positive colonies using M13 primers. Two hundred and eighty-five fragments were sequenced with M13 forward and reverse primers using the UC Berkeley sequencing facility. Sequences were analyzed using Sequencher 4.0 (GeneCodes Corporation). Repeats were identified visually and using the program Microsatellite Repeats Finer (Bikandi 2006).

Primers were designed for ninety-six sequences that were positive for microsatellite repeats and had enough sequence data on either end for primer design of flanking regions. Primers were designed manually and with the aid of the software Primer3 (Rozen and Skaletsky 2000). Synthesized primers (IDT) were optimized for amplification on the source plant's DNA and were visualized on a 1.5% agarose gel. Twenty-nine primer pairs yielded products that were both the expected size and appeared to be polymorphic using visualization on the agarose gel. Fluorescently-labeled forward primers (6-Fam, Hex, and Tet, Sigma) were obtained for these twenty-nine.

To screen for polymorphism we carried out PCR on extracted DNA from 20 individuals from FONR. To test performance across the species' range, DNA from 2 individuals each from BMR, KG, and HNHR was also used for analysis. PCR conditions were as follows: 2.5ul 10XPCR-$MgCl_2$ buffer (New England Biolabs) 25 μg/ml BSA, 4 mM $MgCl_2$, 0.52 mM of each primer, 150 μM of each dNTP, 1 unit Taq DNA Polymerase (New England Biolabs), and 2.5 μl of a 1:20 dilution of extracted DNA in a 25 μl reaction. The thermocycler program was as described in the development of *Lithocarpus densiflorus* microsatellites.

Table 2.2: Primer sequences designed to amplify microsatellite loci in *Lupinus nanus*, and their characterization at FONR. H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, fixation index; *Denotes primer fluorescently labeled with hex; $^+$Denotes primer fluorescently labeled with 6-fam; $^\#$Denotes primer fluorescently labeled with tet.

Locus	GenBank accession number	Sequence 5'-3'	Repeat sequence	Allele range (bp)	Total no. of alleles	H_O	H_E	F_{IS}
Luna1	GU232510	F:CGTATTGAGAGCGTGAG$^+$ R:AGTGAAGGAAAAACTAGCCGTTG	$(GA)_{10}$	142-178	8	0.632	0.698	0.098
Luna2	GU232511	F:GAGAAGAGGGATCATCAGGTTG* R:AAGCAGAGAGATCGGTGTGG	$(TGA)_3(CCA)_3(TC)_{10}$	156-180	7	0.842	0.836	-0.007
Luna3	GU232512	F:AGAATTTGCATACACAAAGCA$^\#$ R:CCCTTTGGGATAGTGTGCAT	$(CTAT)_7$	139-159	5	0.737	0.727	-0.014
Luna4	GU232513	F:TCCAATTCCAAACCCTACCA$^+$ R:TAAGTCCCAATCCAAGG	$((CT)_3)_3$	160-170	3	0.263	0.462	0.436
Luna5	GU232514	F:TGTTGTAATCAATGGAGGGAGA* R:ACAGGCGCAAACTAATCGAG	$((TGTT)_{3-4})_4$	152-180	4	0.333	0.348	0.042
Luna6	GU232515	F:AGCTCATCATCCAAATC$^\#$ R:TCAATGGGAACTCGTCAATG	$((ATC)_{3-7})_4$	167-202	9	0.526	0.812	0.358
Luna7	GU232516	F:TTCCATGGAGGGAAAGTGAG$^+$ R:GTGCGCTTCCGATCTCTAAT	$((AG)_3)_2(GTT)_4(TGA)_6$	177-189	5	0.368	0.778	0.533
Luna8	GU232517	F:CCAAGCCTAGCCTCCATTCT* R:TGTGAAGCTGTGAGAAGTCGTT	$(AT)_5(AG)_{10}$	175-199	6	0.632	0.785	0.200
Luna9	GU232518	F:CGGAGTAGGTTGAAGCGATTA$^+$ R:CGAAACGGACAACTTCAACA	$(GTTA)_7$	176-201	6	0.368	0.602	0.394
Luna10	GU232519	F:CGTTGATGCAATGTAGGTATC* R:CGAAACATTGCGATTCATCT	$(TGT)_5(GAA)_3$	180-193	8	0.368	0.744	0.512
Luna11	GU232520	F:CGATATGGCAGTTACTTGTTTGG$^+$ R:GAAACAGGGAGAACGTGAGC	$(CTCA)_3(CT)_4$	163-198	4	0.412	0.497	0.177
Luna12	GU232521	F:TTTGAACCAAACAACATAGTGAGTT* R:AAAGGGTTACTTGTTGATTGAAGA	$(GA)_4(CT)_9(CA)_3$	186-206	9	0.474	0.882	0.470
Luna13	GU232522	F:ATCCGTACACGACAGGGAAG$^+$ R:TGAATGTGAATGATGCCAATCT	$(AGTG)_5$	200-208	4	0.526	0.679	0.229
Luna14	GU232523	F:AAACAAGCATTAGAAGCAGT# R:CATCCTCTTATGGCGGTGAT	$(AC)_3(TAAC)_4(AG)_3$	205-225	6	0.789	0.751	-0.053
Luna15	GU232524	F:TTTGCCCGGTAAACTAGTCG$^+$ R:GTCGCCTACTTGCTGAAAGC	$(TCT)_6$	212-218	2	0.053	0.309	0.833
Luna16	GU232525	F:CGGATCTTTCAAAGGGAAAT* R:GATCACGGATGTTGGGAGTC	$(ACA)_7(TTC)_3(GAC)_3(CAA)_8(CAT)_3$	226-253	8	0.737	0.885	0.171
Luna17	GU232526	F:CGGATTAGGGTTTGGGTTTT$^+$ R:AAAGATGGATGAGGCAAAGG	$(TTG)_4(GTT)_6$	232-238	3	0.474	0.540	0.127
Luna18	GU232527	F:GAGACATGACCCAACATGTCTATAA* R:CAGAGATTCTTCTTTGGCTTTC	$(AAAG)_3(GAA)_3(AG)_{11}((TTTTCA)_4)_2(ACA)_3$	210-228	8	0.684	0.836	0.186
Luna19	GU232528	F:CGTGGGGATTAATGTTCTGA$^\#$ R:GCAAATGAGCTAAATGGATCG	$((TC)_{3-6})_8$	240-279	13	0.631	0.908	0.310
Luna20	GU232529	F:CAGAAACAGAGTTGTTGTGACG$^+$ R:GAGAAGGACAGGGTCGTTTG	$(TC)_9(AGA)_3(AAT)_4(TCC)_4(CT)_{15}$	235-269	11	0.579	0.899	0.362
Luna21	GU232530	F:GTCGAACCCCCACTCAA* R:GGTTGGTAGCCGCTTCTGTA	$(CAA)_3((GAA)_{3-6})_7((TGA)_3)_2$	278-290	4	0.474	0.700	0.329

Table 2.3: Linkage disequilibrium for Luna microsatellite loci at FONR. Significant ($p<0.05$) linkage disequilibrium indicated by a +.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	*	-	+	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	+	+	+
2		*	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-
3			*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
4				*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5					*	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	+
6						*	-	+	+	+	-	-	-	-	+	-	+	-	-	-	+
7							*	-	+	-	-	-	-	-	+	-	+	+	+	+	-
8								*	-	-	-	+	-	-	+	-	-	-	-	-	+
9									*	-	-	-	+	-	-	-	-	-	-	+	-
10										*	-	-	-	-	-	-	+	-	-	-	+
11											*	-	+	+	-	+	-	+	+	-	+
12												*	-	+	-	-	+	-	+	+	-
13													*	-	+	-	-	-	-	-	-
14														*	-	-	-	-	-	-	+
15															*	+	-	-	-	+	+
16																*	-	+	+	+	-
17																	*	-	-	-	-
18																		*	+	+	-
19																			*	+	-
20																				*	-
21																					*

0.5 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).

Of the twenty-nine tested primers, twenty-one showed polymorphism within the species range examined here and yielded reproducible and scorable bands (Table 2.2). The twenty individuals from a single FONR population were then screened for fragment size variation at these loci. One individual was reliably triploid, and was eliminated from further analysis. Observed and expected heterozygosity in the population, deviation from Hardy-Weinberg equilibrium, and linkage disequilibrium were tested with Arlequin version 3.11 (Excoffier et al 2005). F_{IS} was calculated using Genepop version 4.0.10 (Raymond and Rousset 1995).

Allelic richness across scored individuals ranged from 3 to 18 alleles, while allelic richness within the FONR population ranged from 2 to 13 alleles. Significant linkage disequilibrium was observed between several loci (Table 2.3). Linkage disequilibrium can indicate a history of admixture between two or more populations. This is supported by the detection of a significant deviation from HW equilibrium ($p<0.05$) for Luna4, Luna6, Luna7, Luna9, Luna10, Luna12, Luna15, Luna19, Luna20, and Luna21. These loci also had high (>0.3) F_{IS} values, further supporting the hypothesis of unequal mating or a recent mixture between populations. Analysis of ten microsatellite loci (Luna1, Luna4, Luna6, Luna7, Luna10, Luna12, Luna16, Luna18, Luna19, Luna20) for a different population near FONR also suggests recent admixture (Jessica Shade, personal communication) and might reflect past introduction of genotypes in some ecological restoration project on this former Army base.

Chapter 3: Summer Lake conditions do not favor plants from Summer Lake, Winter Lake, or Winter Upland environments

Introduction:

The capability of populations to invade new habitats depends mostly on their abilities to respond to natural selection in these habitats (reviewed in Lee 2002). If there is both genetic variation for the traits on which selection might act and negligible gene flow from other environments, then populations can quickly adapt to novel habitats and become locally adapted. If the selection gradient is strong, this can occur even with substantial gene flow (Endler 1977). The grass *Anthoxanthum odoratum* adapted to different fertilizer conditions within one hundred generations (Snaydon and Davies 1976). Within 100 generations, natural selection coupled with reproductive isolation in the form of differing flowering times led to population differentiation between grasses growing on polluted mine tailings and those in unpolluted habitats (Antonovics and Bradshaw 1970). Additionally, the sunflower *Helianthus anomalus* adapted within 60 generations to a novel sand dune environment (Ungerer et al 1998).

Plants can migrate from an ancestral environment to a novel environment, and undergo local adaptation to that new environment. If a population of plants is locally adapted, one would expect that these plants would survive conditions in that environment better than plants taken from the ancestral environment (Nosil et al 2005, Hendry et al 2007). This study is designed to address this prediction in the Folsom Lake system. If the Summer Lake plants do not survive better in the Summer Lake environment than do the Winter plants, which are assumed to be ancestral, then the Summer Lake plants are not locally adapted to that environment. This quality of being locally adapted depends on a many things – including selection at a variety of life stages (germination, seedling, adult, reproductive output, plant-plant competition) and also phenotypic plasticity in germination cues and allocation strategy.

The summer environment at Folsom Lake is harsh in many respects. Lake levels drop rapidly, sometimes more than a foot a day. There is no rain in the summer, and temperatures commonly exceed 37 degrees Celsius. In most of the shoreline lupine habitat, there is no shade. These factors contribute to rapid drying of the soil, and many plants that germinate in the summer do not live long enough to flower. If there were local adaptation to the Summer Lake environment, these extreme environmental conditions are some of the factors we would expect would be important in driving selection in traits for Summer Lake plants.

Two methods were used to examine the effects of selection and the potential for local adaptation to the Summer Lake environment: a common garden experiment and analysis of within-season changes in microsatellite allele frequencies after mortality of plants natively growing in the Summer Lake environment.

The common garden experiment, in which plants from Winter Lake, Winter Upland, and Summer Lake locations were grown in the Summer Lake environment, examined how these plants respond to the extreme Summer Lake environment. If the Summer Lake plants come from an isolated population that has adapted locally to selective pressures, we would expect Summer Lake plants to survive better in this environment than either Winter Lake or Winter Upland

plants. If the Summer Lake plants represent a random subset of the Winter population, we would expect no difference in their survival in the Summer Lake environment.

Microsatellite analysis was performed on nineteen loci to compare allele frequencies among seedlings natively germinating in the Summer Lake environment with those among natively germinating plants that survived to reproduce in that environment. If allele frequencies of seedlings differed from those of survivors, this would be consistent with the hypothesis that natural selection had favored nearby non-neutral loci and that the surviving genotypes were better adapted to the summer environment. If the allele frequencies did not change, it would suggest that mortality is random with respect to loci near those markers and that the harsh Summer Lake environment had not imposed natural selection on any traits correlated with the genetic markers.

Materials and Methods:

To establish the common garden experiment, seeds near the Rattlesnake Bar Road entrance to Folsom Lake from Winter Lake (38°45'50"N 121°06'41"W) and Winter Upland (38°45'55"N 121°06'34"W) environments were collected on May 24, 2008, and seeds from the Summer Lake environment (38°45'46"N 121°06'47"W) were collected December 18, 2007. Fifty-four seeds came from the Summer Lake environment, with one to three seeds from each of twenty maternal families. Fifty-one seeds came from the Winter Lake environment, with one to two seeds from each of twenty-seven maternal families. Fifty-three seeds came from the Winter Upland environment, with one to six seeds from each of fourteen maternal families. Records of maternal families were used only to ensure representation from a wide range of families. Maternal family data indicated in Table 3.1.

Seeds were surface-sterilized using a mixture of 50 ml bleach and 500 ul liquid Alconox. Seeds were mixed with 1 ml of the bleach solution for 2 minutes, vortexing once per minute. The bleach solution was pipetted off, and 1 ml sterile water was immediately added. Without vortexing, the sterile water was pipetted off. Five rinses of 1 ml sterile water with vortexing followed. Finally, individual seeds were placed into 300 ul 96-well plates with 200 ul sterile water and left at 15 degrees Celsius overnight.

Seed scarification was carried out in a sterile laminar flow hood, using sterile equipment. Seeds were removed with forceps and placed in a Petri dish where a single-edge razor blade was used to remove the seed coat at the hilum. They were then placed individually into 300 ul 96-well plates with 250 ul sterile water, and left to imbibe at 15 degrees Celsius for four days. Imbibed seeds were placed 1-cm below the surface of sterile Turface ® MVP ® (Profile Products, Buffalo Grove, IL) in Rootrainers (Spencer-Lemaire, Acheson, Alberta, Canada). These were placed in a greenhouse with nighttime lows between 14.5-16.5 degrees Celsius, and daytime highs at 23.5 degrees Celsius, and watered daily for two weeks prior to planting at Folsom Lake. Sixty-seven of the 158 scarified seeds survived to transplant.

A 20.42-m transect was laid out on the shoreline on June 19, 2008 at 38°45'46"N 121°06'47"W. A random number generator was used to randomly order Summer Lake, Winter Lake, and Winter Upland plants placed 35 cm apart along the transect. Moist soil on the shoreline was parted with a spatula to create openings approximately 10 cm deep and 2.5 cm

wide. Plants and Turface were slipped into the openings, and the soil was replaced around them. To protect the plants from sun during the first two days, tan shade cloth (Easy Gardner, Waco, Texas) was placed approximately 15 cm above the plants. Plants were watered once, after two days, using lake water. Transplant survival was measured after two days, and a Chi-square test was performed to detect differences in transplant shock survival between the Summer Lake, Winter Lake, and Winter Upland plants.

Plant survival status was measured after one month, three months, and four months, through the reproductive phase of the normal Summer Lake growing season (Table 3.1). Hazardous fire and smoke conditions during the summer and early fall impeded more frequent assessments of progress. Survival was also assessed at seven months, at which point the surviving plants were growing alongside young Winter Lake plants.

Survival analysis models the length of time before groups of individuals experience an event, such as death, to determine whether a treatment grouping changes survival time. While an ANOVA might be used to partition the variance of mean survival time for plants from different environments, it cannot take into account incomplete data. Survival analysis takes this into account using censorship to remove these individuals from analysis at the time they left the study. When the study ends, some plants may still be alive, and some plants have left the analysis for reasons unrelated to the factor being measured (for example herbivory). Censored individuals' information will not be used after the time of censorship, making a survival analysis more conservative than an ANOVA. Survival analysis has been used successfully to show local adaptation in populations of the fish *Menidia menidia.* Fish native to a polluted habitat performed better in polluted environments than in unpolluted environments (Roark et al 2005).

Survival analysis was used to determine if plants from Summer Lake, Winter Lake, and Winter Upland environments experienced different survival rates. An additional analysis was performed specifically to compare survival of the Summer Lake plants with that of the Winter Lake plants, as these plants vary only in the date on which their parent plants germinated. If the hypothesis that the Summer Lake plants are an independently evolving population were true, the Winter Lake environment would likely be the ancestral environment for the Summer Lake plants, and we would expect plants native to the Summer Lake environment to survive Summer Lake conditions better than plants from the ancestral Winter environment. Survival analysis was performed with GraphPad Prism 5 (GraphPad Software, San Diego, CA) using the Kaplan-Meier product limit method, comparing curves using the Mantel-Cox test.

To compare the allele frequencies of natively germinating Summer plants with those of plants that survived to reproduce, I collected tissue from plants germinating naturally in the Summer Lake environment near the beginning of the season (July 15, 2008) and late in the season, when Summer Lake plants were flowering (October 19, 2008). In both cases, a fifty-meter measuring tape was laid parallel to the water line at the elevation at which Summer Lake plants were growing. A random number generator was used to pick thirty locations along the tape. Three to four young leaves were taken from the plant closest to each location along the tape (within 1 m of the tape).

Table 3.1: Common garden experiment. SL stands for Summer Lake, WL for Winter Lake, and WU for Winter Upland. "Alive" indicates plant was alive. "Flowers" indicates the plant had at least one inflorescence. "Pods" indicates the plant had at least one fully expanded pod.

Parent plant	Source environment	June 21 # cotyledons	June 21 # leaves	July 15 # leaves	Sept. 10 survival	Oct. 19 survival	Oct. 19 # inflorescences	Nov. 7 survival	Feb. 20 survival
08LU0013	SL	2	0	12	alive	alive	0	alive	flowers
08LU0023	SL	2	1	11	alive	flowers	1	pods	
08LU0024	SL	2	0						
08LU0028	SL	2	2	15	alive	alive	9	flowers	alive
08LU0064	WL	2	1	7	alive	alive	1	flowers	
08LU0065	WL	2	2						
08LU0065	WL	2	2	11	alive	alive	5	flowers	flowers
08LU0066	WL	2	3	11	alive	alive	6	flowers	
08LU0070	WL	2	5						
08LU0079	WL	1	0						
08LU0080	WL	2	0						
08LU0083	WL	0	3	10					
08LU0083	WL	1	4						
08LU0084	WL	2	3	25	alive	pods	19	pods	
08LU0084	WL	0	1						
08LU0086	WL	2	0	12	alive	pods	32	pods	alive
08LU0089	WL	2	2	3	flowers	pods	3	pods	
08LU0090	WL	2	3						
08LU0091	WU	0	1						
08LU0092	WU	2	2						
08LU0094	WU	2	3	4	alive	pods	19	pods	
08LU0094	WU	2	3						
08LU0095	WU	2	4	13	alive	alive	0	alive	alive
08LU0095	WU	2	4						
08LU0095	WU	2	4	13	alive	alive	0	alive	
08LU0097	WU	2	1	12					
08LU0099	WU	2	2		alive	flowers	12	flowers	
08LU0099	WU	2	2	11	alive	pods	15	pods	
08LU0101	WU	2	0						
08LU0102	WU	1	0	8	alive	alive	0	alive	alive
08LU0102	WU	2	0	16	alive	flowers	9	pods	
08LU0106	WU	2	0						
08LU0106	WU	2	2	8	alive	alive	0	alive	alive
08LU0106	WU	2	1	8	flowers	pods	7		
08LU0106	WU	2	0						

DNA was extracted using the DNeasy Plant Mini kit (Qiagen) according to their protocol. Microsatellite analysis was performed as described in Chapter Two of this dissertation using nineteen loci (Luna1-4, Luna6-20). Arlequin version 3.11 (Excoffier et al 2005) was used to perform an AMOVA analysis, which partitions the molecular variance into covariance components within individuals, between individuals, within the early and late season collections, and between the early and late season collections. Jensen et al (2008) used AMOVA analysis to examine spatial and temporal differences in selection in brown trout.

F-statistics, which compare the variance among loci within individuals, within the early and late plants, and between early and late plants, were also calculated using Arlequin version 3.11 (Excoffier et al 2005). Comparisons of F_{ST} values calculated for each locus to the global F_{ST} has been used to detect specific microsatellite loci under selection in temporally varying brown trout populations (Jensen et al 2008).

An exact G-test was used to assess the significance of allele frequency changes at each locus. Ryman et al (2006) show that a G-test is an extremely sensitive measure of divergence between populations, so much so that it may detect divergence when none exists (Type I error). A Fisher's exact test, which performs a similar analysis across all loci, was used to test the null hypothesis that the allele frequencies are unchanged. This test is a more robust measure of divergence between groups of organisms, using allele frequencies across a number of loci (Ryman et al 2006). These exact tests were also used by Jensen et al (2008) to detect selection on microsatellite loci linked to TAP (Transporter associated with Antigen Processing) genes in brown trout across spatially and temporally varying populations. Fisher's exact test and the exact G-test were performed with Genepop version 4.1.10 (Raymond and Rousset 1995, Rousset 2008) using option 3 for genotypic population differentiation between pairs of populations. If natural selection is acting on heritably varying traits, we would expect plants with certain alleles to survive better than plants with other alleles, and therefore we would expect to see a difference in allele frequencies between the early and late season plants. Although the microsatellite loci themselves are neutral, they may be linked to genes under selective pressure, and so changes in microsatellite allele frequency can indicate selection on nearby loci.

Results:

In the common garden experiment, survival was generally poor across all conditions. One hundred and fifty-eight seeds were sterilized and scarified (54 Summer Lake, 51 Winter Lake, 53 Winter Upland). Of those, sixty-seven (11 Summer Lake, 25 Winter Lake, 31 Winter Upland) grew into seedlings and were transplanted to the Summer Lake environment. Only four Summer Lake, fourteen Winter Lake, and seventeen Winter Upland seeds survived past two days due to transplant shock. A Chi-square test was performed to compare germination and transplant survival between the Summer Lake, Winter Lake, and Winter Upland plants. It indicated a significant difference ($p < 0.05$) between the groups due to the low germination and survival of the Summer Lake plants. The plants that survived past two days were used for analyses, as it was assumed the other plants died as a result of transplant shock. Table 3.1 shows survival data for these plants.

Survival through the end of the normal Summer Lake season is illustrated in Figure 3.1. There are no error bars on the points on the far right because no new deaths happened on that

day. When the experiment concluded, three of the four Summer Lake plants, five of the fourteen Winter Lake plants, and nine of the seventeen Winter Upland plants were still alive. A Mantel-Cox test shows that plants from the three environments did not survive at different rates (p=0.6779), and that plants from the Summer Lake environment did not survive differently when compared only to the plants from the Winter Lake environment (p=0.2867).

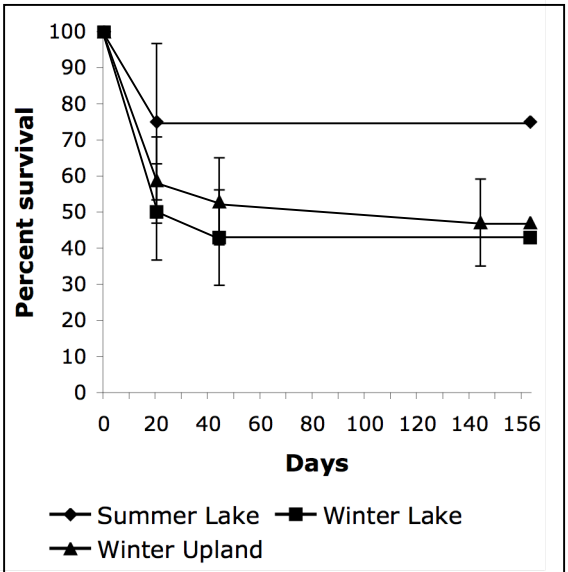

Figure 3.1: Survival analysis using Kaplan-Meier proportions of survivors. Percent is based on original successful transplants. Numerical data for numbers of survivors can be found in Table 3.1

The AMOVA comparing allele frequencies of natively germinating Summer Lake plants to that of plants that survived to reproduce is presented in Table 3.2. The overall F_{IS}=0.29107 (p=0.000), F_{IT}=0.29210 (p=0.000), and F_{ST} =0.00145 (p=1.000). For eighteen of the nineteen loci, F_{ST} calculated by locus was not significantly different from zero. However, F_{ST} was significantly different from zero for the locus Luna9 (F_{ST}=0.047 p=0.077). Of the nineteen loci examined with an exact G-test, only Luna9 showed significant differences in allele frequencies between early and late season plants (p=0.00049). Fisher's exact test found no significant difference in average allele frequencies between early and late season plants (p=0.204).

Discussion:

In the common garden experiment, survival in the Summer Lake environment was very poor for plants from all environments. Only thirty-five out of the original one hundred fifty-eight seeds, or just over 22%, survived the transplant procedure. This is much lower than found in other studies involving transplantation of native California plants. For example, in *Gilia capitata*, 68% of plants survived transplantation after being grown in a greenhouse to the two-leaf stage (Nagy and Rice 1997). The low proportion of plants that survived the transplant was probably due to the extreme conditions at the lake.

Although transplants were put in the ground only a few days after the lake level started to drop, the lake level dropped so rapidly that the plants had to be placed in exposed bare soil. At the high water line, where the population is generally found, germinating seedlings would normally have the protection of shade from the dead Winter Lake lupines that had never been submerged.

Fewer Summer plants survived transplanting than did Winter plants. Only four Summer Lake plants survived the transplant, while more than three times as many Winter Lake and Winter Upland seeds survived. Winter Lake and Winter Upland seeds used in this study were freshly collected, while Summer Lake seeds had been collected nearly a year previously. Seed age may have compromised the vigor of germinated seedlings. Another explanation is that

Source of variation	Sum of squares	Variance components	Percentage variation
Between early and late season plants	7.804	0.00853	0.14547
Among individuals within early and late season collections	423.742	1.70521	29.06487
Within individuals	239.000	4.15317	70.78966
Total	670.546	5.86691	

Table 3.2: AMOVA comparing plants growing early in the season to those that survived Summer Lake conditions through late in the season.

Summer Lake seeds might have germinated more slowly, been less vigorous, and less likely to survive transplant due to maternal effects. The Summer Lake environment is harsh, and parent plants may not be able to allocate as many resources to seed production as plants in the WinterLake environment. To determine the potential maternal environmental effect, we would need to grow Summer Lake and Winter Lake seeds in the greenhouse for a generation before transplantation. Additionally, the transplantation process itself was artificial. Future experiments should provide shade to the transplanted seedlings for a longer time period, or instead use *L. nanus* seeds glued to toothpicks and placed in the germination environment, which has recently been shown to be more successful than transplantation (Moore 2009).

Of those that survived the transplant procedure, 75% (3 out of 4) of the Summer Lake plants, 36% (5 out of 14) of the Winter Lake plants, and 53% (9 out of 17) of the Winter Upland plants survived to the end of the Summer Lake season. Casual personal observations of plants growing natively in the Summer Lake environment suggest approximately 50% survival for plants germinating in that environment. In an annual lupine from Texas, *L. subcarnosus*, 48% of plants germinating natively survive to the middle of the plant's life cycle, with a lower mortality rate between that time and the natural end of the plant's life cycle (Schaal and Leverich 1982).

Survival in the Summer Lake environment did not differ significantly between Summer Lake, Winter Lake, and Winter Upland plants ($p=0.6779$); nor was there a significant difference when Summer Lake and Winter Lake plants were compared ($p=0.2867$). The results of this study were difficult to interpret, however, as survival was generally very poor across all seed sources due to transplant shock and harsh Summer Lake conditions. Most of the plants that did survive the transplant procedure appeared unhealthy and had high concentrations of anthocyanins, suggesting a high level of stress (Chalker-Scott 1999). It is likely that the lack of water and high temperatures of the lake environment caused stress and mortality in plants transplanted to the Summer Lake environment.

Some plants from the common garden experiment survived the Summer Lake season and lived into the Winter Lake season. Two plants (one Summer Lake, one Winter Lake) flowering at the end of the normal Summer Lake season flowered for several more months into the Winter Lake season, while two other plants (both Winter Upland) that were not flowering at the end of the Summer Lake season began flowering shortly thereafter in January, and continued to flower as normal Winter Lake plants began flowering in April. Personal observation of other plants germinating naturally in the Summer Lake environment suggests that some plants can survive their season, and continue to reproduce into the next season, further supporting the idea that the Summer Lake plants can exchange genes with the Winter Lake plants and are therefore not a separate population.

The AMOVA comparing allele frequencies of natively germinating Summer Lake plants to those surviving to reproduce, indicates that the majority of the genetic variation is due to variation within and among individuals. Variation between early and late season plants explains only 0.14547 percent of genetic variation.

F_{ST} is a measure of variance between populations. In this case, the early and late season populations are in fact the same population before and after mortality due to the harsh Summer Environment. F_{ST} is only 0.00145 (p=1.000), suggesting there is no significant variance between the early and late season plants. On the other hand, F_{IS} and F_{IT}, measures of individual variance, are around 0.29 and are highly significant (p=0.000). This suggests that, on average, mortality in the Summer Lake environment does not impose selection on the marker loci.

When F_{ST} is individually calculated for each of the nineteen loci, Luna9 yields a low and marginally significant F_{ST} value of 0.047 (p=0.077), which might suggest that selection may be acting on a gene closely linked to this locus. An exact G-test, which provides an extremely sensitive measure of differentiation, and as a result is prone to type I error (Ryman et al 2006), was performed to test if allele frequencies at each locus changed during the mortality event. Even using this very sensitive test, only one locus out of nineteen examined showed significant differences in allele frequencies between the early and late season plants (p=0.00049). A more robust Fisher's exact test of allele frequencies across all loci suggests the early and late season plants are not significantly different (p=0.204). This further indicates that there is no evidence that mortality changed the frequencies of alleles at any of these loci.

It is expected that by chance a significant result with p=0.077 will be obtained once among every thirteen tests performed (0.077 = 1/13). In this case, nineteen univariate tests were performed, one for each locus, making it likely that at least one test will be significant by chance alone. There might be selection on genes linked to Luna9, but it is more likely a random effect of performing nineteen tests. An NCBI BLAST (Altschul et al 1990) of this locus for somewhat similar sequences (blastn) did not result in any closely related matches. Future studies of local adaptation to the Summer Lake environment should focus on functional genes, however, as the microsatellite loci in this study are assumed to be neutral to selection and so might not show evidence of differentiation early on in the process.

If the Summer and Winter plants are separate, independently evolving populations, we would expect that Summer Lake plants would survive better in the Summer Lake environment than would the Winter Lake and Winter Upland plants. We would also expect microsatellite analysis to show some change in allele frequencies between the populations. If the Summer and Winter plants are part of the same population that is able to respond to germination cues at two times during the year, we would expect no difference in survival between the plants in the Summer Lake environment, and we would expect no population differentiation.

The results in this chapter suggest that either all genotypes are equally affected by the harsh summer conditions, or if selection is occurring, is not strong enough to affect overall survival, or is swamped by gene flow. It is additionally possible that some selection is occurring, but it is not strong enough to be detected by neutral markers used in this study. These

possibilities support the idea that the Summer Lake plants are a part of the same population as the Winter Lake plants, and that Summer Lake plants may simply be individuals of this population that have germinated at a different time. The ability of plants from a single population to survive such a temporal shift in germination suggests that these plants may be able to adjust their phenotype to future anthropogenic change.

Chapter 4: Summer Lake germination occurs in response to environmental cues

Introduction:

Annual California lupines usually germinate in winter and flower in spring. However, in the inundation zone of Folsom Lake*, Lupinus nanus* have been observed germinating in summer and flowering in early autumn. While Winter germinating seeds experience frequent rains and cool temperatures, Summer germinating seeds are inundated by lake water, then exposed in a hot, dry environment. Most Summer and Winter germinating plants have non-overlapping flowering times; hence sharing gametes within a single contemporaneous reproductive period is rare. Variable selective pressures during the year, coupled with reproductive isolation between these plants may lead to genetically differentiated seasonal populations.

Population differentiation in these lupines would be facilitated by genetic variation in specific germination cues. For example, temperature requirements for germination have been shown to be under genetic control in several species (Whittington et al 1970, Stratton 1991). Variation among populations in germination response to soil moisture has been seen in many species (Baskin and Baskin 1998). For example desert plants like *Hordeum spontaneum* C. Koch have been shown to germinate in response 10 mm rain in the winter, but in the summer they require 50 mm rain to germinate (Gutterman and Gozlan 1998). Genetic variation in germination could create a genetic correlation between germination time and flowering time, and select for different traits in the two populations. In contrast, if there is no genetic variation in germination response, or if the response is phenotypically plastic and every genotype is equally likely to germinate in summer if the right cues are provided, then this mechanism could not produce population differentiation.

The possibility of phenotypic plasticity in germination response has been documented in plants with impermeable seed coats like lupines. *L. nanus* has a tough water-resistant seed coat that enforces dormancy. Daily temperature fluctuations can break dormancy and cause germination in many species with hard, impermeable seed coats (Baskin and Baskin 1998). When exposed to temperature fluctuations of 31 degrees Celsius, seeds from *Lupinus digitatus*, *L. luteus*, and related legumes with impermeable seed coats *Medicago tribulus* and *Trifolium subeterraneum,* germinated (Quinlivan 1961). A 15 degree Celsius daily temperature fluctuation regardless of the initial temperature (from 15 to 60 degrees Celsius) has been shown to cause *T. subterraneum* germination (Quinlivan 1966). Since a 15 degree fluctuation can cause germination at a variety of temperatures, it is possible that germination could occur during any season experiencing that amplitude of temperature fluctuation.

Phenotypic plasticity is a major source of genetic variation, and species with greater potential for plasticity are thought to be more likely to survive anthropogenic change (Sultan 2004). High phenotypic plasticity of many traits including germination response in the selfing grass *Bromus tectorum* has allowed nearly identical genotypes of these grasses to live in a wide range of environments, leading the grass to become invasive in Western North America (Novak et al 1991, Meyer et al 1997). Phenotypic plasticity is also thought to be the initial stepping stone towards evolutionary divergence, where phenotypic plasticity allows organisms to live in novel environments to which they can later adapt (West-Eberhard 1989).

Summer and Winter germinating plants at Folsom Lake are either genetically differentiated or they are not. If they are differentiated, Winter and Summer plants would comprise two separate populations reproducing independently, genetically programmed to germinate in response to a particular cue that is unique to that time of year, and adapted to their specific temporal environment. If they are not differentiated, then all the Folsom Lake plants would belong to one population, where seeds produced in one growing season might either germinate immediately or remain in the seed bank, depending on environmental cues.

If Summer and Winter plants are part of the same population, they are likely to share a common germination cue, even though there may be a wide variance around that cue. One potential environmental cue for Summer germination might be provided by lake level fluctuation. Folsom Lake fills with winter rains and spring snowmelt, reaching its peak elevation in May. Lake levels drop as water is used for hydroelectric power and irrigation during the dry season, and are lowest in November (Figure 4.1A). In May, when the water is highest, there are several days when the lake level remains constant. Hourly lake level measurements, however, show that the lake fluctuates, dropping several centimeters during the hot part of the day as water is used to generate hydroelectric power, and refilling at night with cold snowmelt (Figure 4.1B).

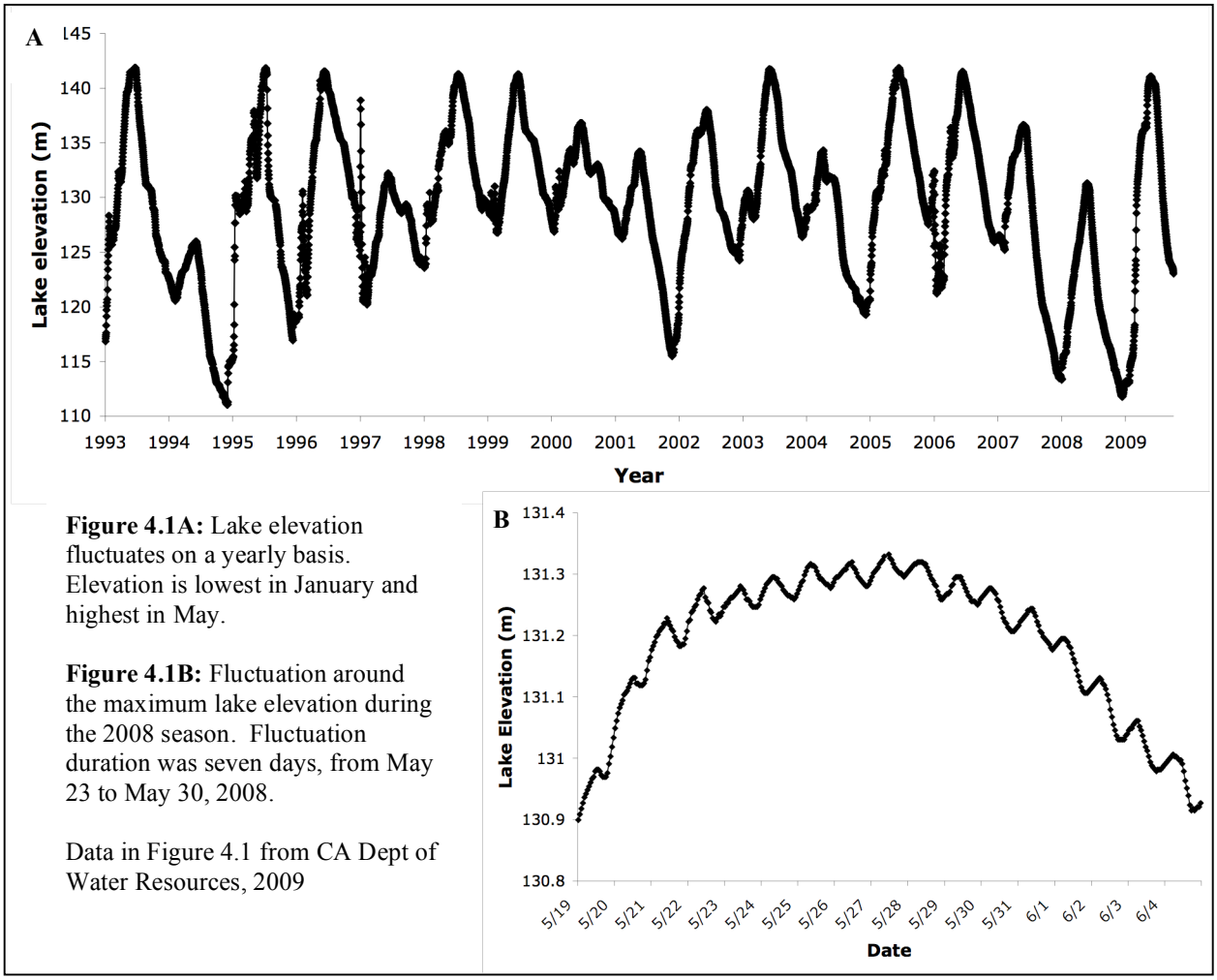

Figure 4.1A: Lake elevation fluctuates on a yearly basis. Elevation is lowest in January and highest in May.

Figure 4.1B: Fluctuation around the maximum lake elevation during the 2008 season. Fluctuation duration was seven days, from May 23 to May 30, 2008.

Data in Figure 4.1 from CA Dept of Water Resources, 2009

During this period, seeds in the fluctuation zone experience wet and cold nights, while their days are dry and hot.

I hypothesized that this wet/cold - dry/hot cycle could be releasing seeds from dormancy, thereby causing summer germination of seeds that happen to be in the fluctuation zone. This hypothesis predicts that Summer Lake plants are a product of Winter Lake seeds that happened to land in the fluctuation zone, and are not a separate population. In this chapter, I will explore the historical record of the fluctuation period and will report the results of experiments designed to test how seed germination responds to the wet/cold – dry/hot cycle.

Materials and Methods:

For historical lake level analysis, data were obtained from the California Department of Water Resources (CA Dept of Water Res). Graphs of hourly lake level elevation in the month surrounding the yearly maximum were examined to find fluctuation periods. A fluctuation period was defined as more than two days during which the majority of hourly lake level measurements overlapped with each other. Graphs of daily lake level elevation were examined to determine the yearly minimum lake elevation.

For the germination experiment, Winter Lake seeds from 38°45'50"N 121°06'40"W were placed in the following four environments: constant wet/cold on filter paper substrate, constant wet/cold on sand substrate, alternating wet/cold – dry/hot cycle on filter paper substrate, and alternating wet/cold – dry/hot cycle on sand substrate. The constant wet/cold environment mimics the environment that submerged seeds would experience. The wet/cold – dry/hot cycle replicates the environment at the high water line in the fluctuation period. The sand substrate more closely mimics the soil at Folsom Lake, while the filter paper substrate was used for ease of visually scoring individuals.

Twenty plastic Petri dishes were used to hold seeds. A hot metal instrument was inserted twelve to fifteen times into the smaller half (top) of each Petri dish pair to create drainage holes. This half of the Petri dish was nested inside the larger half (bottom), with the open sides of both halves facing upward. In ten of these modified dishes, two layers of filter paper were placed over the holes, ten Winter Lake seeds were placed on top of the filter paper layers, and one additional layer of filter paper was used to cover the seeds. In the other ten modified dishes, one layer of filter paper was placed over the holes, an approximately 1-cm thick layer of sand was placed on the filter paper, and ten Winter Lake seeds were placed on top of the sand. All Petri dish assemblies were then filled with distilled water. Each Petri dish assembly represented an experimental unit.

Five of the filter paper Petri dishes and five of the sand Petri dishes were placed in a constant wet/cold environment at 12 degrees Celsius inside a dew chamber. This temperature reflects the water temperature of the lake during the fluctuation period. Petri dishes were checked daily to monitor the status of the seeds, and to add more water when necessary to keep the seeds inundated. Observations of the seeds ceased after twelve days, one day longer than the longest fluctuation period observed since 1993.

The other five filter plate Petri dishes and the other five sand Petri dishes were placed in the same nightly wet/cold environment. Each morning after assessing the germination status of the seeds, the smaller Petri dish was removed from its larger base to drain, and was placed in a dry/hot environment at 32 degrees Celsius for 5-6 hours. This temperature reflects a normal daily high temperature around the lake during the fluctuation period. After being returned to the Petri dish base, additional distilled water was added as needed to cover the seeds.

Germination was assessed daily for each Petri dish. Survival analysis was used to determine if seeds exhibited different germination rates. Traditionally, survival analyses have been performed to compare treatment effects on subjects, with the event of interest being failure, most commonly death. Here, germination was used as the event of interest. Survival analysis is one of the recommended data analysis methods for germination studies (Scott et al 1984) and has been used to assess germination response to environmental conditions in bromeliads (Winkler et al 2005). A survival analysis is designed to be a very conservative estimate of difference, as it includes censorship. When a germination study ends, some seeds may not have germinated because of the germination condition being tested, while others may not have germinated because they are inviable. Censorship, which removes these data from analysis, takes this into account.

Three survival analyses were performed: one with the sand and filter paper data combined, one for the sand substrate only, and one for the filter paper substrate only. If the hypothesis that the Summer and Winter plants are part of the same population is correct, the fluctuation period environment (wet/cold – dry/hot cycle) is expected to cause germination. Survival analysis was performed with GraphPad Prism 5 (GraphPad Software, San Diego, CA) using the Kaplan-Meier product limit method, comparing curves using the Mantel-Cox test.

A Chi-square test on the average germination date was also performed. This test provides a liberal estimate of difference, as it cannot take into account incomplete, or censored data. Using this test requires the assumption that all the seeds in the study were viable. The Chi-square test was performed to confirm the results from the more conservative survival analysis. While not a recommended test for survival analysis, this test has been used in germination studies when the assumption can be made that all seeds were viable (Scott et al 1984).

Results:

The California Department of Water Resources has collected hourly lake level data since 1993. These data have been summarized in Table 4.1. There has been a fluctuation period in every year except 1995, with two fluctuation periods occurring in 2009. The location of the fluctuation zone changes among years, with an average elevation of 137.73 m. Each fluctuation period lasts on average 6.59 days, with an average change in lake level elevation of 9.14 cm. The average yearly change in lake elevation is 16.98 m.

The data for the germination experiment sorted by substrate and environmental treatment are shown in Figure 4.2. The wet/cold – dry/hot sand substrate condition had the most germination overall, with seeds germinating throughout the experiment for a total of eight seeds out of fifty germinated. Seeds on sand in the constant wet/cold environment germinated second most, with a total of four seeds out of fifty germinating by the end of the experiment. Three

Table 4.1: Historical lake level data summary since 1993, the start of hourly elevation measurements. There was no fluctuation period in 1995, and two fluctuation periods in 2009. Data from CA Dept of Water Resources, 2009.

Year	Fluct. period starts	Fluct. period ends	Duration of fluct. period (days)	Min. elev. during fluctuation period (m)	Max. elev. during fluctuation period (m)	Elevation change during fluctuation period (cm)	Yearly min. elevation (m)	Yearly max. elevation (m)	Yearly change (m)
1993	Jun 16	Jun 24	8	141.76	141.92	16.15	120.56	141.89	21.33
1994	Jun 3	Jun 7	4	125.92	126.00	8.53	111.04	126.00	14.97
1995							119.04	141.86	22.82
1996	Jun 8	Jun 13	5	141.51	141.58	7.62	120.22	141.58	21.36
1997	Jun 17	Jun 25	8	131.79	131.92	13.41	123.57	132.22	8.65
1998	Jul 15	Jul 19	4	141.27	141.30	2.44	126.79	141.30	14.51
1999	Jun 23	Jun 26	3	141.25	141.33	7.92	126.88	141.33	14.45
2000	Jun 16	Jun 23	7	136.79	136.88	9.14	126.22	136.88	10.66
2001	May 18	May 27	9	134.16	134.25	9.14	115.47	134.25	18.78
2002	Jun 6	Jun 14	8	137.89	138.07	17.98	124.22	138.07	13.85
2003	Jun 1	Jun 12	11	141.65	141.77	11.28	126.38	141.77	15.39
2004	Mar 26	Apr 2	7	134.08	134.19	10.36	119.27	134.36	15.08
2005	Jun 26	Jul 5	9	140.78	140.85	6.40	121.20	141.86	20.65
2006	Jun 11	Jun 14	3	141.50	141.54	3.96	125.21	141.54	16.33
2007	May 24	Jun 2	9	136.58	136.68	9.45	113.34	136.68	23.34
2008	May 23	May 30	7	131.25	131.33	8.53	111.78	131.33	19.55
2009	May 23	May 28	5	141.06	141.13	6.40		141.13	
2009	Jun 4	Jun 9	5	140.61	140.68	6.71			
Average			**6.59**	**137.64**	**137.73**	**9.14**	**120.70**	**137.89**	**16.98**
Std Dev			**2.37**	**4.73**	**4.73**	**3.94**	**5.41**	**4.80**	**4.31**

seeds out of fifty on filter paper in the wet/cold – dry/hot environment germinated. No seeds out of fifty germinated on filter paper in the constant cold/wet environment. Viability of seeds that did not germinate during the experiment was not assessed.

Survival analysis indicated that overall seeds in the wet/cold – dry/hot treatments germinated at a greater rate than those in constant wet/cold. Figure 4.3 shows survival over time for all seeds included in the study and the standard error as calculated in the survival analysis. The last point for the constant wet/cold line has no standard error bars because there were no new events (germinations) at that time point. Forty-two sand wet/cold – dry/hot seeds, forty-six sand constant wet/cold seeds, forty-seven filter paper wet/cold – dry/hot seeds, and all fifty filter paper constant wet/cold seeds remained ungerminated at the end of the study. The combined data and the filter-paper only data showed marginally significant differences in event occurrence when compared to their respective wet/cold – dry/hot treatments ($p=0.096$, $p=0.080$ respectively using a Mantel-Cox test).

There were no germination events in the filter paper constant wet/cold treatment. Therefore further analysis was performed only on the combined constant wet/cold and wet/cold – dry/hot data. The statistical power of the combined germination experiment is 0.37 as calculated using SSP (http://cct.jhsph.edu/javamarc/index.htm), which means that to achieve $p < 0.05$, I would have needed to start the experiment with 299 seeds, instead of the 200 seeds actually used,

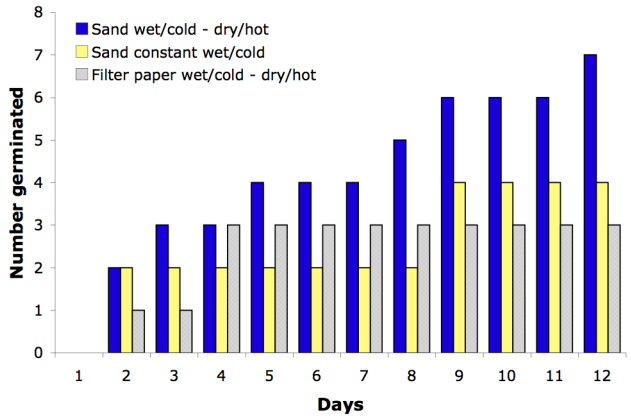

Figure 4.2: Germination over time, by treatment and substrate. No seeds germinated for the filter paper and constant wet/cold condition.

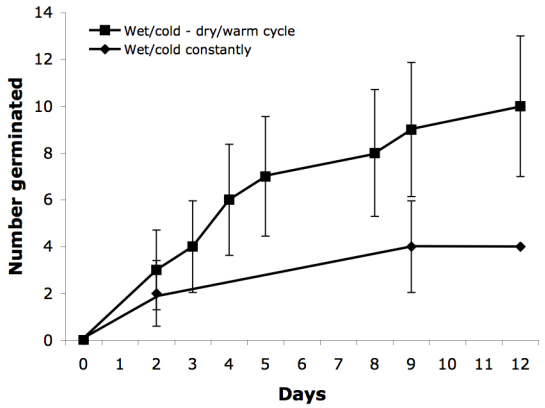

Figure 4.3: Germination of all seeds in wet/cold – dry/hot cycle compared to all seeds in constant wet/cold conditions.

assuming the variance between and within treatments remained the same (SSP, http://cct.jhsph.edu/javamarc/index.htm).

A survival analysis is designed to be a very conservative estimate of difference. When a more liberal Chi-square test was performed, requiring the assumption that all seeds were viable, the combined data indicated highly significant differences between the wet/cold – dry/hot and constant wet/cold treatments ($p < 0.0001$).

Discussion:

Two predictions exist for the source of Summer germinating plants at Folsom Lake. Winter and Summer plants could comprise two separate populations reproducing independently; genetically programmed to germinate in response to a particular cue that is unique to that time of year. Alternatively, all the Folsom Lake plants could belong to one population, where Summer plants represent a subset of the Winter seeds which have experienced a local environmental cue that stimulates germination.

If Summer and Winter plants are part of the same population, the environments must share a germination cue. I have hypothesized that the wet/cold - dry/hot cycle in the lake level fluctuation zone may be the germination cue for Summer plants. This hypothesis predicts that Summer plants are a product of Winter Lake seeds that happened to land in the fluctuation period, and are not a separate population. If the Summer and Winter plants were separate populations, we would not expect Winter seeds to respond to the Summer germination cue.

The fluctuation period has been regularly observed since at least 1993, and most likely has existed since Folsom Dam was built in 1955. One reason Folsom dam was built was to control seasonal flooding in the American River valley, and it is possible that similar fluctuation periods around these natural floods occurred, with water levels rising during the night as water came down from the mountains, and water levels dropping from evaporation during the day. The average change in elevation during the fluctuation period is small (9.14 cm), but enough to provide seeds in the fluctuation zone with a wet/cold – dry/hot cycle that might stimulate

germination. This is further supported by personal observations that Summer *L. nanus* grow almost exclusively in an extremely narrow band at the high water line that matches the fluctuation zone elevation.

Survival analyses show significant differences in germination rates between constant wet/cold and wet/cold – dry/hot treatments for both the combined substrates ($p=0.0961$) and for the filter paper substrate alone ($p=0.0802$). A more liberal Chi-square test on the combined substrates also detected significant differences between constant wet/cold and wet/cold – dry/hot treatments ($p<0.0001$). These results suggest that germination is stimulated by the wet/cold – dry/hot cycle in the fluctuation period. However, since only seeds from the Winter environment were tested, we can not say if Summer seeds would have responded similarly. Additionally, genetic components of germination were not assessed.

Normally, impermeable lupine seeds must be manually scarified to break dormancy and germinate in lab conditions. Studies are lacking in regards to breaking dormancy in field conditions. None of the seeds used in this experiment had been scarified, yet the wet/cold – dry/hot cycle was able to break dormancy and cause germination. Daily temperature fluctuations can break dormancy and cause germination in many species with hard, impermeable seed coats (Baskin and Baskin 1998). A 15 degree Celsius daily temperature fluctuation, regardless of the initial temperature, has been shown to cause germination of a related legume, *Trifolium subterraneum* (Quinlivan 1966). The Folsom Lake wet/cold – dry/hot cycle results in a 20 degree Celsius change in daily temperature, which would be of sufficient amplitude to cause germination.

More seeds germinated in the sand constant wet/cold environment than in the filter paper wet/cold – dry/hot environment. This may be because the addition of water to the plates caused the seeds to rub against the sand, and over time this abrasion was able to break through the seed coat. Abrasion against sandpaper is commonly used to penetrate impermeable seed coats in lab conditions to cause germination (Baskin and Baskin 1998).

Repetition of this experiment should be performed to confirm the results, with both Summer Lake seeds and Winter Lake seeds. An additional control of a wet/cold - dry/cold environment should be performed to confirm it is the temperature fluctuation that provides the germination cue. Some seeds should be manually scarified before the experiment starts to determine if the cycle is causing scarification of the seed coat, or is providing some alternate cue for germination. Finally, seeds should be placed in the Summer Lake and Winter Lake environments to compare germination rates in the field to those in experimental conditions.

The two overarching hypotheses in this dissertation are that either the Summer and Winter Lake plants are independently evolving populations with germination under genetic control, or Summer and Winter Lake plants are all part of the same population, with seeds that happen to be in the fluctuation period germinating in the Summer. The existence of an environmental cue in the Summer Lake environment that causes germination of Winter Lake seeds supports the second hypothesis. While a genetic control of germination was not tested, the ability of Winter seeds to respond to the wet/cold – dry/hot cycle suggests that Summer Lake

plants may be a subset of the Winter Lake population that have experienced this unique wet/cold - dry/hot cycle.

If Summer and Winter plants represent two independently evolving populations, it indicates that *L. nanus* can adapt rapidly to environmental change. This type of rapid adaptation and genetic differentiation has been seen in other species. A novel summer larval population of the pine processionary moth has shown significant genetic differentiation from the normal winter larval population, even though the summer population has only been in existence since 1997 (Santos et al 2007). The Madeiran storm-petrel also has shown rapid independent evolution in response to a novel nesting season (Friesen et al 2007). If, however, the Summer and Winter *L. nanus* at Folsom Lake are part of the same population, it indicates that they are able to live in environments that have been subjected to anthropogenic change because they already possess the phenotypic plasticity to do so. This has been shown to occur in *Bromus tectorum,* which, due to phenotypic plasticity of many traits including in germination response, can live in a wide range of environments and has become widespread and invasive in Western North America (Novak et al 1991, Meyer et al 1997).

Chapter 5: Summer Lake, Winter Lake, and Winter Upland plants show no evidence of population structure

Introduction:

Lupinus nanus normally germinates in the winter and flowers in the spring. At Folsom Lake, *L. nanus* also germinates in the summer at the high water line, and flowers in the fall. Since they flower at different times, Summer and Winter plants cannot directly share gametes within the same reproductive season. Summer and Winter plants also experience different selective pressures, which coupled with their temporal reproductive isolation, could lead the two populations to undergo divergent selection. Alternatively, Summer and Winter plants may be both from a single population, and Summer germinating plants derive from Winter seeds that happen to land in the fluctuation zone where they experience the appropriate germination cue.

There exist several examples of observable genetic differentiation as a result of temporal variation in reproductive period. For example, in *Howea* palms on an isolated oceanic island, growth on a novel substrate caused a six-week difference in flowering time. This reproductive isolation between plants growing on different substrates, combined with the differing selective pressures (as shown by F_{ST} of AFLPs) has been cited as a major factor of their speciation (Savolainen et al 2006, Babik et al 2009). The Madeiran storm-petrel provides another example. In this seabird, cool-season and warm-season nesting populations use the same burrows. Microsatellite data demonstrated that this temporal difference in reproductive timing has drastically reduced gene flow between these populations (Friesen et al 2007). In the pine processionary moth, larval development normally occurs in winter; however in 1997, it was discovered that larvae also develop during the summer. Microsatellite data shows that summer larvae exhibit much less allelic richness, and are significantly genetically differentiated from winter developing larvae, suggesting that they represent a distinct population that has recently experienced a genetic bottleneck (Santos et al 2007).

However, if gene flow is high between populations, genetic differentiation may never occur even if there is selection for a particular phenotype (Slatkin 1987). In general, if the selection coefficient is less than the migration rate, selection will not be able to cause population divergence (Storfer 1999). As few as one migrant per year between the islands and the mainland in the Lake Erie common garter snake have been found to balance out natural selection for color pattern (Bittner and King 2003). Recurrent migration in *Timema cristinae* walking-stick insects obscured effects of selection on insects living on two host plant species (Bolnik and Nosil 2007). In the threespine stickleback fish, high gene flow between lake and outlet stream populations constrains adaptation in the stream by 80-86% (Moore et al 2007).

Phenotypic plasticity is also a major source of genetic variation, and species with greater potential for plasticity are thought to be more likely to survive anthropogenic change (Sultan 2004). High phenotypic plasticity of many traits in the selfing grass *Bromus tectorum* has allowed nearly identical genotypes of these grasses to grow in a wide range of environments, leading the grass to become invasive in Western North America (Novak et al 1991, Meyer et al 1997). Phenotypic plasticity is also thought to be the initial stepping stone towards evolutionary divergence, where phenotypic plasticity allows organisms to live in novel environments to which they can later adapt (West-Eberhard 1989).

Determining whether the Summer Lake, Winter Lake, and Winter Upland patches of *L. nanus* at Folsom Lake are genetically distinct, and how much gene flow occurs between them, would indicate their potential for divergent evolution in response to spatially (Lake vs. Upland) or temporally (Summer vs. Winter Lake) specific selection pressures. A high degree of gene flow would support the hypothesis that the Winter and Summer plants are members of the same population, and can respond to germination cues throughout the year. Little gene flow would suggest that the plants could be separate populations, independently evolving in response to anthropogenic lake level fluctuations, with germination time providing the necessary reproductive isolation. It may also be the case that since Folsom Lake is only fifty-four years old, that analysis of neutral markers like microsatellites may not detect any divergence, whereas future analysis of function sites might provide greater detail.

Materials and Methods:

To determine the extent of the Summer Lake phenotype around Folsom Lake, in December of 2007, twenty-three people walked thirty miles of the approximately forty-five mile Folsom Lake shoreline to record flowering plants from Summer germinating seed. The only sections of the lake not surveyed were those inaccessible by roads, or too hazardous to walk.

Plant tissue for genotyping was collected from Summer Lake, Winter Lake, and Winter Upland locations near the Rattlesnake Bar Road entrance to Folsom lake on the North Fork of the lake. Additional Summer Lake and Winter Lake plant material was collected from the South Fork of the lake at Old Salmon Falls Road (Figure 5.1, Table 5.1). For sampling the Summer Lake sites, a random number generator was used to pick thirty locations along a fifty-meter

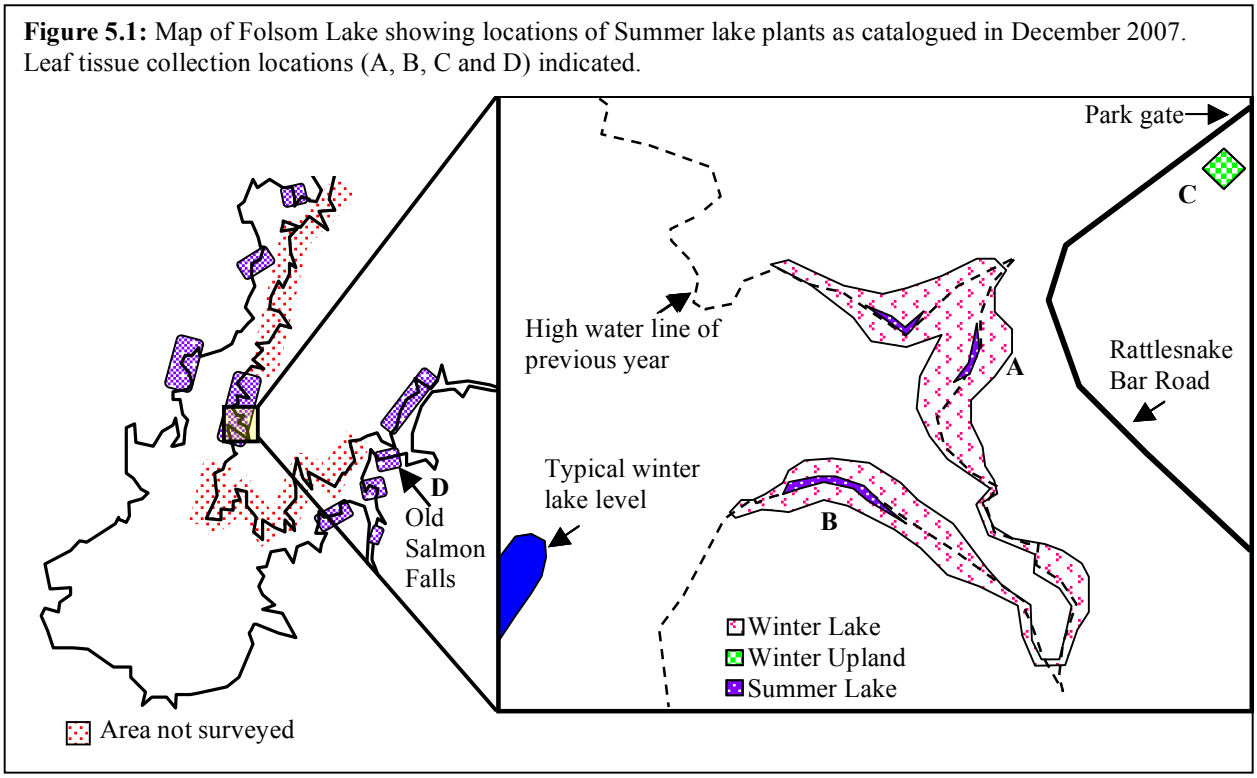

Figure 5.1: Map of Folsom Lake showing locations of Summer lake plants as catalogued in December 2007. Leaf tissue collection locations (A, B, C and D) indicated.

Table 5.1: Collection data for plants in microsatellite analysis. Code refers to places indicated in Figure 5.1.

Environment	Code	GPS coordinates	Location on map	Date	Num. collected
Summer Lake	A	38°45'50"N 121°06'40"W	Rattlesnake Bar Road	November 24, 2007	30
Summer Lake	B	38°45'46"N 121°06'48"W	Rattlesnake Bar Road	November 24, 2007	30
Winter Lake	A	38°45'50"N 121°06'41"W	Rattlesnake Bar Road	April 20, 2008	30
Winter Lake	B	38°45'47"N 121°06'45"W	Rattlesnake Bar Road	April 29, 2008	30
Winter Upland	C	38°45'55"N 121°06'34"W	Park Gate	April 20. 2008	20
Summer Lake	B	38°45'47"N 121°06'47"W	Rattlesnake Bar Road	July 15, 2008	30
Summer Lake	B	38°45'46"N 121°06'47"W	Rattlesnake Bar Road	October 19, 2008	30
Summer Lake	D	38°45'16"N 121°03'32"W	Old Salmon Falls	October 4, 2008	30
Winter Lake	D	38°45'15"N 121°03'30"W	Old Salmon Falls	February 20. 2009	30

measuring tape extended parallel to the waterline at the level at which Summer Lake plants were growing. Three to four young leaves were taken from the plant closest to each location along the tape (up to 1 m away). For sampling the Winter Lake sites, a random number generator was used to pick six locations along a fifty-meter measuring tape extended across the widest part of the population parallel to the lake. At each of these six locations, another fifty-meter measuring tape was laid out perpendicular to the original tape, and five locations were chosen along second tape using a random number generator. Three to four young leaves were collected from the plant nearest to each of these thirty locations (up to 10 cm away). Winter Upland plants were scarce; therefore three to four young leaves were collected from all plants at the site.

Fresh plant tissue was immediately placed in silica and dried for at least three days. DNA extraction was performed using the DNeasy Plant Mini Kit (Qiagen) according to manufacturer's protocol. Microsatellite analysis was performed as described in Chapter Two of this dissertation using nineteen loci (Luna1-4, Luna6-20) for most analyses, or nine loci (Luna1, Luna4, Luna6, Luna10, Luna12, Luna16, Luna18-20) for analyses involving the Winter Lake plants at Old Salmon Falls (location D).

Structure version 2.2 (Pritchard et al 2000) was used to estimate the number of populations to which individuals could be assigned and again to assign individual plants to populations. To determine the percentage of variation seen in the plants that can be ascribed to among-population or between-population differences, an Analysis of Molecular Variance (AMOVA) was used (Arlequin version 3.11, Excoffier et al 1992). This method partitions the variance into covariance components based on differences within an individual, between individuals, within a population, and between populations.

Table 5.2: Number of alleles at each locus across populations surveyed. Loci in Winter Lake D without allele numbers were not analyzed for this population. A and B locations are at Rattlesnake Bar Road. Location C is at the Park Gate, and location D is at Old Salmon Falls.

Locus	Summer Lake A 11/07	Summer Lake B 11/07	Winter Lake A 4/08	Winter Lake B 4/08	Winter Upland C 4/08	Summer Lake B 7/08	Summer Lake B 10/08	Summer Lake D 10/08	Winter Lake D 2/09
Luna1	9	7	9	9	10	9	10	7	7
Luna2	4	3	4	4	2	2	5	4	-
Luna3	4	3	3	3	3	5	3	4	-
Luna4	3	4	4	3	3	4	4	4	3
Luna6	10	6	11	9	9	11	9	10	-
Luna7	5	7	5	6	5	6	6	5	7
Luna8	10	9	10	7	6	10	9	8	-
Luna9	7	7	6	6	5	5	6	4	-
Luna10	5	6	7	7	5	5	6	7	4
Luna11	7	6	5	5	5	5	8	5	-
Luna12	10	12	12	13	12	13	13	13	8
Luna13	4	3	3	3	3	3	3	3	-
Luna14	6	7	6	5	7	7	7	6	-
Luna15	2	1	1	2	1	3	2	1	-
Luna16	9	7	8	7	8	7	5	9	10
Luna17	2	3	3	4	4	5	2	3	-
Luna18	11	9	12	11	8	9	7	9	11
Luna19	17	16	17	19	13	16	16	22	16
Luna20	20	19	21	18	16	18	18	20	17

Table 5.3: Observed heterozygosity at each locus across populations surveyed. Loci in Winter Lake D without heterozygosity information were not analyzed. Other missing heterozygosity information is for loci with only one allele in the population. A and B locations are at Rattlesnake Bar Road. Location C is at the Park Gate, and location D is at Old Salmon Falls.

Locus	Summer Lake A 11/07	Summer Lake B 11/07	Winter Lake A 4/08	Winter Lake B 4/08	Winter Upland C 4/08	Summer Lake B 7/08	Summer Lake B 10/08	Summer Lake D 10/08	Winter Lake D 2/09
Luna1	0.867	0.933	0.667	0.733	0.850	0.900	0.667	0.724	0.367
Luna2	0.500	0.433	0.333	0.267	0.300	0.400	0.400	0.539	-
Luna3	0.267	0.333	0.400	0.233	0.550	0.133	0.241	0.450	-
Luna4	0.367	0.167	0.433	0.133	0.200	0.133	0.167	0.458	0.000
Luna6	0.667	0.551	0.867	0.533	0.600	0.690	0.533	0.825	-
Luna7	0.400	0.533	0.379	0.400	0.684	0.300	0.642	0.557	0.267
Luna8	0.551	0.800	0.783	0.636	0.667	0.750	0.583	0.742	-
Luna9	0.633	0.667	0.467	0.423	0.600	0.400	0.551	0.623	-
Luna10	0.333	0.267	0.267	0.133	0.300	0.233	0.233	0.726	0.067
Luna11	0.379	0.414	0.300	0.233	0.250	0.333	0.292	0.639	-
Luna12	0.267	0.400	0.400	0.433	0.350	0.233	0.267	0.890	0.167
Luna13	0.467	0.500	0.367	0.533	0.350	0.433	0.433	0.581	-
Luna14	0.567	0.517	0.517	0.533	0.450	0.567	0.300	0.672	-
Luna15	0.033	-	-	0.000	-	0.067	0.000	-	-
Luna16	0.793	0.733	0.733	0.500	0.750	0.633	0.600	0.738	0.333
Luna17	0.067	0.167	0.133	0.333	0.300	0.267	0.133	0.186	-
Luna18	0.733	0.767	0.667	0.567	0.550	0.633	0.600	0.858	0.233
Luna19	0.897	0.862	0.867	0.633	0.750	0.400	0.800	0.944	0.433
Luna20	0.900	0.933	0.767	0.733	0.950	0.833	0.828	0.924	0.367
Mean	0.510	0.525	0.492	0.421	0.497	0.439	0.435	0.442	0.248
S. D.	0.263	0.263	0.240	0.205	0.241	0.240	0.227	0.209	0.146

F-statistics, which compare heterozygosity of individuals (I), subpopulations (S), groups of subpopulations (C), and the total population (T), were also used (Arlequin version 3.11, Excoffier et al 1992). The inbreeding coefficient, F_{IS}, compares the heterozygosity of an individual relative to the subpopulation. The coefficient F_{IT} compares heterozygosity of individuals to the total population heterozygosity. Each coefficient can range from -1, where every individual is highly heterozygous, to +1, where all individuals are highly inbred. F_{ST} measures the heterozygosity of a subpopulation relative to the total population. F_{SC} compares subpopulations to their groupings in Arlequin, while F_{CT} compares the groups to the total population. For these statistics, a value of 0 means there is no difference between the subpopulations or groups, where a value of +1 means subpopulations or groups are totally different.

Results:

Within each locus, the number of alleles remained fairly constant across populations (Table 5.2). Heterozygosity also remained fairly constant across populations for each locus, with the exception of the Winter Lake plants from Old Salmon Falls, location D (Table 5.3).

When Structure was used to assign all plants to populations, the model with two populations was found to be most likely, with an alpha of 0.311. Assignment of individuals is illustrated in Figure 5.2. It was hypothesized that individuals clustering in the second (blue) population in Figure 5.2 were actually members of a closely related self-fertilizing congener, *Lupinus bicolor*. Pedicel length is a one of the few relatively reliable indicators of species identity among sympatrically growing individuals, and was analyzed to test this hypothesis. Pedicel lengths were not recorded from Winter or Summer plants at Old Salmon Falls (location D), nor from Summer Lake B plants collected in July 2008, but were collected for all other plants in the analysis. Two of the three plants that were very highly likely to be clustered with the blue group had 2-mm pedicels, whereas the other had 5-mm pedicels. Only three other individuals assigned to the *L. nanus* (yellow) group of the total 170 analyzed had pedicel lengths

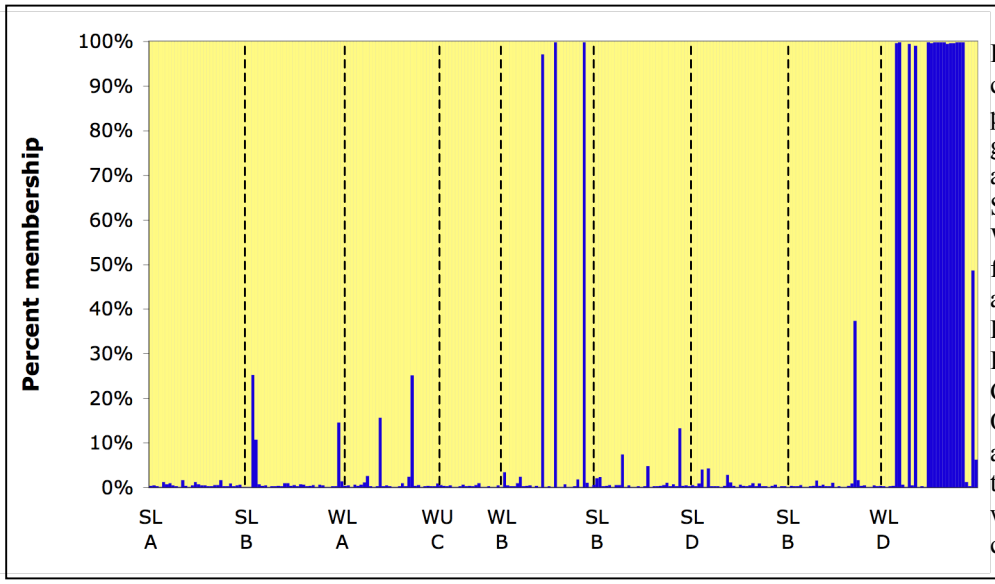

Figure 5.2: Plants cluster into two populations. Collection groups are on the x-axis. SL stands for Summer Lake, WL for Winter Lake, and WU for Winter Upland. A and B locations are Rattlesnake Bar Road. Location C is Park Gate, and location D is Old Salmon Falls. Y-axis shows percent of trials in which the plant was assigned to that cluster. Alpha = 0.311.

Source of variation	d. f.	Sum of squares	Variance component	Percentage of variation
Between lake and upland groups	1	7.383	-0.004	-0.07
Between populations within groups	1	7.836	0.021	0.39
Among individuals within populations	74	491.728	1.215	22.31
Within individuals	77	324.500	4.214	77.36
Total	153	831.474	5.447	

Table 5.4: AMOVA of Rattlesnake Bar (locations A and B) Winter Lake and Park Gate (location C) Winter Upland plants.

of less than 3 mm. Additionally, all plants identified as belonging to the second (blue) population were homozygous and shared alleles at almost all loci. As further elaborated in the discussion section, these data support the hypothesis that the plants in the blue population are actually members of *L. bicolor*. Consequently, the Winter Lake D plants and the other three individuals identified as *L. bicolor* were removed from further analyses. The Structure analysis was repeated without the *L. bicolor* individuals and only one population was detected.

To examine if plants from lake and upland sites differed genetically, an AMOVA was performed to compare Winter Upland and Winter Lake plants collected in April 2008 from the Park Gate (location C) and Rattlesnake Bar Road (locations A and B) (Table 5.4). The inbreeding coefficient comparing the observed heterozygosity of individuals from the heterozygosity expected within their subpopulations, F_{IS}, is 0.224 (p=0.000). The deviation of the observed heterozygosity of individuals from that expected across the total population, F_{IT}, was 0.226 (p=0.000). Comparison of the heterozygosities of the subpopulations to that expected from their groupings as Lake or Upland, F_{SC}, was 0.004 (p=0.155). Comparison of heterozygosities of Lake and Upland groups versus heterozygosity expected from the total population reveals F_{CT} was -0.001 (p=1.000).

To determine if Summer Lake plants on the North Fork (Rattlesnake Bar Road, location B) and the South Fork (Old Salmon Falls, location D) of the lake are part of the same population, an AMOVA was performed using plants collected from these locations in October of 2008 (Table 5.5). To exclude any possible difference based on year of collection, only plants collected in 2008 were used. Individual F- statistics F_{IS} and F_{IT} were 0.300 (p=0.000) and 0.309 (p=0.000) respectively. F_{ST}, a comparison of the North and South fork subpopulations to the total population, was 0.013 (p=0.299).

To examine the relationship between Summer Lake and Winter Lake plants growing at Rattlesnake Bar Road (locations A and B), an AMOVA was performed using Summer Lake

Source of variation	d. f.	Sum of squares	Variance component	Percentage of variation
Between North fork and South fork populations	1	10.650	0.066	1.27
Among individuals within populations	58	318.117	1.546	29.66
Within individuals	60	216.000	3.600	69.07
Total	119	614.767	6.212	

Table 5.5: AMOVA of Summer Lake at the North fork (Rattlesnake Bar Road, location B) and at the South fork (Old Salmon Ralls, location D).

Source of variation	d. f.	Sum of squares	Variance component	Percentage of variation
Between Summer Lake and Winter Lake groups	1	6.026	-0.038	-0.71
Among collections within Summer and Winter Lake groups	2	20.905	0.071	1.32
Among individuals within populations	113	713.911	0.981	18.28
Within individuals	117	509.500	4.354	81.11
Total	233	1250.342	5.159	

Table 5.6: AMOVA of Summer Lake and Winter Lake plants at Rattlesnake Bar Road (locations A and B).

plants collected in November 2007 and Winter Lake plants collected in April 2008 (Table 5.6). Individual F statistics F_{IS} and F_{IT} were 0.184 (p=0.000) and 0.189 (p=0.000) respectively. F_{SC}, which compares the subpopulations to the Summer and Winter groupings was 0.013 (p=0.000). Comparison of the Summer and Winter groups to the total population F_{CT} was -0.007 (p=1.000).

Discussion:

Allele number and heterozygosity were surprisingly similar across all populations, with the exception of the heterozygosity of the Winter Lake plants at Old Salmon Falls (location D), which includes a high proportion of *L. bicolor* individuals. The similarities in these basic genetic parameters suggest that the plants from these spatiotemporal locations may be interbreeding. Indeed, while the heterozygosity is low among Winter Lake plants at Old Salmon Falls, the number of alleles is similar, and there is only one new allele in these plants. This result suggests very recent gene flow among these sites.

Using the Structure program to assign plants to populations, some of the plants in the Summer Lake at Old Salmon Falls (location D) clustered with plants from other environments around the lake. Most of these plants had pedicel lengths of 2mm, and were homozygous at almost all loci. *Lupinus bicolor* is very closely related to *L. nanus*, and they can be difficult to distinguish. While *L. nanus* has an average outcrossing rate of 0.72, *L. bicolor* is a selfing species, with an average outcrossing rate of 0.04 (Harding et al 1974). One of the few reliable ways to distinguish these plants is that *L. bicolor* has a pedicel length of 1-3 mm, while *L. nanus* has a pedicel length of 3-7 mm (Dunn 1955). These facts suggest that the only observed segregation was between *L. bicolor* and *L. nanus*.

There were no *L. bicolor* collected or observed in the Summer Lake environment, but they were more common in the Winter Lake environment, especially at Old Salmon Falls. It may be that *L. bicolor* lacks the phenotypic plasticity in germination response that apparently occurs in *L. nanus*, with the result that they are not found growing in the Summer Lake environment. The higher proportion of *L. bicolor* in Winter Lake at Old Salmon Falls, as compared to Winter Lake at Rattlesnake Bar Road, may be due to collection error. The leaf samples at Old Salmon Falls were collected very early in the season, when the plants were small and not yet flowering. All Winter Lake plant samples from other locations were collected later in the season, when the plants were tall and flowering. Since *L. nanus* usually has larger and showier flowers than *L. bicolor*, it could be that when collecting during flowering, *L. bicolor*

plants were overlooked in favor of the larger flowered *L. nanus*. Finally, the Old Salmon Falls location might be more suitable for *L. bicolor* due to some undiscovered environmental factor.

All of the AMOVA analyses showed that the majority of variation in allele frequencies occurred within or among individuals rather than among locations or germination seasons. The low and insignificant values of F_{ST} and F_{CT}, which reflect differences between subpopulations or groupings, suggest there is no difference between the subpopulations or groups. This lack of population differentiation is likely due to high gene flow between the plants. Many studies have demonstrated that high gene flow can prevent population differentiation even when natural selection is occurring (Storfer 1999, Bittner and King 2003, Bolnik and Nosil 2007, Moore et al 2007).

After removing *L. bicolor* from the analyses, it is clear that there is no genetic differentiation among *L. nanus* collected from the Summer Lake, Winter Lake, and Winter Upland environments. The similarities in the genetic parameters of these plants suggest that the plants are not reproductively isolated and are sharing genetic information among seasons. This supports the hypothesis that Summer and Winter plants are part of the same population, and can respond to germination cues that occur at least twice during the year. The presence of phenotypic plasticity in germination response may be adaptive, allowing these plants to live in a variety of environments. Alternatively, it may eventually lead to adaptation and population differentiation of the Summer and Winter plants.

Conclusion

When faced with anthropogenic change, individuals may experience phenotypic plasticity in response to different environments, or the species may go extinct, migrate, or undergo natural selection. The water level fluctuations at Folsom Lake are associated with a novel Summer germinating phenotype. This dissertation has tested whether these populations have merely undergone phenotype plasticity or have undergone genetic changes allowing adaptation to this novel environment. If the Winter and Summer phenotypes represent evolved responses, then Winter and Summer plants would be considered separate populations, reproducing independently, and likely possessing genetically-determined germination cues at a particular time of year, as well as other morphological and/or physiological traits that are adapted to their specific spatiotemporal environment. If they have merely undergone phenotypic plasticity, all the Folsom Lake plants could instead belong to one population, in which seeds produced in one growing season may either remain dormant or germinate immediately for the next growing season, according to environmental cues. Most of the experiments presented are consistent with the hypothesis that the Summer and Winter germinating plants are part of the same population that responds to multiple germination cue events during a single year.

I found no evidence that Summer Lake plants possess a selective advantage in the Summer Lake environment. In a common garden experiment growing plants from Summer Lake, Winter Lake, and Winter Upland environments in the harsh Summer Lake conditions, survival was very poor across all source locations. There was also no genetic evidence for selection on natively growing Summer Lake plants during the course of a season, although only neutral markers were used. Microsatellite analysis comparing natively growing Summer Lake plants from early in the season to those that survived to reproduce showed no genotypic differences between these plants, suggesting that there is no differential selection based on genotype for plants germinating in this environment. This result is not definitive because microsatellite polymorphism is assumed to be neutral; further work is needed to measure selection on phenotypes.

Next, I reproduced the wet/cold – dry/hot cycle caused by the fluctuation zone, and placed untreated Winter Lake seeds in this environment. Seeds germinated in response to this wet/cold – dry/hot cycle, showing that it might provide a germination cue that allows Winter Lake seeds to germinate in the Summer Lake environment. Further experiments with additional controls and scarification of some of the seeds will elucidate the germination cues.

Finally, I used microsatellite markers to examine population structure among plants growing in various environments around the lake. I detected a related species, *Lupinus bicolor*, only in the Winter Lake populations, and removed this species from further analysis. Analysis of the *L. nanus* around the lake strongly suggests that all Summer Lake, Winter Lake, and Winter Upland *L. nanus* at Folsom Lake belong to the same population, and that there is no genetic distinction between these plants.

These results support the hypothesis that the Summer and Winter plants at Folsom Lake are all part of the same population and that individuals can respond to germination cues twice during the course of a year. There is an additional alternative hypothesis that the Summer and Winter Lake plants are just beginning the process of becoming separated populations, because

they experience different selective pressures and are likely to have increased reproductive isolation due to different phenology. Were this to happen, it would likely take several centuries for the populations to become completely separate, given the probable size of the seed bank (Moore 2009) and the fact that there is no sign of such isolation occurring after fifty-four years of lake level fluctuations at Folsom Lake. Analysis of functional genes likely to be under selection, such as genes involved in drought tolerance or flooding tolerance, might provide a more detailed look at this separation in progress if it is occurring.

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