

THESIS

EXAMINING MATERNAL EFFECTS AND GENETIC DIFFERENTIATION IN *P. FLEXILIS*
AND *P. ARISTATA* TO IMPROVE SUCCESS OF CONSERVATION ACTIONS

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ABSTRACT

EXAMINING MATERNAL EFFECTS AND GENETIC DIFFERENTIATION IN *P. FLEXILIS* AND *P. ARISTATA* TO IMPROVE SUCCESS OF CONSERVATION ACTIONS

As the climate changes and invasive species continue to spread, proactive management may be needed to conserve native plant populations. Selecting appropriate plant material for restoration or other actions that will sustain populations is an integral part of any such plan and must take into account genetic differentiation to limit maladaptation. Common garden studies are used to determine the genetic basis of trait variation among populations from different geographic sources. However, maternal effects, the effect of environment during offspring development, can also affect performance, complicating the interpretation of these studies. Growing one generation in a common environment can help correct for maternal effects, but is often not practical with long-lived species. Using limber pine (*Pinus flexilis*) and Rocky Mountain bristlecone pine (*Pinus aristata*) as model species, I explored the contribution of maternal effects to early seedling growth among populations in a greenhouse common garden study. I grew offspring sourced over multiple years from the same mother trees, comparing growth traits between source years. Additionally, I collected five twig clippings from the upper canopy of each mother tree and measured characteristics indicative of the relative vigor of the tree during each seed source year.

There were significant ($p < 0.05$) differences in year-to-year variation in twig growth characteristics, seed size, and seedling performance. For bristlecone pine, there was a significant positive relationship between the relative inter-annual (RIA) variation in seed mass and seedling

total dry mass and a negative relationship between the RIA variation in seed mass and needle growth at 210 days. For limber pine, there were significant positive relationships between RIA variation in seed mass and cotyledon length, stem height, stem diameter, and needle length at 20, 120, and 190 days. These results a) support the hypotheses that maternal effects are evident in both *P. flexilis* and *P. aristata* and that these effects translate into variation in early seedling growth and b) suggest possibilities for statistically correcting for maternal effects in genetic differentiation common garden studies involving long-lived species.

Using these data I then conducted a common garden greenhouse study to determine the degree of genetic differentiation in limber pine populations in the Southern Rockies. Mid-summer precipitation varies greatly along a latitudinal gradient throughout this region, potentially selecting for local adaptation of populations to their native moisture regime. I evaluated the differential response of seed sources from northern and southern portions of the range to different moisture regimes during early seedling growth. To test whether seedling growth traits, which are often adaptive, differed between northern and southern seed sources, I measured primary needle length, stem diameter, water potential, and biomass allocation between root and shoot before, during, and after treatments. To test for differentiation in the adaptive traits associated with water use, I also measured carbon isotope ratios ($\delta^{13}\text{C}$) as a proxy for water use efficiency. To account for maternal effects I used cotyledon length as a covariate, which I found in the previous study to be a good indicator of maternal year-to-year variation in seedling growth.

There were significant ($p < 0.05$) effects of source region for root length, stem diameter, needle length, and total dry mass, where seedlings from southern sources were bigger than those from northern sources. Seedlings from the north had a higher probability of mortality than those

from southern populations, as did seedlings in the dry treatment. The only significant interaction between growth response and source region, signifying the possibility of local adaptation of populations, was with the carbon isotope ratio ($\delta^{13}\text{C}$, $p < 0.1$). All seedlings in the dry treatment regardless of origin had higher $\delta^{13}\text{C}$, while seedlings in the wet treatment varied between slightly higher (southern populations) and lower (northern populations) values of $\delta^{13}\text{C}$. These data indicate that genetic differentiation exists among populations in the Southern Rockies, potentially increasing the risk of maladaptation when moving seed far from its source.

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INTRODUCTION

Background

As invasive pests continue to thrive and the climate changes from historical conditions in which species have evolved, many plant populations are becoming increasingly threatened. In some cases, supplemental plantings can enhance the genetic diversity of plant populations and shift the frequency of key adaptive traits to help ensure future success (Schoettle and Sniezko 2007, Schoettle et al. 2009). In other cases, assisted migration may be a desirable way to manage for the potential loss of range of a particular species (McLane and Aitken 2011, Williams and Dumroese 2013). In either case, research is needed to identify the patterns of genetic differentiation across specific plant populations to ensure successful future management. Research aimed at determining the presence of differentiation often involves common garden studies where populations from throughout a species' range are grown together in a common environment to better distinguish genetic differentiation among populations from differences due to environment.

Maternal effects, or the effect of the environment during development on offspring performance, can complicate the interpretation of common garden studies (Roach and Wulff 1987). Maternal effects can influence offspring directly through seed provisioning or indirectly, by influencing gene expression (Roach and Wulff 1987, Galloway 2005). Provisioning has been found to impact seed size, germination, and early growth of many plant species (Roach and Wulff 1987, Sultan 1996) while the emerging field of epigenetics is finding that conditions during seed development can act like a 'memory' (Johnsen et al. 2005), affecting gene expression in the offspring. In short-lived species, the effects of the maternal environment can be

minimized by raising at least one generation of all the source material under the same conditions (Bischoff and Müller-Schärer 2010) and using the F1 seeds in further studies of genetic differentiation. However this technique is often not feasible in long-lived species that do not reach reproductive maturity for decades. Before common garden studies can be accurately interpreted, prior research is needed to determine the magnitude of environmental maternal effects present and find potential statistical or procedural methods to control for them.

To examine maternal effects in this study, I grew seed sourced over multiple years from the same mother plant in a greenhouse common garden to evaluate the difference in seed and seedling growth traits between years. Using this technique allowed me to keep genetics as constant as possible to more closely decipher the role environmental maternal effects play in the early growth of two pine species, *Pinus flexilis* (limber pine) and *P. aristata* (Rocky Mountain bristlecone pine). Additionally, I collected twig clippings from each mother tree to better detect the differences in growth environments during the years of seed collections. With these data, it was possible to test for differences among twig growth characteristics and seed size between years, and then determine if those differences affected seedling performance. This study tested the technique of using seed from multiple years to evaluate the role of maternal effects in early seedling growth while providing evidence for growth metrics that could help correct for maternal effects in future common garden studies.

Taking the maternal effects data into account, I then conducted a greenhouse common garden study with *P. flexilis* to determine the degree of genetic differentiation among populations in the southern Rocky Mountains to better prevent maladaptation when seed is moved from its native source for conservation efforts. Limber pine is currently threatened and will require active management to conserve, which may require moving seed far from its native environment. High-

elevation five-needle pine ecosystems of western North America, such as those with whitebark, limber, and bristlecone pines, are vulnerable to the impacts caused by the non-native fungus *Cronartium ribicola* that causes the lethal disease white pine blister rust (WPBR). Since its accidental introduction to the Vancouver, British Columbia area around 1910, WPBR continues to spread and is now threatening southern Rocky Mountain limber pine forests (Fig. 1). WPBR has contributed to high mortality in whitebark pine populations in the northern Rockies and has been a factor in petitioning that species to be listed as endangered under the Endangered Species

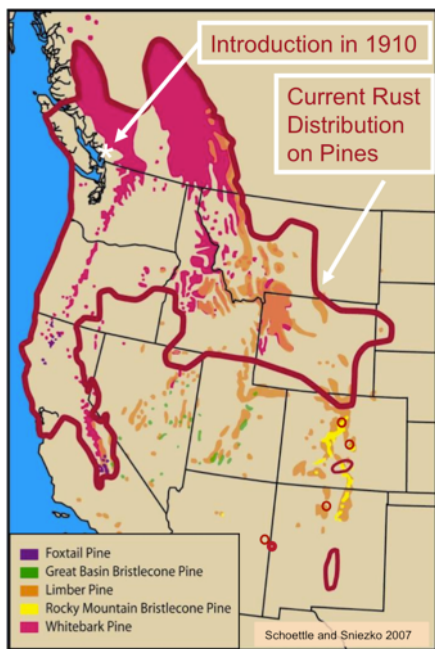


Fig. 1 Current distribution of WPBR, circled in red, overlaid with the distribution of five-needle pines. Limber pine distribution is in orange (from Schoettle and Sniezko 2007).

Act (NRDC 2008). It is also infecting increasingly more limber pine in Canada, causing the tree species to be listed as endangered in 2008 in Alberta under *The Wildlife Act* (Government of Alberta 2010).

In the Southern Rockies limber pine has been infected in the field with *C. ribicola* in central Wyoming since the 1970s, southern Wyoming since the mid-1990s and was first detected in Colorado in 1998 (Johnson and Jacobi 2000). The disease has since been found in several other National Forest lands along the Colorado Front Range, in the Great Sand Dunes National Park and Preserve in 2004, and in Rocky Mountain National Park in 2009 (Blodgett and Sullivan 2004, Schoettle et al. 2011). Risk assessment reveals that more than

half of these high elevation forests have environmental conditions annually that are conducive to the disease while the other half have environments that support the disease less

regularly, suggesting that WPBR will continue to spread throughout the five-needle pine forests of the Southern Rockies (Howell et al. 2006, Kearns et al. 2013).

The effects of WPBR are compounded by threats from mountain pine beetle (*Dendroctonus ponderosae*) and climate change. Whereas blister rust can quickly kill young limber pine trees, mountain pine beetle kills mature trees, reducing reproductive ability of the stand. Limber pine forests are often in marginal habitats and are among those ecosystems that are suspected to experience vast changes as the climate becomes warmer and drier in the western United States, increasing the likelihood of fire and drought stress (IPCC 2007).

The potential for widespread mortality of this species is concerning because of the ecological importance of limber pine. Limber pine seeds provide an excellent source of nutrition for Clark's Nutcrackers (*Nucifraga columbiana*) and black bears (*Ursus americanus*), contributing to the health of those animal populations (Tomback and Achuff 2010). Limber pines tend to dominate xeric regions that cannot support other tree species; their presence prevents erosion, creates habitat, and caches snow (Schoettle 2004a, Coop and Schoettle 2011). Those growing near treeline help retain snow and slow its melt in the spring (Schoettle 2004a). The loss of this species would have profound ecological effects.

Research Basis

Work has already begun in the management and restoration of whitebark pine forests devastated by WPBR in the Northern Rockies (Keane and Arno 2000). Seed transfer guidelines have been developed based on results from common garden studies (Bower and Aitken 2008), planting guidelines have been established and refined (McCaughey et al. 2009), and management guidelines are in place to protect whitebark pine forests now and into the future (Hunt et al. 2010, Schwandt et al. 2010, Keane et al. 2012).

Work has only just recently begun in the management and conservation of limber pine in the Southern Rockies, however (Burns et al. 2008). The research proposed here enhances a project that began in 2001 to conserve this species by supporting the natural recovery of limber pine from the threats of WPBR (Schoettle 2004b). Past research has confirmed the presence of genetic resistance to WPBR in limber pine in the Southern

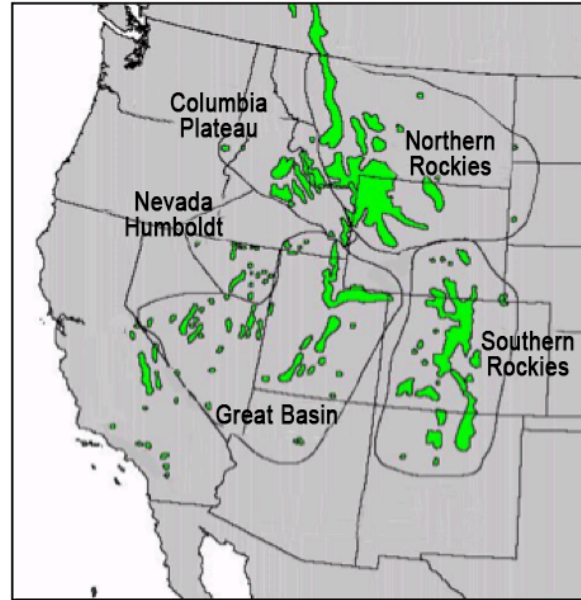


Fig. 2 Seed transfer guidelines for limber pine split into five zones (from top clockwise): Northern Rockies; Southern Rockies; Great Basin; Nevada Humboldt; and Columbia Plateau (Mahalovich 2006).

Rockies (Schoettle et al. 2009; Schoettle et al. 2013). The resilience of five-needle pine ecosystems to this invasive disease can be increased through targeted restoration with resistant seed stock and regeneration opportunities (Schoettle and Sniezko 2007). In preparation for planting, resistance mechanisms and their geographic distributions are being investigated (Schoettle et al. 2009, Schoettle et al. 2013), outplanting tests have been conducted (Casper et al. 2011), and guidelines are being prepared. Survival of nursery-grown seedlings in the field has been high during the first two years after planting at all test locations from southern Colorado to southern Wyoming. However, the long-term success of management treatments involving proactive restoration depends not just on the establishment of the planted seedlings, but also on their continued ability to succeed in the varied climates where outplanting is needed.

Seed zones were developed to account for the fact that plants are often locally adapted to their native climate based on generations of selection on adaptive traits and thus perform best

close to where they evolved. Current seed transfer guidelines for limber pine delineate the Southern Rockies as one seed zone with elevation restrictions of +/- 200m (Fig. 2, Mahalovich 2006). While this zone was defined using the best available information, including limited genetic studies and examination of morphological traits, it has not yet been tested. Because patterns of resistance to WPBR are not uniform across the landscape (Schoettle et al. 2013) and northern Colorado populations have a greater frequency of resistance than those in southern Colorado (Schoettle et al. in prep), long-distance movement of seed throughout the zone will likely be needed to supplement resistance in some populations. The potential for maladaptation with long-distance movement of seed sources is a distinct possibility given the broad elevational (1600-3400m) (Schoettle and Rochelle 2000) and latitudinal (33-43°N) range within this region. Mid-summer moisture availability varies greatly along this latitudinal gradient and local adaptation to the timing of water events is likely to have evolved among populations.

The second common garden study explored the presence of genetic differentiation among limber pine populations in the southern Rockies, providing insight into the likelihood of maladaptation following movement of seeds from their native source. Together, the maternal effects and genetic differentiation studies help provide the scientific basis to improve the success of conservation efforts of five-needle pines in the Southern Rockies.

CHAPTER 1: UNDERSTANDING MATERNAL EFFECTS IN LONG-LIVED PLANT SPECIES TO IMPROVE INTERPRETATION OF COMMON GARDEN STUDIES

INTRODUCTION

As climate shifts away from historical norms and invasive species continue to spread, more research is being done to better understand methods to conserve threatened plant species. Conservation actions like targeted restoration or assisted migration must take into account the source population to ensure successful establishment and continued growth. Common garden studies are often used to test for genetic differentiation, local adaptation, and trait plasticity among populations that could lead to maladaptation in conservation actions. However, it is well established that maternal effects, the influence of the environment during seed development on offspring performance, can influence early seedling growth, complicating the interpretation of common garden studies (Roach and Wulff 1987, Bischoff and Müller-Schärer 2010). Maternal effects can be both genetic and environmental in origin (Roach and Wulff 1987, Rossiter 1996); environmental maternal effects will be the focus in this study.

Maternal effects can influence offspring directly through seed provisioning or indirectly, through influencing gene expression (Roach and Wulff 1987, Galloway 2005). In gymnosperms, both the seed coat and the megagametophyte, which provides food for the germinating seedling, are of maternal origin (Kramer and Kozlowski 1979). Larger seeds are generally associated with larger seedlings due to the fact that there are more available reserves for the seedling to use in its early life (Reich et al. 1994, Westoby et al. 1996). Provisioning due to maternal effects has been found to impact seed size, germination, and early growth of many plant species (Roach and

Wulff 1987, Sultan 1996), including trees (Castro 1999, Castro et al. 2008, Oliver and Borja 2010, Gonzalez-Rodriguez et al. 2012). The emerging field of epigenetics explores how the environment during seed development influences gene expression, unrelated to changes in DNA, in offspring (Wolffe and Matzke 1999). Epigenetics can be thought of as a ‘memory’ of environmental conditions during seed development (Johnsen et al. 2005). For example, a study found that temperature during seed development affected timing of bud set and cold hardiness in Norway spruce progeny (Johnsen et al. 2005). This study focuses primarily on the provisioning aspect of maternal effects.

Maternal effects can vary among closely related species and so far, no clear trends have been found to predict their influence (Roach and Wulff 1987), causing them to remain a source of uncertainty in common garden studies of long-lived organisms. It is generally accepted that maternal effects are strongest in the earliest stages of a plant’s life and then diminish over time, often lasting at least one growing season (Roach and Wulff 1987, Reich et al. 1994). Growing one or more generations from random crosses in one common environment can minimize variation due to maternal environment (Bischoff and Müller-Schärer 2010), though this technique is often not feasible for long-lived species that do not reach reproductive maturity for decades.

Rather, the technique proposed here is to examine the magnitude of maternal effects through raising seeds across multiple growing seasons from the same maternal plant (Carles et al. 2009). Very few studies have used this method to examine maternal effects (Castro 1999, Castro et al. 2008, Oliver and Borja 2010) and none have highlighted the effect of the maternal environmental, rather than variations due to family effects that could be genetic or environmental in nature, on offspring growth. By using seed from the same maternal plant over various years,

genetics are held as constant as possible in an open pollinated system and it is possible to test if the environment had any effect on seed or seedling characteristics by exploring correlations between the change in environment, seed traits, and seedling performance between years.

This study assessed the effect of the environment during seed development on seed characteristics and early seedling growth using seed from multiple maternal trees from at least two different collection years from *Pinus aristata*, Rocky Mountain bristlecone pine, and *Pinus flexilis*, limber pine. The objectives were 1) to determine if year-to-year differences in environment during seed maturation affect offspring seed characteristics, 2) to evaluate whether those differences in seed traits between years correspond to a difference in early seedling growth, and 3) to evaluate the multiple year sampling method for assessing maternal effects in long-lived plants. It was predicted that greater variation in twig growth between years would correspond to greater variation in seed mass, which would relate in turn to more variation in seedling growth.

METHODS

Seed Source

Both *P. aristata* and *P. flexilis* seeds had been previously collected and stored at -20°C at the USDA Agricultural Research Service National Center for Genetics Resources Preservation lab in Fort Collins, Colorado. Seed sources originated across northern Colorado and southern Wyoming (Fig. 3) from multiple open-pollinated mother trees per population. *P. aristata* seeds from twelve seedtrees and three populations sourced from 2008 and 2009 were used. *P. flexilis* seeds from eighteen seedtrees and seven populations sourced from 2003, 2008, or 2009, were

also used (Table 1). Seeds were x-rayed to ensure the presence of a full embryo before thirty seeds per family per year were randomly selected for inclusion in the study.

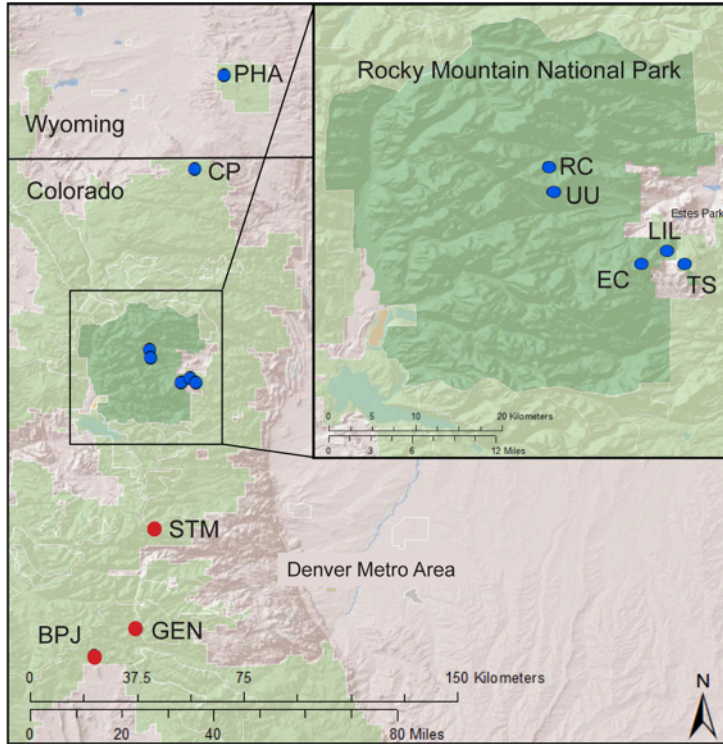


Fig. 3 Site locations for *P. aristata* (red) and *P. flexilis* (blue).

Table 1. Seed sources included in the maternal effects study. Thirty seeds per family per source year were sown.

Species	Population	# of seed trees	Source years
<i>P. aristata</i>	STM	7	2008, 2009
	GEN	2	2008, 2009
	BPJ	3	2008, 2009
<i>P. flexilis</i>	PHA	2	2003, 2009
	CP	2	2003, 2009
	RC	5	2008, 2009
	UU	2	2008, 2009
	EC	3	2008, 2009
	LIL	3	2008, 2009
	TS	1	2008, 2009

Experimental design

Maternal plants from at least two years from twelve open pollinated *P. aristata* families and eighteen *P. flexilis* families from the Southern Rockies were included in this study. Twig clippings were collected from each seedtree to observe the differences in mother tree growth between years. Thirty seeds from each seedtree and each source year were randomly assigned to ten replicate blocks in a greenhouse common garden to monitor differences in early seedling growth between source years. Seedling stem height (measured from soil to the base of cotyledons), cotyledon length, and primary needle length were measured throughout the eight months of the study before seedlings were destructively harvested for stem diameter, root, and shoot biomass measurements.

Twig Clippings

Twig growth traits were included in the study to detect environmental differences experienced by each seedtree between seed collection years. It is often assumed that changes in weather measured regionally predictably impact trees on a local level, but microsite can greatly influence what weather conditions are actually experienced by a plant. To detect environmental effects on mother tree growth during seed development, five twig samples from the upper canopy of each seedtree were collected during the summer of 2012. Straight twigs with no branching or cone development were collected from the south side of each tree in full sun. Measurements were taken on each year's twig growth including increment length (twig extension), average needle length, number of fascicles, and specific leaf area (SLA) using the methods of Schoettle (1994). SLA was calculated from the projected leaf area of that year's needle cohort (Delta-T Area Meter System, Cambridge, England) divided by the dried mass of

the needle cohort (65°C, 48h). Twig data from 2003 were limited by length of clippings and abscised needles. The purpose of including the twigs was to characterize mother tree growth during seed provisioning, so tree twig data from the year of seed collection was used in analyses for all traits except number of fascicles, which is determined in the branch the year prior to cone maturity. See Table 2 for a list of all traits measured for twigs and seedling.

To see if twig growth was related to local weather conditions, I used precipitation and temperature data collected daily by the USDA Natural Resources Conservation Service at Snowpack Telemetry (SNOTEL) stations throughout the region. Accumulated precipitation (Sensotec 100" Transducer) and average temperature (Extended Range) data were gathered from the Bear Lake SNOTEL site 322, which is roughly central to study sites.

Table 2 Traits measured from mother tree twigs (left), seeds (center) and seedlings (right).

Twig traits measured	Seed traits measured	Seedling traits measured
Increment length	Seed mass	Stem height
Twig needle length	Seed length	Cotyledon length
Specific leaf area	Seed width	Primary needle length
Number of fascicles		Relative growth rate
		Stem diameter
		Total dry biomass
		Root-to-shoot ratio

Common Garden

P. aristata and *P. flexilis* seeds sourced over multiple years from the same mother tree were grown in a greenhouse to compare growth traits between source years. Each seed was weighed and seed length and width were measured and recorded. *P. flexilis* seeds underwent a 1-2°C moist stratification period of six weeks in a growth chamber (Precision Scientific LT-105, Perry, IA). Seeds were kept separate in labeled mesh bags strung over a sealed plastic tub filled

with approximately one inch of water. Seeds were rinsed weekly and checked for mold. Moldy seeds were rinsed with a 1% solution of H₂O₂. After stratification seeds were sown in ten randomized complete blocks per species with three replicates per block. The planting medium was composed of a 1:1 ratio of 4P mix (Conrad Fafard Inc, Agawam, MA) and sand to increase drainage; pots used were 656ml deepots D40h (Stuewe and Sons, Inc, Tangent, OR). Once sown, seeds were watered daily until germination was complete, then watered 2-3 times per week and fertilized weekly with Jack's Professional 20-8-20 Forestry (JR PETERS, Inc, Allentown, PA) at 100ppms. Greenhouse temperatures varied between 17-22°C with a 16H/8H photoperiod.

Seedling performance was assessed by measuring stem height, cotyledon length, primary needle length, relative growth rate, stem diameter, and dry biomass (Table 2). Relative growth rate (RGR) was calculated as follows:

$$[1] \quad RGR = \frac{\ln(s_2 - s_1)}{(t_2 - t_1)}$$

where s is equal to the length of needles in millimeters and t is equal to time in days. Date of emergence was recorded for each seedling. Stem and cotyledon length were measured once at twenty days after emergence as these did not change over time, while monthly measurements of primary needle length and mortality were also made. After eight months, all seedlings were harvested for diameter, root, and shoot biomass measurements (65°C, 7d).

Data Analysis

I tested 1) whether twig characteristics, seed size, and seedling performance varied between years within each family, 2) whether the difference in twig characteristics between years was related to the difference in seed size within each family, and 3) whether the difference in seed size between years was related to the difference in seedling performance within each

family. To determine if there were variations in twig growth, seed size, and seedling performance, a mixed model analysis of variance (ANOVA) was used with random effects of site, family nested within site, and year nested within family and site. The significance of each random effect was tested through likelihood ratio tests where the model was run with and without each term. The effect of variation in twig growth between years on variation in seed size between years was analyzed through a linear regression. Similarly, linear regression was used to examine the effect of variation in seed size on variation in seedling performance. Prior to regression analyses, relative inter-annual (RIA) variation in each trait was calculated according to the following equation

$$[2] \quad RIA = 1 - \left(\frac{\text{Trait}_2 - \text{Trait}_1}{\text{Trait}_1} \right)$$

where Trait_2 is the average trait value in seed source year 2 and Trait_1 is the average trait value in seed source year 1 for a given family. Using the *relative* inter-annual variation takes into account the fact that twig growth, seed size, and seedling growth vary among families, allowing for a more accurate comparison of inter-annual variation, independent of differences in mean traits among families. The regression model used to determine the effect of twig growth differences during seed provisioning on seed size differences was

$$[3] \quad RIA_{\text{seed}} = RIA_{\text{twig}}$$

where RIA_{seed} is the relative inter-annual difference in seed mass between years and RIA_{twig} is the relative inter-annual difference in twig trait between years. The regression model used to determine the effect of seed size differences on seedling growth differences observed in the greenhouse was

$$[4] \quad RIA_{\text{seedling}} = RIA_{\text{seed}}$$

where $RIA_{seedling}$ is the relative inter-annual difference in seedling trait. Transformations were needed to obtain a normal distribution for some traits (Table 3). Normality was monitored through the Shapiro-Wilk test and the distribution of residuals in qqplots and histograms. The families with seed from 2003 and 2009 were analyzed separately from the families with seed from 2008 and 2009 because a) residuals could not be normalized when 2003 was coded as “year 1” along with 2008 and b) including 2003 masked significant differences in traits between 2008 and 2009. Data for each species was analyzed separately using SAS 9.3 (SAS Institute, Cary, NC).

Table 3 Transformations of seedling growth traits needed to achieve normality in regression analyses.

Species	Trait	Transformation	Species	Trait	Transformation
Bristlecone	Needle length at 20 days	Tan(x)	Limber	Diameter	Sin(x)
	RGR between 80-140 days	Tan(x)		Cotyledon length	1/x
	RGR between 140-210 days	Sqrt(x)		Needle length at 190 days	1/x
				RGR between 20-60 days	Sqrt(x)
				RGR between 60-120 days	1/x

RESULTS

Weather data indicate that the temperature was fairly consistent in difference source years and that 2003 was the wettest, followed by 2009, then 2008 (Fig. 4). The highest and lowest temperatures occurred in 2003. These weather patterns were reflected in other SNOTEL sites throughout the area and are within the normal range of weather over the last fifteen years.

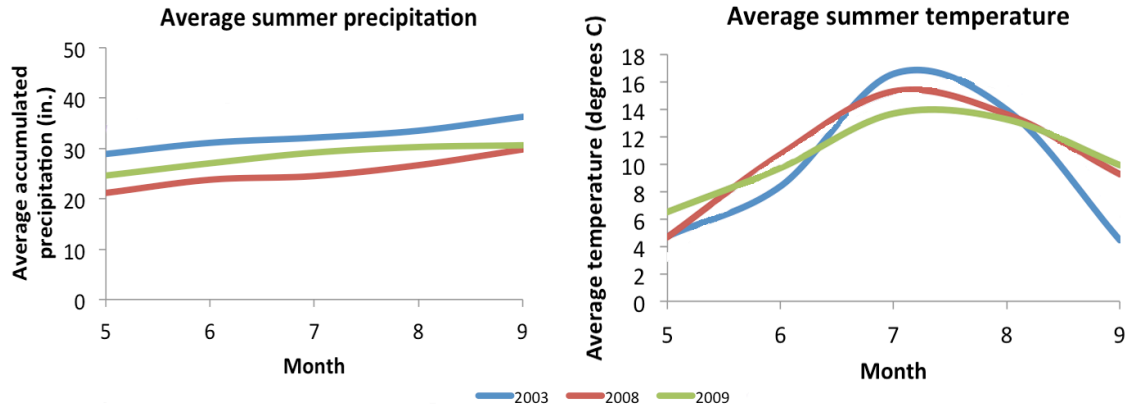


Fig. 4. Average accumulated precipitation (left) and temperature (right) during the growing season for seed collection years (from wettest to driest) 2003 (blue), 2009 (green), and 2008 (red). The highest and lowest temperatures occurred in 2003.

Data for seed length, width, and mass were used in a principal component analysis; the resulting factor PC1 had seed mass accounting for 91% of the total variance in bristlecone and 82% in limber, so seed mass was used in future analyses for ease of interpretation. Results from the ANOVA revealed between-year variation in twig traits, seed mass, and seedling performance based on site, family, and year. For the twig traits, the year effect was significant for needle length in bristlecone pine while it was significant for increment length in limber pine (Table 4). For seed mass, site and year contributed significantly to variation in both species (Table 5), where 2008 seeds were generally larger than 2009 seeds. For seedling performance, the year effect was significant in all traits except for stem diameter (bristlecone only) and root-to-shoot ratio (Table 6).

Table 4. Covariance parameter estimates of random effects for bristlecone (top) and limber pine (bottom) mother tree twig traits. The random effect year(site*family) was significant for needle length (bristlecone) and increment length (limber pine), denoted by an asterisks ($p < 0.05$).

Bristlecone Pine

CovParm	Covariance parameter estimates			
	Increment length	Needle length	SLA	Fascicle number
Site	0.03215*	1.59E-19	3.0948	0
Family(site)	0.01575*	0	3.6434	49.5686*
year(site*family)	0	0.08269*	3.3776	0
Residual	0.1076	0.1599	18.0774	230.15

Limber Pine

CovParm	Covariance parameter estimates			
	Increment length	Needle length	SLA	Fascicle number
Site	0.0775	0.1847	10.4782*	3.5644
Family(site)	0.06600*	0.08104	3.3363*	79.5171*
year(site*family)	0.02990*	0.05426	0	8.0614
Residual	0.1363	0.3721	6.2013	88.8067

Table 5 Covariance parameter estimates of random effects for bristlecone (top) and limber pine (bottom) seed mass. The year effect was significant in both species, denoted by asterisks ($p < 0.05$).

Bristlecone Pine

CovParm	Covariance parameter estimates
	Seed mass
Site	14.5571*
Family(site)	3.8797
year(site*family)	6.1723*
Residual	6.9223

Limber Pine

CovParm	Covariance parameter estimates
	Seed mass
Site	211.04*
Family(site)	67.3442
year(site*family)	228.99*
Residual	220.62

Table 6. Covariance parameter estimates of random effects for bristlecone pine (top) and limber pine (bottom) for each seedling growth trait. Asterisks denote a significant source of variation indicated by a likelihood ratio test ($p < 0.05$).

Bristlecone Pine

CovParm	Estimate				
	Stem height	Cotyledon length	Stem diameter	Total dry mass	
Site	2.9735*	4.8368*	0.006002	0.04876*	
Family(site)	0	0	0.00341	0.002393	
year(site*family)	2.1479*	4.9351*	0	0.01108*	
Residual	7.5049	13.4184	0.1057	0.1852	
	Root: shoot	RGR (30-60 days)	RGR (100-140 days)	Needle length (20 days)	Needle length (140 days)
Site	0.005430*	1.63E-6*	0	3.3595*	11.9440
Family(site)	0.000772	0	5.567E-8	0	5.2840
year(site*family)	0	1.647E-6*	1.518E-7	3.3373*	1.6456
Residual	0.03922	0.000028	3.365E-6	27.0814	83.8198

Limber Pine

CovParm	Estimate				
	Stem height	Cotyledon length	Stem diameter	Total dry mass	
Site	4.2600*	36.7667*	0.02143*	0.04919*	
Family(site)	0.976	0	0.0053	0.008379	
year(site*family)	4.2416*	24.5492*	0.00885*	0.02359*	
Residual	10.4805	32.2421	0.09264	0.1137	
	Root: shoot	RGR (30-60 days)	RGR (120-190 days)	Needle length (20 days)	Needle length (190 days)
Site	0.003339	2.22E-06	0	13.7844*	33.0101
Family(site)	0.001118	1.25E-23	1.808E-8	1.2224	22.7787*
year(site*family)	0.001271	4.879E-6*	0	6.3846*	10.7080*
Residual	0.08224	0.000035	3.436E-6	24.8365	179.94

The regression analyzing the effect of inter-annual variability in mother tree twig growth on variation in seed mass found no significant relationships for any twig traits. For bristlecone pine there was a positive relationship between the RIA variation in seed mass and variation in total seedling dry mass after eight months ($p=0.0475$ $r^2=0.272$) and a negative relationship between the RIA variation in seed mass and variation in seedling needle length measured at

seven months ($p=0.0498$ $r^2=0.266$) (Fig. 5). For limber pine there was a positive relationship between the RIA variations in seed mass and cotyledon length ($p=0.0027$ $r^2=0.6612$), stem diameter measured at eight months ($p=0.0457$ $r^2=0.3041$), stem length ($p=0.0334$ $r^2=0.3460$), and needle length measured at 20 days ($p=0.01012$ $r^2=0.4867$), 120 days ($p=0.0329$ $r^2=0.3479$), and 180 days ($p=0.0193$ $r^2=0.4147$) (Fig. 6). There were no significant relationships between the relative inter-annual variation in seed mass and any measurement of seedling growth for the families with seed sourced from 2003 and 2009.

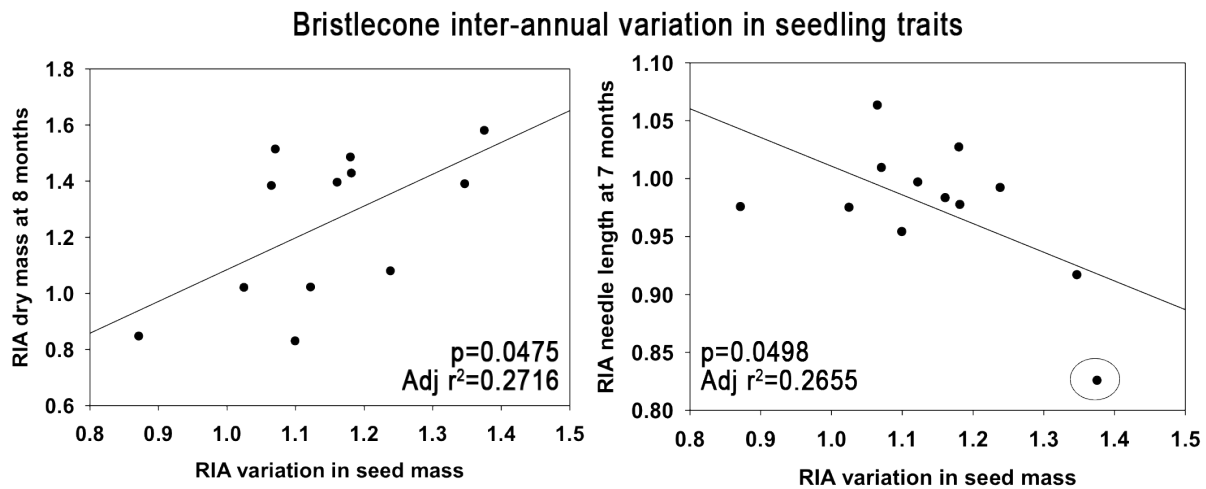


Fig. 5 Graphs of bristlecone pine depicting the relationship between the relative inter-annual (RIA) variations in seed mass and seedling dry mass (left, $p<0.05$) and needle length at 7 months (right, $p<0.05$). Trend lines represent the best fit from the linear regression analysis. The circled point in the right graph highlights the outlier that drives the negative relationship; when removed from the analysis, no significant trend is present. RIA values > 1 indicate 2008 was bigger while RIA values < 1 indicate 2008 was smaller.

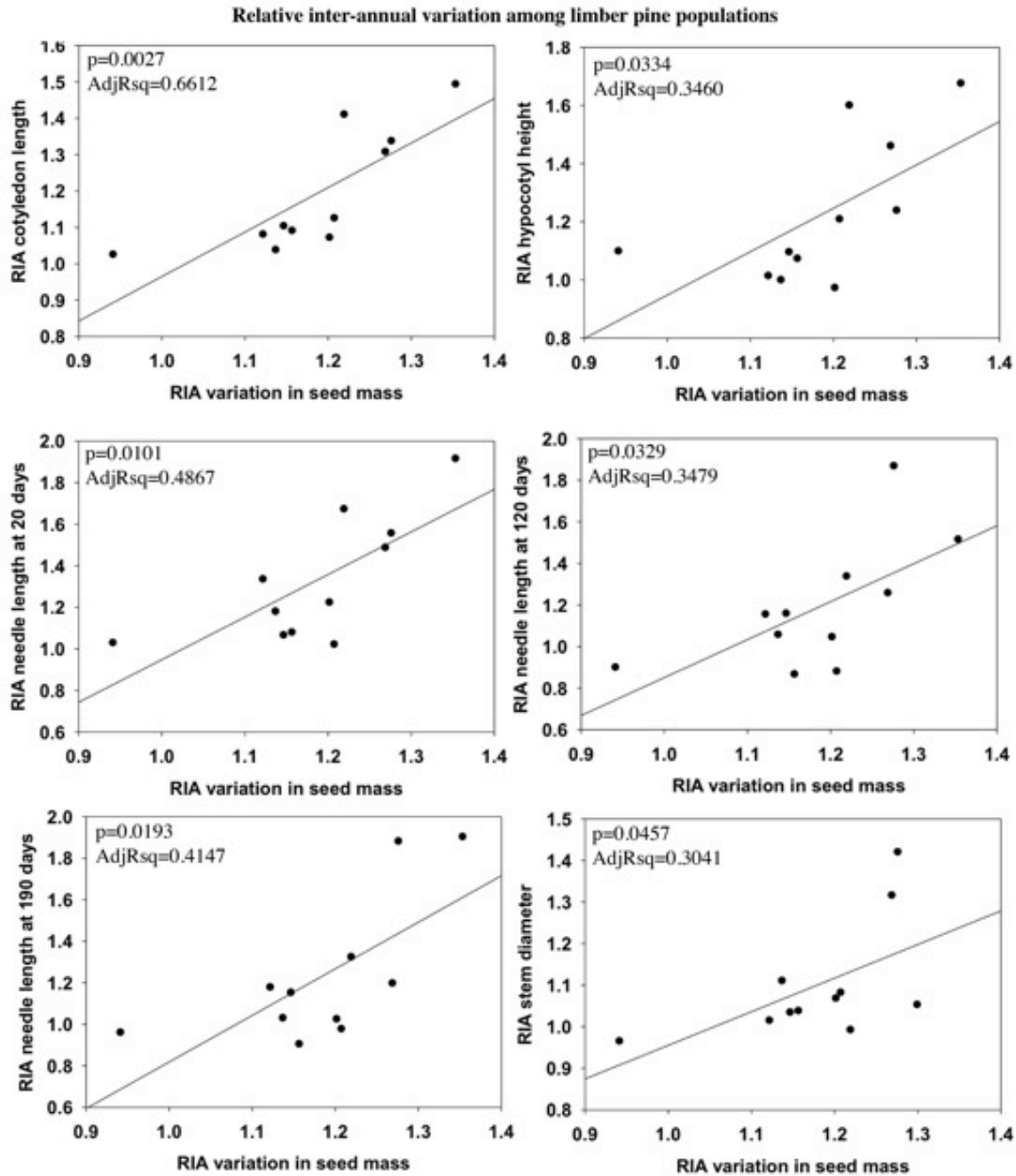


Fig. 6 Graphs depicting the relationships between the relative inter-annual (RIA) variations in seed mass and seedling traits of limber pine from top left: cotyledon length, stem height, needle length at 20 days, 120 days, and 190 days, and diameter. The positive relationship in each case signifies that years with larger seeds produced larger seedlings on average. Trend lines represent the best fit from linear regression analyses. RIA values > 1 indicate 2008 was bigger while RIA values < 1 indicate 2008 was smaller.

DISCUSSION

The goals of this study were to explore if weather during development affects seed size and whether that difference translates into variation in early seedling growth. The objectives were to 1) determine if year-to-year differences in environment during seed maturation affect offspring seed characteristics, 2) evaluate whether those differences in seed traits between years correspond to a difference in early seedling growth, and 3) to evaluate the multiple year sampling method for assessing maternal effects in long-lived plants.

The purpose of including twig clippings was to detect environmental differences in seedtree growth during seed development between seed source years. The effect of seed collection year was a significant source of twig growth variation in needle length (bristlecone) and increment length (limber), with more growth occurring in 2008, the drier year. Due to lack of twig data from 2003, there were only two observations comparing 2003 to 2009 limber pine twig growth, which was not enough to see a trend. Given that only one twig trait per species reflected the different environment during growing season, using twig clippings was not fully successful in detecting tree responses to inter-annual variability in weather. Given the lack of variation in twig traits, it is unsurprising that it was not effective in predicting variation in seed size. It could be that the relatively small sample size of twigs and the similar temperature and precipitation trends between the years with the largest samples, 2008 and 2009, prevented the ability to detect significant trends in this relationship. So while there are many other factors that contribute to the variation in seed size among years, temperature and precipitation during seed development should not be ruled out as a potential source of variation.

The regression using the relative inter-annual difference between years to examine the relationship between seed size and seedling traits did indeed find significant differences. In bristlecone pine, a greater variation in seed mass between years was associated with a greater variation in total dry mass of seedlings, but less variation in needle length of seedlings, driven by a single outlier. In limber pine, relative inter-annual variation in seed mass was positively associated with variation in cotyledon length, stem height, stem diameter, and needle length at 20, 120, and 180 days. These positive relationships indicate that the years with larger seeds were associated with larger seedlings on average. In the strongest relationship, seed mass variation explained 66% of variation in cotyledon length, suggesting cotyledon length is a good indicator of maternal effects.

This study looked at maternal environment as a source of variation, though paternal effects might have also played a role in offspring variation. The mother trees were open pollinated, meaning the pollen cloud responsible for fertilization could have come from a variety of pollen sources and likely differed year-to-year. Offspring trait variation could therefore have been influenced both by the environment during pollen development and the fact that different pollen sources (and thus paternal genotypes) may have fertilized the maternal plant in different years. However, while paternal effects may also have contributed to offspring variation, they tend to be less influential than maternal effects (Roach and Wulff 1987).

The years included in this study were determined by what seed had already been collected and was available for use and happened to be from years where the weather did not differ drastically. The study duration was also relatively short in relation to the species' lifespans, lasting less than one year. Time and resource permitting, it would be interesting to see how the

difference in maternal environment affects offspring growth over the first several years of seedling life using seed that developed in environmental conditions that differed more.

In cases like this with prolonged maternal effects, visible throughout the eight months of this study, the influence of the maternal environment on seedling performance needs to be accounted for in common garden studies. As no pattern has been found yet to predict maternal effects in any given species (Roach and Wulff 1987), common garden studies hoping to determine the presence of genetic differentiation should account for the difference in seedling size statistically. Bischoff and Müller-Schärer (2010) found in their study with angiosperms that using seed mass as a covariate was not effective in reducing maternal effects and tended to underestimate genetic differentiation in common gardens. They suggested instead using initial plant size to better account for maternal provisioning. We found in this study that cotyledon length in both species detected the year-to-year variation in maternal plant and that the years with larger seeds were associated with larger cotyledons, a finding mirrored in Reich 1994. Growth of cotyledons is related to the size of megagametophyte reserve in the germinating seedling (Sasaki and Kozłowski 1970), so larger seeds are able to produce seedlings with longer cotyledons. Therefore, we propose using cotyledon length as a covariate to account for any residual maternal effects in greenhouse common garden studies with *P. flexilis*.

CONCLUSION

It was predicted that greater variation in twig growth would correspond to greater variation in seed mass, which would relate in turn to more variation in seedling growth. Though inter-annual variability was found in twig growth, seed size, and seedling growth, changes in twig growth were not significantly related to changes in seed mass. For many traits, however,

relative inter-annual seed mass variation was related to variation in seedling performance, indicating that maternal effects influence early seedling growth in *P. aristata* and *P. flexilis*. Consequently when conducting common garden studies to evaluate genetic differentiation, studies should account for differences in plant growth using cotyledon length as a covariate representing the year-to-year variation in seed provisioning.

In this study, examining differences in twig growth between years was not effective in predicting seed size. However using multiple years of seed from the same maternal plant was effective in determining the influence of maternal effects on offspring growth traits. Using this technique to study the impact of maternal effects in other long-lived species will help to clarify the interpretation of common garden studies.

CHAPTER 2: TESTING FOR GENETIC DIFFERENTIATION AMONG SOUTHERN ROCKY LIMBER PINE POPULATIONS ALONG A LATITUDINAL GRADIENT

INTRODUCTION

Active management is needed to sustain healthy limber pine (*Pinus flexilis*) forests in the Southern Rockies as they are threatened by the interaction of the mountain pine beetle epidemic, climate change, and the spread of the non-native pathogen *Cronartium ribicola* that causes the lethal disease white pine blister rust (WPBR). The frequency of genetic resistance to WPBR is not distributed uniformly across the Southern Rockies landscape (Schoettle et al. 2013); northern Colorado limber pine populations have greater frequency of WPBR resistance than those to the south (Schoettle et al. in prep). As a result, long-distance movement of seed within the Southern Rockies will be needed to supplement resistance in some populations to bolster forest resiliency against increased blister rust pressure.

Seed zones are developed to account for the fact that plants are often locally adapted to their native climate based on generations of selection on adaptive traits and thus perform best close to where they evolved. The idea of seed zones began in commercial forestry as a way to improve productivity of timber species and has since expanded to include non-commercial tree species as well as grasses, shrubs, and forbs (Johnson et al. 2004). Information for developing seed zones historically came from decades-long provenance trials, where plant material from a range of locations is grown in multiple testing locations and compared, and more recently through common garden studies (Johnson et al. 2004). Seed zones are generally delineated by identifying variation in plant traits among populations and mapping them to environmental factors like temperature or moisture (Campbell 1986, Sorensen 1992, Rehfeldt 1994, St. Clair et

al. 2005). Variation in molecular markers can also be used to inform seed zones, but because neutral markers are rarely correlated with adapted traits, those studies should be confirmed through common gardens (Johnson et al. 2004).

Currently the Southern Rockies are considered one seed zone for limber pine (Fig. 2) (Mahalovich 2006). The delineations for these seed zones were based on limited genetic studies and variation in morphological traits and have not been tested. The Southern Rockies Seed Zone covers large elevational (1600m-3,400m), latitudinal (33-43°N), and climatic (temperature, precipitation) gradients (Schoettle and Rochelle 2000), causing speculation that some level of genetic differentiation among populations may exist. Previous population genetic studies have found patterns of genetic differentiation using neutral markers among northern Colorado limber pine populations based on glacial refugia (Latta and Mitton 1997) and elevation (Schuster et al. 1989). Others have looked at geographic variation of phenotypes (Steinhoff et al. 1971, Van Haverbeke 1983) and genetic diversity (Jørgensen et al. 2002) across the range. As of yet, no one has examined genetic differentiation of adaptive traits along a latitudinal gradient in the Southern Rockies as a tool to test the current seed zone delineation.

Mid-summer precipitation in this region is highly correlated with latitude (Fig. 7). Since water availability is generally considered to be a limiting factor in plant growth in arid regions, this difference in precipitation could act as a selective force causing local adaptation of populations and contributing to the maintenance of genetic variation within the species. Other studies examining genetic differentiation in relation to climatic gradients for the purpose of devising seed zones have found precipitation (Rehfeldt 1990, O'Brien et al. 2007, Johnson et al. 2010) and latitude (Sorensen 1992, Campbell 1986) to be highly correlated with variation in adaptive traits.

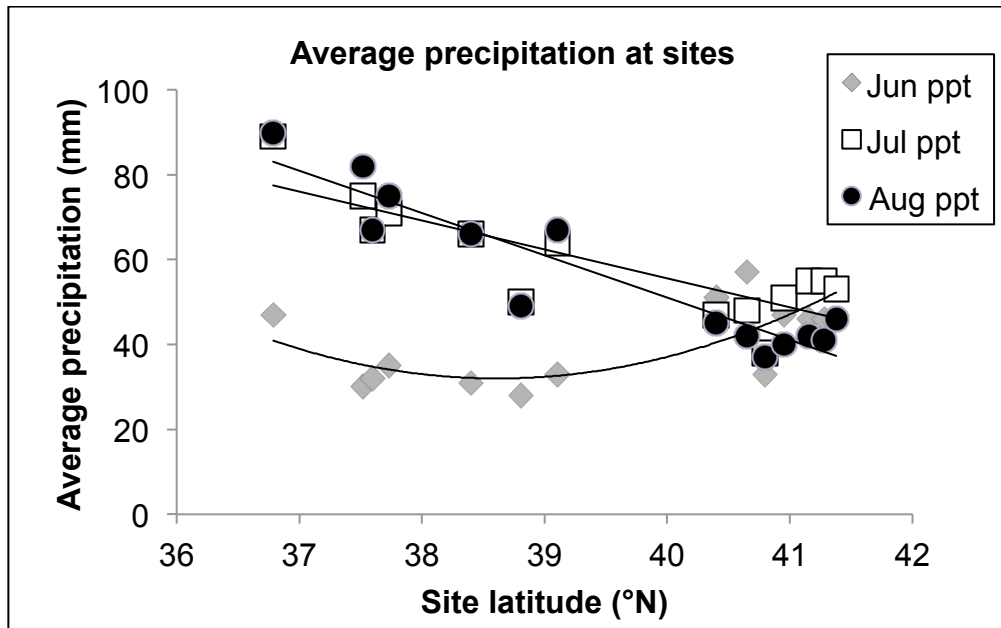


Fig. 7. Mean monthly precipitation from the Southern Rockies limber pine seed collection sites included in this study (n=14 sites; see Table 7). Precipitation modeled by Hutchinson's thin plate splines (Rehfeldt 2006).

The aim of this study was to determine the degree of genetic differentiation among populations of *Pinus flexilis* in the Southern Rockies. A greenhouse common garden study evaluated the differential response of seed sources from northern and southern portions of the range to different moisture regimes during early seedling growth. Differences in seedling performance between northern and southern seedlings would provide evidence for genetic differentiation among limber pine in the Southern Rockies. An interaction between region of seed source and drought treatment, with seedlings performing better in the treatment representing their native moisture regime, would support the hypothesis that populations are locally adapted to differences in summer water availability. Seedling performance was evaluated through primary needle length, stem diameter, root length, and biomass allocation between root and shoot. These traits were measured before, during, and after water treatments to determine whether temporal responses of seedlings from different locations responded differently to

drought. Carbon isotope ratios ($^{13}\text{C}/^{12}\text{C}$) were also measured to evaluate a difference in water use efficiency among populations. I predicted that 1) there would be a differential response of seedling growth based on region of seed origin and 2) the response of seedlings to treatment would depend on their source region, where northern seedlings would perform better in the dry treatment and southern seedlings would perform better in the wet, indicating local adaptation to their home mid-summer precipitation regime.

METHODS

Seed Source

Seed was collected from seven northern (40-41° latitude) and six southern (36-39° latitude) populations in 2012. For balance, seed from an additional southern site collected in 2003, 2005, and 2006 was used as well. Each population was represented by 28 seeds from each of three open-pollinated mother trees, spaced at least 60m apart (Fig. 8, Table 7). 2012 had a relatively poor cone crop across the southern Rockies, therefore sites were chosen largely based on the presence of mature cones in the appropriate geographic location.

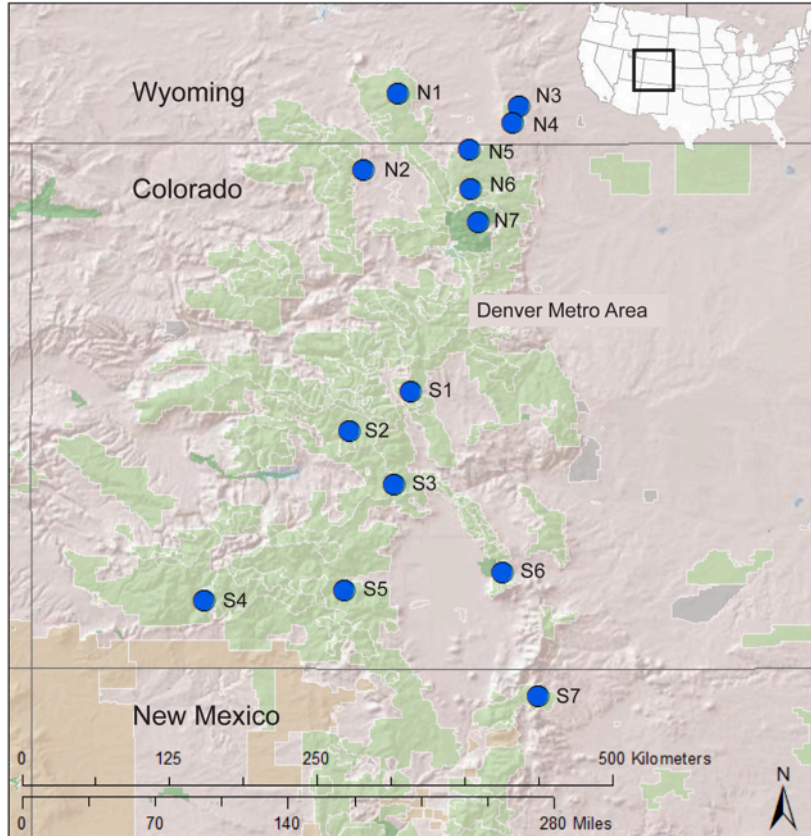


Fig. 8 Location of cone collecting sites. Southern sites are labeled S1-S7. Northern sites are labeled N1-N7. Seeds were collected from three trees (families) per site.

Table 7 *P. flexilis* seed sources showing the latitude, region, and number of seeds sown from each site. Twenty-eight seeds from each of three trees were sampled from each population.

Population	Name	Latitude	Region/state	# seeds sown
S1	BUP	39.1047	South / CO	84
S2	MAP	38.3938	South / CO	84
S3	TAC	38.8116	South / CO	84
S4	DUR	37.5173	South / CO	84
S5	BCR	37.5934	South / CO	84
S6	MSL	37.732	South / CO	84
S7	USF	36.7864	South / NM	84
N1	GLE	41.3782	North / WY	82*
N2	LAJ	40.7947	North / CO	84
N3	TEL	41.2773	North / WY	53*
N4	VED	41.1539	North / WY	84
N5	80C	40.9520	North / CO	84
N6	BL	40.6458	North / CO	84
N7	HIV	40.3991	North / CO	84

* poor germination from one or more families

Experimental Design

The study design was a full factorial with variables of seed source latitude (north and south) and watering treatment (wet simulating southern monsoons and dry simulating northern drought). Seedlings from each family were randomly assigned a water treatment level. Treatments were applied in a split-plot design to 28 blocks containing one replicate seedling per family (total n=1,143). All blocks and seedlings within blocks were spatially randomized in the greenhouse. Before the treatment began, all seedlings were measured to obtain cotyledon and primary needle length and two blocks were harvested to determine stem diameter, root length, root, shoot, and total dry biomass, and water potential. Subsequent harvests were made mid-way through the treatment and then again at the end of the treatment to assess changes in seedling response.

Common Garden

All seeds underwent a six week cold stratification at 1-2°C in an incubator (Precision Low Temperature Incubator 815, Thermo Scientific, Waltham, MA). Seeds were soaked in a 1% hydrogen peroxide solution, rinsed with water, and placed in plastic bags with moistened vermiculite to maintain moisture. Seeds were checked weekly for mold; moldy seeds were discarded. After six weeks, seeds were germinated on moistened filter paper in 8x8cm plastic trays (day 18°C day/16°C night, 12 hour photoperiod, Precision Low Temperature Illuminated Incubator 818, Thermo Scientific, Waltham, MA). Trays were sprayed weekly with a 1% solution of hydrogen peroxide to prevent mold. After three days seeds were checked daily for germination (defined as the radicle protruding at least 2mm) and immediately transplanted upon germination.

Twenty-eight germinated seeds per mother tree were transplanted into 656ml deepots D40h (Stuewe and Sons, Inc, Tangent, OR) in a mixture of 20% forest soil, 50% Fafard 4P mix potting soil (Conrad Fafard Inc, Agawam, MA), 20% sand, and 10% pea gravel for drainage. Forest soil was included to better represent actual growing conditions and provide beneficial mycorrhizae. It was collected in a mixed conifer forest containing limber pine in Centennial, Wyoming on October 3, 2012. Each pot included Osmocote Classic 14-14-14 control release fertilizer (Everris International B.V, the Netherlands). Greenhouse temperatures varied between 17-22°C with 16H day/8H night photoperiod.

Water treatments began two months after germination and continued for two months. *P. flexilis* grow substantially between 60-120 days old before growth levels off; by targeting treatment during this time of intense growth, a visible effect is more likely. At the commencement of treatments, all seedlings were watered to saturation. Half of the seedlings (drought simulation) underwent three 2-4 week dry-down cycles while the other half (wet simulation) were watered weekly with 50ml de-ionized water per pot (the average weekly precipitation in the south). The length of dry-down periods was determined through a pre-study of drought effect on two-month old limber seedlings (see appendix). The original plan of two one-month drought cycles was amended due to high mortality. The first drought cycle lasted one month with total water exclusion while the second was punctuated by a watering event after two weeks (Table 7).

At each harvest (mid-treatment and post-treatment), survival was monitored and growth of primary needle length, stem diameter, root length, dry root and shoot biomass, and relative growth rate (RGR) was measured. RGR was calculated as follows:

$$[3] \quad RGR = \frac{\ln(s_2 - s_1)}{(t_2 - t_1)}$$

where s is equal to the length of needles in millimeters and t is equal to time in days. During all harvests, roots and shoots were separated below the cotyledons, placed in separate labeled coin envelopes, and dried (60°C, 7d). Once dry, roots and shoots of each seedling were weighed to the nearest hundredth of a milligram (Sartorius LE225D, Germany). Water potential was measured on a subset of seedlings with a pressure bomb (PMS Instrument Company Model 600, Corvallis, OR) before (one seedling per family) and during (one seedling per family per treatment) water treatment to ensure the treatment had the desired effect. At the conclusion of the treatment, all remaining seedlings were harvested (Table 8).

Table 8 Schedule of events including seed treatment, germination, transplanting, seedling measurements and watering schedule. During dry-down cycles, seedlings in the wet treatment were watered weekly with 50ml deionized water per pot while seedlings in the dry treatment were not watered. At the end of each dry-down cycle, all seedlings were watered to capacity.

Week	Task	Measurements taken
1	Cold stratification	
6	Seed germination	Date of germination
6-7	Seed transplanted to pots	
16	Measured all seedlings <i>Beginning of 1st dry-down cycle</i>	Cotyledon length, primary needle length
	Harvested 2 blocks	Root and shoot biomass, diameter, root length
19	Measured all remaining <i>Beginning of 2nd dry-down cycle</i>	Primary needle length
20	Harvested 10 blocks	Root and shoot biomass, diameter, root length
22	Harvested 2 blocks <i>Beginning of 3rd dry-down cycle</i>	Water potential, root and shoot biomass, diameter, root length
23	Measured all remaining	Primary needle length
24	Harvested 14 blocks	Root and shoot biomass, diameter, root length

Carbon isotopes

At the end of the two-month drought treatment and after dry mass was recorded for individually harvested seedlings, primary needles of seedlings from each family and each treatment (7 wet blocks and 7 dry blocks) were pooled for carbon isotope ratio measurements.

During photosynthetic carbon assimilation, C_3 plants discriminate against the heavier ^{13}C isotope, but to a lesser extent when CO_2 is more limiting as a photosynthetic substrate (e.g., when stomata are closed or photosynthetic capacity is high). Therefore, the amount of ^{13}C relative to ^{12}C found in plant reflects the balance between carbon gain and water loss (Ehleringer 1990). Variation in the carbon isotope ratio ($\delta^{13}\text{C}$) indicates changes in either variation in photosynthetic capacity and/or stomatal conductance (Ehleringer 1990). For instance, stomata closing due to drought stress to minimize water loss would also minimize carbon intake, resulting in less discrimination (O'Leary 1988, Farquhar et al. 1989). Generally $\delta^{13}\text{C}$ in plant tissue reflects overall plant water use, and has been shown to be a good predictor of water use efficiency (Farquhar et al. 1989). Here, $\delta^{13}\text{C}$ was used as a metric of water use efficiency (WUE) for determining the presence of genetic differentiation in these populations and provided insight into the link between biomass allocation, growth, and water stress.

To complete these analyses, samples were dried in a 60°C oven, ground using the reciprocal saw method (Alexander et al. 2007), and analyzed at the Colorado State University EcoCore lab using a VG Isochrom continuous flow isotope ratio mass spectrometer (Isoprime Inc., Manchester, UK) coupled to a Carlo Erba NA 1500 elemental analyzer (Milan, Italy). Sixteen random duplicates were run along with a control sample with known isotope composition every twelve samples to document machine precision (0.2‰). Sample analysis was done randomly over two days, represented as block 1 and 2.

Since the atmospheric carbon dioxide levels in a greenhouse differ from those outside (-8‰ relative to PDM standard) and the relative difference between seed source regions and wet and dry treatments was the focus in this study, samples were analyzed as the carbon isotope ratio ($\delta^{13}\text{C}$) and not the standardized isotope discrimination (Δ) using the following equation:

$$[2] \quad \delta^{13}\text{C} = \frac{(R_{\text{sample}})}{[(R_{\text{standard}} - 1) \times 1000]}$$

where $R = {}^{13}\text{C}/{}^{12}\text{C}$ and units are in per mil (‰).

Data Analysis

A mixed model analysis of variance (ANOVA) was used to evaluate differences in seedling performance using fixed effects of region, treatment, and their interaction and random effects of site nested within region, family nested within site and region, and block. Cotyledon was included in the model as a covariate to account for maternal effects (see chapter 1). Response variables included total dry mass, root-to-shoot ratio, stem diameter, root length, relative growth rate (RGR), water potential, and $\delta^{13}\text{C}$. Data from each harvest were analyzed separately. Mortality data were analyzed through a logistic regression with predictors of region, treatment, and their interaction. In cases where there was no significant interaction between region and treatment, a follow-up analysis was conducted using the same ANOVA model, but substituting the continuous variable latitude for the categorical variable region in an attempt to detect any discernible interaction. Assumptions of normality were met in all cases based on the normal and homoscedastic distribution of residuals and all analyses were run using SAS version 9.3 (SAS Institute, Cary, NC).

RESULTS

There were no significant interactions between treatment and seed source region for any response variable. There were, however, significant region and treatment effects for many seedling growth traits. Seedling data from the first harvest before the beginning of treatment

showed a significant difference in primary needle length ($p=0.0391$), cotyledon length, ($p=0.0013$) and root-to-shoot ratio ($p=0.0036$) between seedlings sourced from the south versus the north; southern seedlings had longer needles and cotyledons and northern seedlings had a higher root-to-shoot ratio (Table 9, Fig. 9). Other seedling growth traits including root length, dry biomass, and stem diameter were not significantly different. By the mid-treatment harvest one month after drought was induced, differentiation based on seed source region was evident for needle length, total dry mass, and stem diameter, where southern seedlings were larger, while none of the seedling growth traits were affected by treatment except for RGR (Table 9).

Table 9. ANOVA table showing fixed effects of seed source region, treatment, and cotyledon (covariate to account for maternal effects) for harvest data. No interactions between treatment and region were significant except for $\delta^{13}\text{C}$ ($p=0.0778$) analyzed using latitude (continuous) in place of region (categorical).

* Significant at $p<0.05$

† Estimate represents the estimated difference of least square means between north and south. Negative numbers indicate larger values for southern seedlings.

Response	Effect	Num DF	Pre-treatment		Estimate†	p value
			Den DF	F Value		
Root length	region	1	37	0.11		0.7378
Needle length	region	1	12.3	5.33	-5.52 mm	0.0391*
Stem diameter	region	1	37	0.67		0.4171
Total dry mass	region	1	11.9	3.16		0.1013
Cotyledon length	region	1	38	12.16	-4.91 mm	0.0013*
Root-to-shoot	region	1	37	9.66	0.17	0.0036*

(continued on next page)

Response	Effect	Mid-treatment			Estimate†	p value
		Num DF	Den DF	F Value		
Needle length	region	1	14.2	13.94	-4.34 mm	0.0022*
	trt	1	8.02	3.93		0.0826
	cot	1	275	9.79		0.0019*
Stem diameter	region	1	15.2	4.8	-0.07 mm	0.0445*
	trt	1	8	0.62		0.4549
	cot	1	214	26.45		<0.0001*
Total dry mass	region	1	14.8	9.77	-34.25 mg	0.0070*
	trt	1	7.92	4.39		0.0699
	cot	1	241	40		<0.0001*
RGR between pre- and mid-treatment harvests	region	1	14.6	0.27		0.6139
	trt	1	25.7	29.46	-0.0041	<0.0001*
	cot	1	559	3.62	mm/mm*d	0.0578

Response	Effect	Post-treatment			Estimate†	p value
		Num DF	Den DF	F Value		
Root length	region	1	15.4	8.4	-2.01 cm	0.0108*
	trt	1	12.4	35.26	-4.42 cm	<0.0001*
	cotyledon	1	184	8.01		0.0052*
Stem diameter	region	1	15.4	6.36	-0.12 mm	0.0231*
	trt	1	11.4	27.24	-0.17 mm	0.0003*
	cot	1	331	22.92		<0.0001*
Needle length	region	1	49.1	16.11	-5.74 mm	0.0002*
	trt	1	12.1	56.07	-7.93 mm	<0.0001*
	cot	1	364	10.04		0.0017*
Total dry mass	region	1	14.8	10.16	-56.29 mg	0.0062*
	trt	1	12.1	29.21	-68.94 mg	0.0002*
	cot	1	304	51.49		<0.0001*
Root-to-shoot ratio	region	1	15	0.04		0.8386
	trt	1	12.6	27.22	-0.17	0.0002*
	cot	1	258	0.27		0.6066
RGR between mid- and post-treatment harvests	region	1	15.4	1.78		0.2014
	trt	1	11.8	81.15	-0.00997	<0.0001*
	cot	1	228	0.87	mm/mm*d	0.3521
$\delta^{13}\text{C}$ (categorical)	region	1	13.9	0.98		0.3384
	trt	1	64.7	138.94	1.6845‰	<0.0001*
$\delta^{13}\text{C}$ (continuous)	latitude	1	19.9	1.93		0.1799
	trt	1	63.3	1.73	1.6798‰	0.1930
	lat*trt	1	63.5	3.21		0.0778

Soon after this point water potential measurements were taken. Water potential values in the dry treatment were significantly lower than the pre-treatment levels ($p < 0.05$), indicating increased levels of water stress for seedlings from both northern and southern populations (Fig. 10). There was no significant difference in water potential between seedlings from northern and southern regions within each treatment nor was there an interaction between region and treatment. Water potential measurements were relatively low compared to the pre-study (see appendix), likely due to the time of day and location measurements were taken.

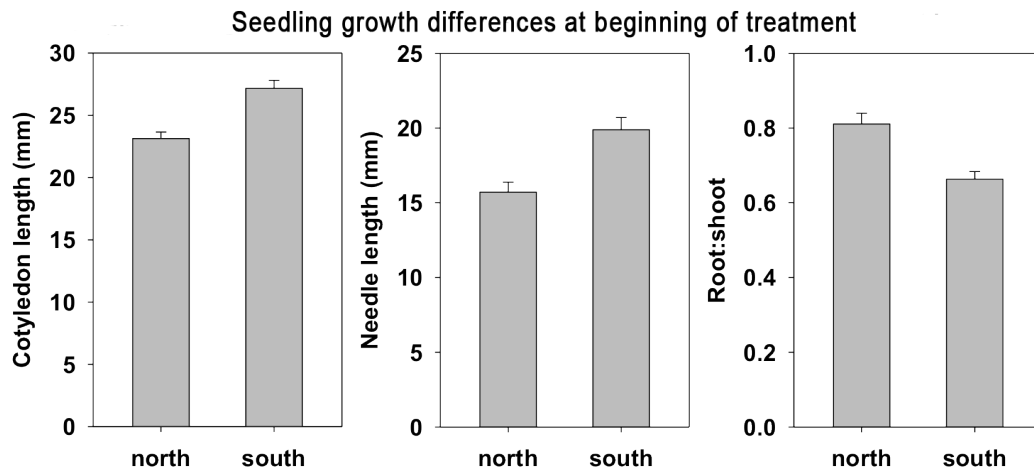


Fig. 9. There were statistically significant ($p < 0.05$) differences in needle length, cotyledon length, and root-to-shoot ratio between seedlings from northern (blue) and southern (red) regions. These measurements, taken before the drought treatment commenced, show that growth differences between northern and southern sources existed prior to treatment.

The ANOVA results for the final harvest at the end of the two-month treatment revealed growth differences based on source origin for root length, needle length, diameter, and total dry mass, where southern seedlings were generally larger. Additionally there was a treatment effect for every seedling growth metric with seedlings in the wet treatment outperforming those in the dry treatment ($p < 0.05$). There was no significant interaction between region and treatment for

any growth trait (Table 9). By the study’s end, 17.6% of all seedlings were dead or declining. Significantly more northern seedlings and seedlings from the dry treatment died, though there was no significant interaction between region and treatment (Table 10).

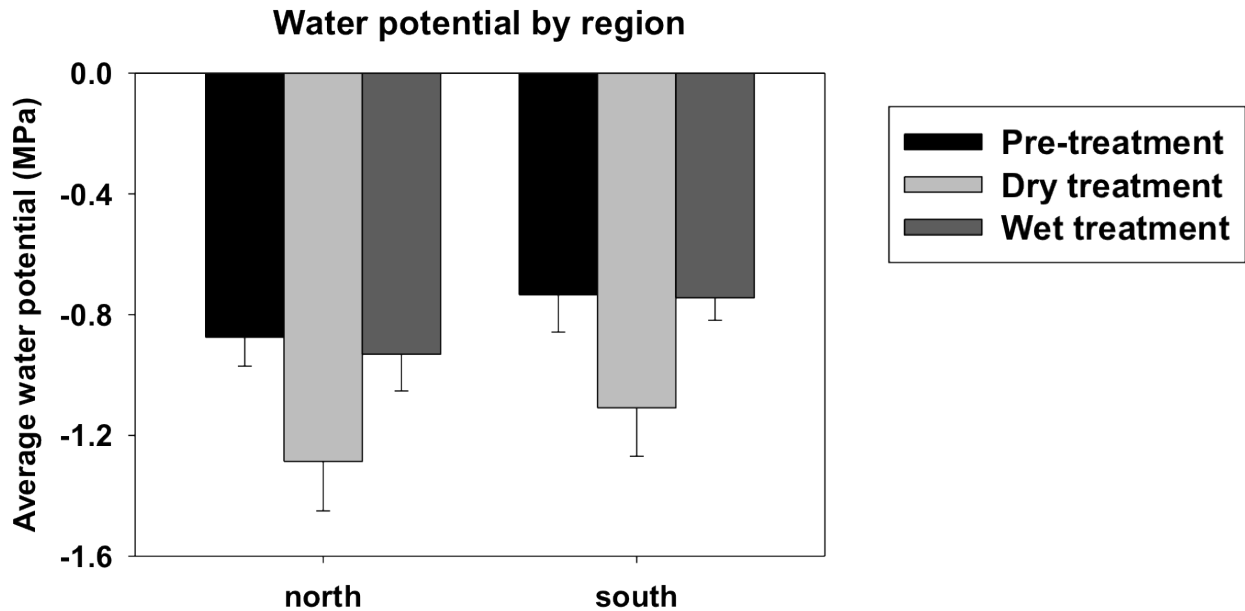


Fig. 10 Chart of mean water potentials with standard error before and during water treatment for southern and northern seedlings. Larger negative values indicate increasing levels of water stress. Seedlings in the dry treatment (light grey) were significantly more stressed than seedlings measured before the treatment began (black) and those in the wet treatment (dark grey) ($p < 0.05$).

Table 10 Mortality results from the logistic regression. Main effects of region and treatment were highly significant. Seedlings from the north or in the dry treatment were more likely to die.

Analysis of Maximum Likelihood Estimates						
Parameter		DF	Estimate	Standard Error	Wald Chi-Square	Pr>Chi Sq
Intercept		1	-1.8889	0.0983	368.9790	<0.0001
Region	north	1	0.4382	0.0919	22.7542	<0.0001
Treatment	dry	1	0.5481	0.0953	33.0673	<0.0001
Odd Ratio Estimates						
Effect			Point Estimate		95% Wald Confidence Limits	
Region	north vs south		2.402		1.676	3.444
Treatment	dry vs wet		2.993		2.060	4.349

Results from the $\delta^{13}\text{C}$ analysis also showed no significant interaction of treatment and seed source region ($p=0.40$). Main effect of region was not significant yet the treatment did have a significant effect ($p<0.0001$) with a difference in least square means showing those in the dry treatment had an estimated 1.6845 higher $\delta^{13}\text{C}$. However, when replacing region with the continuous variable latitude in the model, an interaction was moderately significant ($p=0.0778$, Table 8, Fig. 11). In that case, seedlings that underwent the dry treatment had less discrimination (higher $\delta^{13}\text{C}$) than those receiving more moisture, with little latitudinal variation. With seedlings in the wet treatment, seeds of a more southern origin had a slightly higher $\delta^{13}\text{C}$ than northern seeds (Fig. 11).

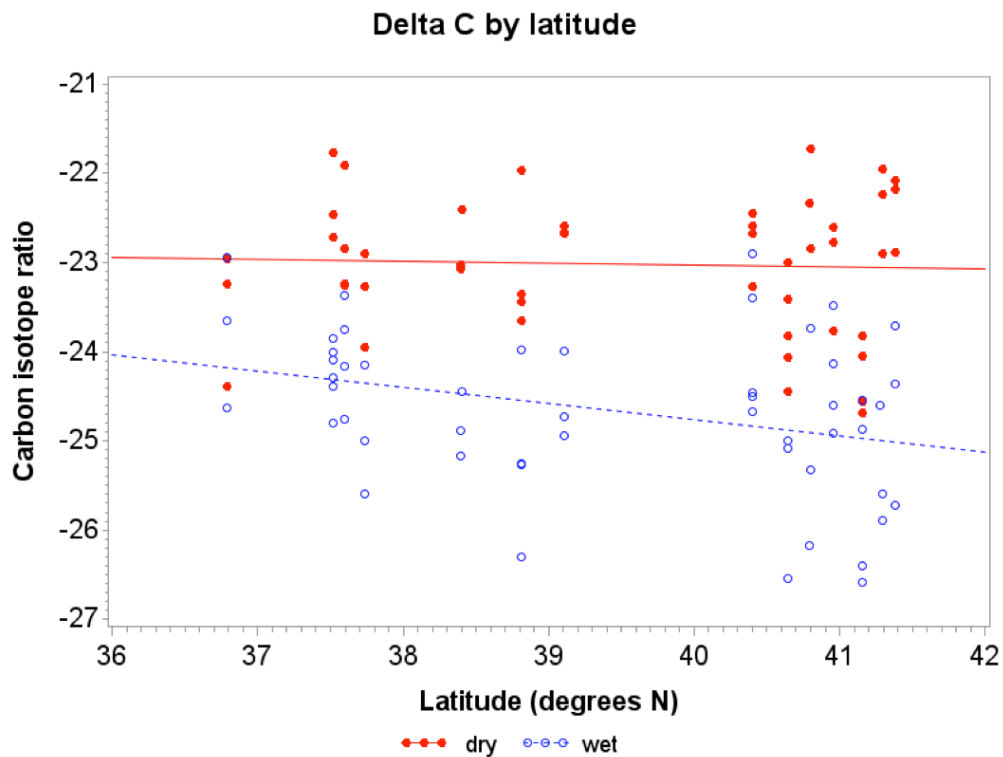


Fig. 11 Graph showing the values of $\delta^{13}\text{C}$ from the model with trend lines using the least square means at a given latitude for both dry and wet treatments. Larger negative values (lower on y axis) indicate more discrimination against ^{13}C and therefore lower WUE. Open, blue circles represent seeds in the wet treatment and closed, red circles represent the dry. The interaction of treatment and latitude was significant at the 0.1 level.

DISCUSSION

The goals of this study were to determine if there was genetic differentiation among *P. flexilis* populations in the Southern Rockies and whether local adaptation to the variation in mid-summer water availability could be documented. A greenhouse common garden study evaluated the differential response of seed sources from throughout the Southern Rockies to moisture regimes representing the wetter south and drier north during early seedling growth.

Pre-treatment harvest results at two months after germination already revealed differentiation of some growth traits between seed source regions. Southern seedlings had longer needles and cotyledons than northern seedlings (by roughly 5mm) while northern seedlings had a 0.17 higher root-to-shoot ratio (Table 9). By the mid-treatment harvest, growth differences between northern and southern seedlings were also observed in total dry mass, where southern seedlings were an estimated 34mg larger and had stem diameters 0.07mm larger than northern seedlings. At this point there were no longer any differences in root:shoot among northern and southern seedlings. Despite the fact that only relative growth rate exhibited an effect from the drought one month into the water treatment, water potential measurements and observations of seedling characteristics confirmed that the drought cycles had an effect on seedling physiology and growth. By the final harvest, two months after the start of water treatments, all metrics of seedling growth were affected by treatment and differentiation between source regions was seen in root length, needle length, stem diameter, and total dry mass, with more growth in southern seedlings (Table 9). This pattern of differentiation extended to mortality as well, where seedlings in the drought treatment were almost 3 times more likely to die than seedlings in the wet

treatment while northern seedlings were 2.4 times more likely to die over the course of the treatment (Table 10).

Carbon isotope composition suggests that southern seedlings had less discrimination (higher $\delta^{13}\text{C}$). It would be unexpected if southern seedlings had higher water use efficiency, being in a region where water is not as limiting as in northern regions, but there are at least two explanations for this pattern. First, others have found that positive correlations between $\delta^{13}\text{C}$ and dry mass usually indicate that the higher carbon isotope ratio is due to higher photosynthetic capacity rather than variations in stomatal conductance (Hubick et al. 1986, Ehleringer 1990, Silim 2001). WUE has also been found to vary due to many complex factors, including temperature, irradiance, humidity (Warren et al. 2001), stand density (Lajtha and Getz 1993), the presence of beneficial mycorrhizae (Hobbie and Colpaert 2004), and elevation (Marshall and Zhang 1994, Warren et al. 2001), so factors unrelated to precipitation of the source environment may be at play in determining WUE. All seedlings regardless of origin had lower discrimination (increased $\delta^{13}\text{C}$) under the drought stress, a trend that has been found in other plants (Ehleringer and Cooper 1988, Lajtha and Getz 1993, Zhang and Marshall 1994, Warren et al. 2001) and is consistent with the mechanisms that contribute to ^{13}C discrimination in arid regions (Ehleringer and Cooper 1988, Lajtha and Getz 1993, Zhang and Marshall 1994, Warren et al. 2001). The higher $\delta^{13}\text{C}$ under drought stress can be explained by stomata closing with decreased moisture availability to prevent water loss (Pataki et al. 1998).

Though there was not differentiation based on region for relative growth rate, there was a highly significant ($p < 0.0001$) treatment effect, a fact to consider in restoration efforts in dry climates. If seedlings grow slower in drier areas (RGR mean of seedlings in the dry treatment

was about 1/3 lower than those in the wet), they may be out-competed by other species more easily than if they are not water limited.

The robust growth of southern populations growing near the southern edge of the limber pine range was surprising; they were considerably bigger and healthier than northern seedlings. There is evidence of hybridization with *P. strobiformis* (southwestern white pine) at the southern edge of the range (Steinhoff et al. 1971), which could perhaps explain this vigor, though results from this study do not address hybridization explicitly. Besides their size and vigor, southern seedlings appeared similar to known pure limber pine seedlings with no clear statistical outliers among southern families.

Though there was little evidence for local adaptation to water availability as experimentally tested in this study, it is possible that other factors could lead to local adaptation of populations. The study was short in duration in relation to the longevity of the species; most common garden studies examining genetic differentiation in conifers last 2-3 years (Johnson et al. 2004). Because of these reasons, the differences in growth may be adaptive and local adaptation of populations to water availability should not be ruled out. Additionally, for many seedlings initial taproot elongation was much shorter and primary needle growth more stunted than expected based on previous work with this species. It is possible that a small percentage of seedlings experienced transplant shock when newly germinated seeds were sown in pots, a planting technique not used in the previous maternal effects study.

CONCLUSION

I predicted that 1) there would be a differential response of seedling growth based on region of seed origin and 2) the response of seedlings to treatment would depend on their source region, indicating local adaptation to their native mid-summer precipitation regime. Most seedling growth traits measured and patterns of seedling mortality demonstrated differential growth based on region of seed origin. Seedling response across treatments did not depend on region (i.e. there was no significant interaction), except when using latitude as a fixed effect instead of region. In that case there was an interaction between latitude and treatment in carbon isotope ratios. Seedlings under the drought treatment had less discrimination (a higher $\delta^{13}\text{C}$) regardless of latitude of origin, possibly due to stomatal closure under stress. In the wet treatment where water stress was not an issue, southern seedlings had a higher $\delta^{13}\text{C}$, likely due to a higher photosynthetic capacity. Results from this study support the hypothesis that genetic differentiation in growth traits exists among seed sources from northern and southern regions of the Southern Rockies Seed Zone, providing evidence to partition the current seed zone into northern and southern sections.

NEXT STEPS

The two studies reported here comprise a small part of a larger effort to provide the scientific basis for successful proactive conservation of five-needle pines in the Southern Rockies. These forests are threatened by the interaction of increased WPBR pressure, mountain pine beetle, and more frequent drought and fire events due to climate change. Plans for pinpointing high priority areas for management and selecting appropriate actions for a given region are being developed (introduced in Schoettle 2004, Burns et al. 2008). The data presented here will help increase the success of any future outplanting by helping scientists and land managers better understanding the role of maternal effects in early seedling growth and by providing information on the likelihood of maladaptation due to genetic differentiation among limber populations when moving seed throughout the current Southern Rockies Seed Zone.

The results from the maternal effects study indicated that environmental maternal effects were present in both limber and bristlecone pine. Although examining differences in twig growth between years was not effective in predicting seed size, using multiple years of seed from the same maternal plant was effective in determining the influence of maternal effects on offspring growth traits. Using this technique to study the impact of maternal effects in other long-lived species will help clarify the interpretation of common garden studies. There were limitations to its use in this study, however. Since seed sources in the maternal effects study were from a small latitudinal range (39-41°N) and were all generally small compared to more southern families, it would be interesting to see the magnitude of this maternal effect if larger seeds were included in the study. Since there is a desire in this field to find a reliable method to account for seed size differences due to maternal effects in common gardens with long-lived species, it would also be

helpful to fully test the cotyledon covariate technique with seeds of all sizes from throughout the range of *P. flexilis*.

The latitudinal study results supported the hypothesis of genetic differentiation among populations of *P. flexilis* in the Southern Rockies. This study used a common garden framework with treatment of two moisture levels representing the two regions to compare genetic variation in adaptive traits related to growth. Though significant trait variation was found in most metrics measured, there was not evidence for local adaptation to mid-summer water availability as we tested it. Since there could be factors other than precipitation selecting upon population traits and possibly leading to local adaptation, this study should be field-tested via a reciprocal transplant. After discovering the traits that vary among populations, the next step in designating seed zones is to correlate those traits with environmental factors, or “genetic mapping” (Campbell 1986, Sorensen 1992, Rehfeldt 1994). The differentiated traits in this study were correlated with latitude, but the importance of other environmental factors like elevation, frost-free days, slope, and others, could be explored as well.

Testing for genetic differentiation using molecular tools would also help address differentiation. Previous genetic studies indicated current patterns of limber pine genetic diversity rangewide were related to repopulation from glacial refugia (Latta and Mitton 1997, Jorgensen et al. 2002), but very few of these molecular studies included adequate sampling from populations in southern Colorado and northern New Mexico (Mitton et al. 2000). Modern molecular techniques can help reveal more about the evolutionary history of white pine species and patterns of genetic diversity and gene flow to help us better manage them for the future.

To best ensure the future resilience of high elevation five-needle pine forests, data predicting the effects of climate change on these threatened ecosystems should be integrated into

any future management discussions. The Alpine Treeline Warming Experiment based outside Boulder, Colorado is studying conifer recruitment in the alpine above current distributions given future warmer temperatures (Reinhardt et al. 2011). Better understanding the probable distribution shifts of limber pine will help improve the accuracy of climate modeling. Incorporating projections from these models into risk assessments and future habitat suitability predictions will only improve the success of proactive management.

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APPENDIX

Pre-study to determine effective drought treatments

Before beginning watering treatments, it was necessary to determine a) the target drought threshold to ensure seedling physiology and growth were affected while avoiding large-scale mortality and b) the water potential of the seedlings throughout the treatment to guarantee the target drought level was being met without destructively harvesting them. This pre-study included 25 seedlings from each of eight families (four southern and four northern populations) that were roughly two months old.

All seedlings were watered to capacity and the soil moisture content of each pot was measured both with a probe (VG200, Vegetronix Inc, Riverton, UT) and gravimetrically to test the accuracy of the instrument. Then all water was excluded and mid-day water potential of seedling and soil moisture were measured periodically for two months (Fig. 12). The resulting chart of the relationship between water potential and soil moisture provided a way to approximate the stress of the seedling through soil moisture (Fig. 13).

A recent study with limber pine found a mid-day water potential of -1.4MPa to be moderately stressed, while severe stress was associated with -4.0MPa (Moyes et al. 2013). These seedlings reached an average of -1.4MPa after about a week and did not consistently reach -4.0MPa throughout the duration of water exclusion. Seedlings were moderately stressed when soil moisture content reached approximately 10-15%, which consequently became the target soil moisture level for the full study.

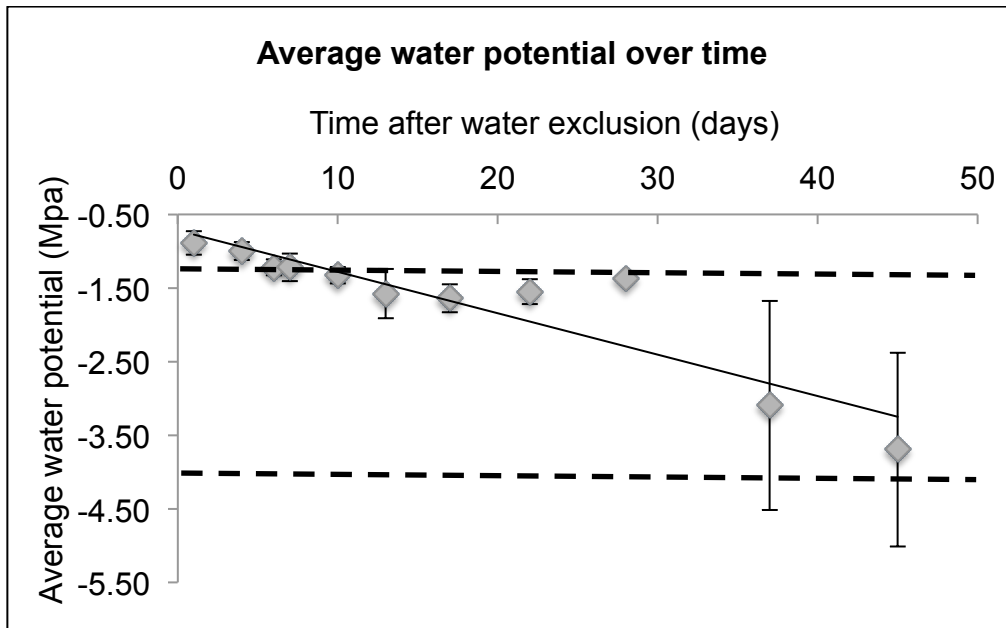


Fig. 12 Graph showing the average water potential of seedlings after water exclusion over time. The top and bottom dotted lines represent what is considered moderate and severely stressed seedlings, respectively (Moyes et al. 2013).

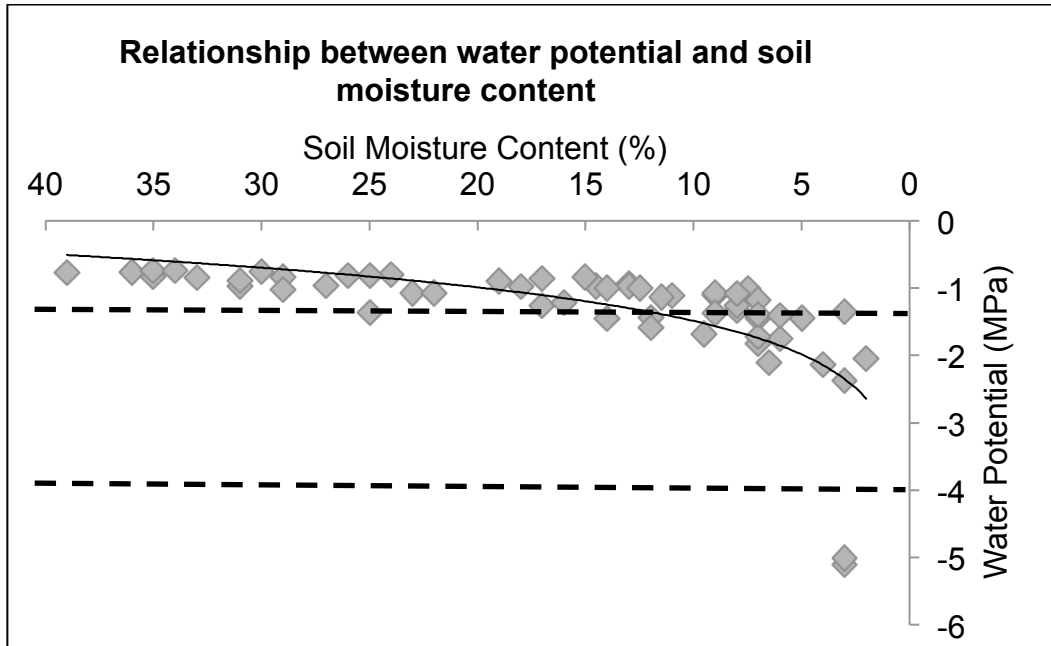


Fig. 13 Graph showing the relationship between soil moisture content and water potential after water exclusion. The top and bottom dotted lines represent moderate and severely stressed seedlings, respectively (Moyes et al. 2013)