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ARTICLE

Assessing the potential for maladaptation during active management of limber pine populations: a common garden study detects genetic differentiation in response to soil moisture in the Southern Rocky Mountains

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Abstract: Active management is needed to sustain healthy limber pine (*Pinus flexilis* E. James) forests in the Southern Rocky Mountains (henceforth, Southern Rockies), as they are threatened by the interaction of the mountain pine beetle (*Dendroctonus ponderosae* Hopkins) epidemic, climate change, and the spread of the non-native pathogen that causes white pine blister rust disease (*Cronartium ribicola* A. Dietr.). Appropriate source material for restoration and proactive introduction needs to be selected, taking into account potential genetic differentiation that would hamper management success. We conducted a common garden study in a greenhouse to determine the degree of genetic differentiation among limber pine populations in the Southern Rockies. We evaluated the differential responses of populations from northern and southern portions of the Southern Rockies to different moisture regimes during early seedling growth by measuring primary needle length, stem diameter, water potential, biomass allocation between root and shoot, and carbon isotope ratios (δ^{13}C; a proxy for water-use efficiency). There were significant ($p < 0.05$) effects of source region for root length, stem diameter, needle length, and total dry mass, with seedlings from southern sources bigger than seedlings from northern sources. Furthermore, there was a marginally significant interaction between soil moisture regime and source region for carbon isotope ratio ($p = 0.0778$), suggesting possible local adaptation. 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 for active management.

Key words: common garden, genetic differentiation, limber pine, *Pinus flexilis*, white pine blister rust.

Résumé : Il faut pratiquer un aménagement actif pour conserver des forêts saines de pin flexible (*Pinus flexilis* E. James) dans les Rocheuses du sud où elles sont menacées par l'épidémie du dendroctone du pin ponderosa, les changements climatiques et la propagation de l'agent pathogène exotique qui cause la rouille vésiculeuse du pin blanc. Le matériel source approprié pour la restauration et l'introduction proactive doit être sélectionné en tenant compte d'une possible différenciation génétique qui pourrait nuire au succès de l'aménagement. Nous avons réalisé une étude comparative en serre pour déterminer le degré de différenciation génétique parmi les populations de pin flexible dans les Rocheuses du sud. Nous avons évalué la différence de réaction des populations septentrionales et méridionales dans les Rocheuses du sud à différents régimes hydriques durant les premiers stades de la croissance des semis en mesurant la longueur des aiguilles primaires, le diamètre de la tige, le potentiel hydrique, l'allocation de biomasse aux racines et aux pousses ainsi que le rapport isotopique du carbone (δ^{13}C; une mesure indirecte de l'efficience de l'utilisation de l'eau). La région source a un effet significatif ($p < 0,05$) sur la longueur des racines, le diamètre de la tige, la longueur des aiguilles et la masse anhydre totale; les semis d'origine méridionale étaient plus gros que les semis d'origine septentrionale. De plus, il y avait une interaction marginalement significative ($p = 0,0778$) entre le régime hydrique du sol et la région source, ce qui indique qu'il y a possiblement une adaptation locale dans le cas du rapport isotopique du carbone. Ces données indiquent qu'il existe une différenciation génétique parmi les populations dans les Rocheuses du sud, ce qui pourrait accroître le risque de mésadaptation si des graines sont déplacées loin de leur point d'origine dans le cadre d'un aménagement actif. [Traduit par la Rédaction]

Mots-clés : jardin commun, différenciation génétique, pin flexible, *Pinus flexilis*, rouille vésiculeuse du pin blanc.

Introduction

Active management is needed to sustain healthy limber pine (*Pinus flexilis* E. James) forests in the Southern Rocky Mountains (henceforth, Southern Rockies), as they are threatened by the interaction of the mountain pine beetle (*Dendroctonus ponderosae* Hopkins) epidemic, climate change, and the spread of the non-native pathogen *Cronartium ribicola* (A. Dietr.) that causes the lethal disease white pine blister rust (WPBR; Burns et al. 2008). WPBR was detected in the Southern Rockies in the early 1990s and in Colorado in 1998 (Johnson and Jacobi 2000). Although many populations have not yet been invaded, risk assessment suggests that WPBR will continue to spread throughout the five-needle pine forests of the Southern Rockies (Howell et al. 2006; Kearns et al. 2013). This is of major concern because WPBR has contributed to high mortality in populations of another five-needle pine, whitebark pine (*Pinus albicaulis* Engelm.), in the Northern Rockies and was a factor in its listing as protected

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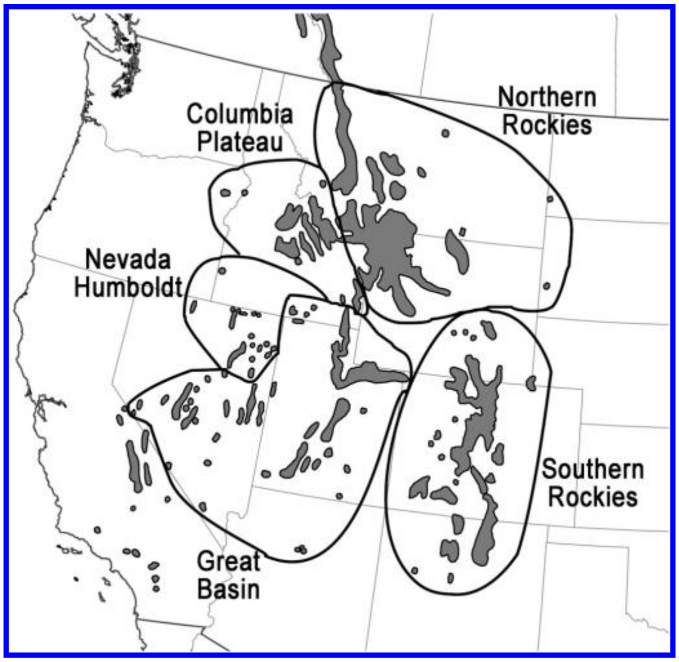
under the federal Species at Risk Act (SARA) in Canada (Government of Canada 2014) and as warranting its endangered species status under the Endangered Species Act in the United States (U.S. Fish and Wildlife Service 2011). Both whitebark and limber pine are listed as endangered by Alberta's Wildlife Act (Government of Alberta 2014).

Work has recently begun to proactively manage and conserve limber pine in the Southern Rockies (Schoettle and Sniezko 2007; Burns et al. 2008; Schoettle et al. 2011*a*) in an effort to prevent healthy limber pine populations from following the same trajectory as those of whitebark pine. Genetic resistance to WPBR has been identified in limber pine, with heritability consistent with a single, dominant R gene named *Cr4* (Schoettle et al. 2014). Promoting regeneration and targeted restoration of stands with disease-resistant seed stock can increase the resilience of five-needle pine ecosystems to this invasive disease (Schoettle and Sniezko 2007). 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). However, the frequency of genetic resistance to WPBR is not uniformly distributed across the Southern Rockies landscape (Schoettle et al. 2014). Limber pine populations from northern Colorado have greater frequency of WPBR resistance than those from southern Colorado (A.W. Schoettle and R.A. Sniezko, personal communication, 2014). 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 delineated to account for the fact that populations are often locally adapted to their native climate and thus individuals perform best closest to their site of origin. Seed zones began in commercial forestry to improve productivity of timber species and have since expanded to include noncommercial tree species, as well as grasses, shrubs, and forbs (Johnson et al. 2004). Historically, information for developing seed zones came from decades-long provenance trials in which plant material from a range of locations is compared side-by-side in multiple testing locations (i.e., common gardens). Variation in molecular markers can also be used to inform seed zones, but because neutral markers are rarely correlated with adapted traits, inferences from those studies should be confirmed through common garden studies (Johnson et al. 2004). Advances also are being made in the application of genomic data to refine seed zone delineation (De Kort et al. 2014). However, common garden studies remain a robust method for delineating seed zones (Johnson et al. 2004).

Currently, the Southern Rockies are considered one seed zone for limber pine (Fig. 1) (Mahalovich 2006). The delineations for these seed zones were based on limited genetic studies and variation in morphological traits, and they have not been tested. The Southern Rockies seed zone covers large elevational (1600–3400 m), latitudinal (33°N–43°N), and climatic (temperature and precipitation) gradients (Schoettle and Rochelle 2000), causing speculation that some level of genetic differentiation and local adaptation among populations may exist. Previous population genetic studies have found patterns of genetic differentiation using neutral markers among northern Colorado limber pine populations related to glacial refugia (Latta and Mitton 1997) and elevation (Schuster et al. 1989). Others have looked at geographic variation of phenotypes in common gardens (Steinhoff et al. 1971; Van Haverbeke 1983) and genetic diversity in the field (Jørgensen et al. 2002) using material from 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. Midsummer precipitation in this region is highly correlated with latitude (Fig. 2*a*). Because water availability is generally considered to be a limiting factor for plant growth in arid regions, this variation in precipitation may be an important selective factor that results in local adaptation of populations and contributes 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. Such information has been useful in the development of seed transfer guidelines and zones to reduce the risk of maladapted seed sources in reforestation activities.

Fig. 1. Seed transfer guidelines for limber pine split into five zones for the Interior West (adapted from Mahalovich (2006)). Limber pine range from U.S. Geological Survey 1999.



The aim of this study was to evaluate genetic differentiation in adaptive traits among populations of limber pine from the Southern Rockies to assess the potential for maladaptation resulting from long-distance seed movement. A greenhouse common garden study examined growth traits and carbon isotope ratios (a proxy for water-use efficiency (WUE)) of seedlings from the northern and southern portions of the Southern Rockies under two different moisture regimes that reflect their source locations. 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 moisture 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. We predicted that the response of seedlings to different watering treatments would depend on their source region, i.e., northern seedlings would perform better in the dry treatment and southern seedlings would perform better in the wet treatment, suggesting that seedlings from the respective latitudinal regions were locally adapted to their home midsummer precipitation regime.

Methods

Seed source

In 2012, seed was collected from each of seven northern (40°N–41°N) and six southern (36°N–39°N) populations. Sites varied by the length of the frost-free period (from 19 to 101 days), growing season precipitation (from 209 to 370 mm), and elevation (from 2425 to 3341 m) (Table 1). For balance, seed from an additional southern site was used as well, collected from three families in 2003, 2005, and 2006. Each population was represented by seeds

Fig. 2. (*a*) Mean monthly precipitation of each of the Southern Rockies limber pine seed collection sites (°N) included in this study ($n = 14$ sites; see Table 1). Climatic variables for each seed source were modeled based on Rehfeldt's (2006) climate surface (http://forest.moscowfsl.wsu.edu/climate/customData/). June (Jun ppt), July (Jul ppt), and August (Aug ppt) precipitation levels are represented by grey diamonds, unfilled squares, and filled circles, respectively. (*b*) Location of seed collection sites. Sites from the southern region are labeled S1–S7, and sites from the northern region are labeled N1–N7 (Table 1).

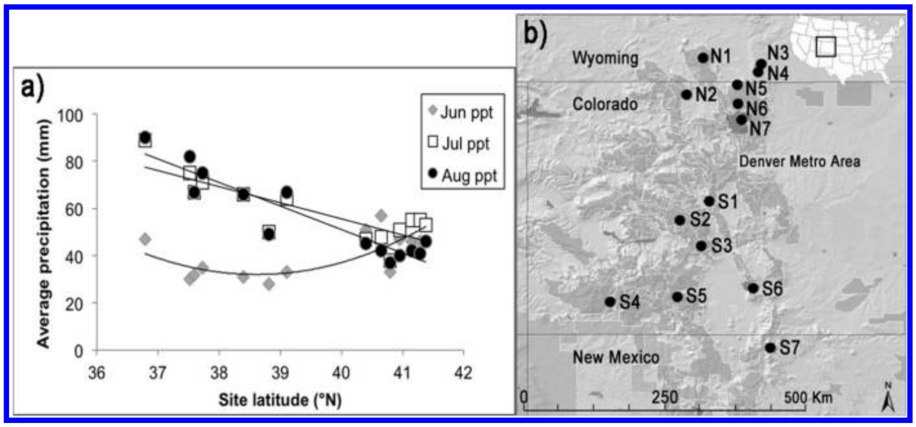


Table 1. *Pinus flexilis* seed sources.

Site	State	Name	Latitude (°N)	Longitude (°W)	Elevation (m)	Frost-free period (days)	Growing season precipitation (mm)
Southern region							
S1	Colorado	Buffalo Peak	39.1047	–106.1531	3341	43	297
S2	Colorado	Marshall Pass	38.3938	–106.2494	3175	31	286
S3	Colorado	Taylor Canyon	38.8116	–106.6192	2813	44	238
S4	Colorado	Durango	37.5173	–107.7355	2965	48	331
S5	Colorado	Beaver Creek	37.5934	–106.6614	2808	19	296
S6	Colorado	Mosca Pass	37.7320	–105.4740	2900	55	261
S7	New Mexico	Upper Shuree	36.7864	–105.1867	2924	60	370
Northern region							
N1	Wyoming	GLEES	41.3782	–106.2502	3324	45	282
N2	Colorado	Lake John	40.7947	–106.5208	2581	52	209
N3	Wyoming	Telephone Road	41.2773	–105.3437	2425	101	232
N4	Wyoming	Vedauwoo	41.1539	–105.3769	2534	99	227
N5	Colorado	80 C	40.9520	–105.7179	2627	62	235
N6	Colorado	Brown's Lake	40.6458	–105.6960	3237	27	302
N7	Colorado	Hidden Valley	40.3991	–105.6454	2798	32	285

Note: Growing season is from April to September. Environmental variables were derived from Rehfeldt's (2006) climate surface (http://forest.moscowfsl.wsu.edu/climate/customData/ [accessed 26 July 2012]). GLEES, Glacier Lakes Ecosystem Experiments Site.

from each of three open-pollinated mother trees, spaced at least 60 m apart (Fig. 2*b*; Table 1). In 2012, there was 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.

Plant culture

Limber pine seeds were cold stratified for 6 weeks at 1–2 °C in a low temperature incubator (Precision Refrigerated Incubator 815, Thermo Scientific, Waltham, Massachusetts). A total of 1500 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 and discarded if mold was found. After 6 weeks, seeds were germinated on moistened filter paper in 8 cm × 8 cm plastic trays (18 °C in the light and 16 °C in the dark, 12-h photoperiod; Precision Low Temperature Illuminated Incubator 818, Thermo Fisher Scientific Inc., Waltham, Massachusetts). Trays were sprayed weekly with a 1% solution of hydrogen peroxide to prevent mold. After 3 days, seeds were checked daily for germination, which was defined as the radicle protruding at least 2 mm. Once germinated, 28 seeds per mother tree were immediately transplanted into 656 mL Deepots D40h (Stuewe and Sons, Inc., Tangent, Oregon) in a mixture of 20% forest soil, 50% Fafard 4P mix potting soil (Conrad Fafard Inc., Agawam, Massachusetts), 20% sand, and 10% pea gravel for drainage. Forest soil was included to better represent actual growing conditions and provide native mycorrhizae. It was collected in a mixed conifer forest containing limber pine in Centennial, Wyoming (41.2981°N, –106.1375°W), on 3 October 2012. Each pot included Osmocote Classic 14–14–14 controlled-release fertilizer (Everris International B.V., the Netherlands). Greenhouse temperatures varied between 17 °C and 22 °C, with supplemental lighting providing a 16 h light – 8 h dark photoperiod.

Experimental design

The study design was a full 2 × 2 factorial with factors of seed source region (north or south) and watering treatment (wet, simulating southern summer monsoons; dry, simulating northern midsummer drought). Seedlings from each of the three seed trees (families) per population were randomly assigned a water treatment level. Treatments were applied in a split-plot design to 28 blocks containing one replicate seedling per family (2 water levels × 2 regions × 7 sites·region^{-1} × 3 families·site^{-1} × 14 seedlings·family^{-1}·water level^{-1}; total $n = 1176$). Half of the seedlings from both northern and southern

Table 2. Time line describing when seedling trait measurements and water treatments occurred during the study.

Postgermination time period (months)	Experimental activity	Traits measured
0–2	Pretreatment	Cotyledon length; needle length; water potential
2	Pretreatment harvest	Root dry mass; shoot dry mass; root length; stem diameter
2–3	Drought cycle 1	Needle length
3	Midtreatment harvest	Root dry mass; shoot dry mass; root length; stem diameter
3–4	Drought cycle 2	Needle length; water potential
4	Posttreatment harvest	Root dry mass; shoot dry mass; root length; stem diameter; carbon isotope ratio

regions received a wet treatment, while the other half of the seedlings received the dry treatment. All blocks and seedlings within blocks were spatially randomized in the greenhouse.

Before the treatment began, when seedlings were 2 months old, all seedlings were measured to obtain cotyledon and primary needle length, and two blocks were harvested to determine stem diameter, root length, root dry biomass, shoot dry biomass, total dry biomass, and water potential (Table 2). Subsequent harvests of 14 blocks (seven dry and seven wet) were made midway through the treatment (3 months old) and then another 14 blocks were harvested at the end of the treatment (4 months old) to assess changes in seedling response. Water treatments began 2 months after germination and continued for 2 months. Because limber pine grow substantially between 60 and 120 days old before growth levels off (Borgman et al. 2014), the watering treatment was targeted during this time of intense growth. At the commencement of treatments, all seedlings were watered to saturation. Half of the seedlings (drought simulation) underwent three 2- to 4-week dry-down cycles, while the other half of the seedlings (monsoon simulation) were watered weekly with 50 mL of deionized water per pot (the mean weekly precipitation in the south). The appropriate length of dry-down periods was explored through a prestudy of drought effect on 2-month-old limber seedlings. Seedlings were moderately stressed with a water potential of approximately –1.4 MPa (Moyes et al. 2013) when soil moisture content reached approximately 10%–15%.[1] The goal was to provide measurable drought stress but avoid high seedling mortality. The first drought cycle consisted of total water exclusion for 1 month. Due to higher than anticipated mortality, the second drought cycle was punctuated by a watering event after 2 weeks before resuming the drought treatment.

At each harvest (midtreatment and posttreatment), survival and primary needle length, stem diameter, root length, and dry biomass of roots and shoots were recorded. Relative growth rate (RGR) was calculated as follows:

$$(1)\qquad \mathrm{RGR} = \frac{\ln(l_2 - l_1)}{(t_2 - t_1)}$$

where l is the length of needles in millimetres, and t is time in days. During all harvests, roots and shoots were separated below the cotyledon node, placed in separate, labeled coin envelopes, and dried (60 °C, 7 days). Once dry, roots and shoots of each seedling were weighed to the nearest hundredth of a milligram (Sartorius LE225D balance, Germany). Water potential was measured on a subset of seedlings with a pressure chamber (model 600, PMS Instrument Company, Corvalis, Oregon) before (one seedling per family) and during (one seedling per family per treatment) water treatment to ensure that the treatment had the desired effect. After 8 weeks of treatment, all remaining seedlings were harvested.

Carbon isotopes

At the end of the 2-month drought treatment and after dry mass had been recorded for individually harvested seedlings, primary needles of seedlings from each family and each treatment (seven wet blocks and seven dry blocks) were pooled for carbon isotope ratio. Generally, the carbon isotope ratio (δ^{13}C) in plant tissue reflects the balance between carbon gain and water loss and has been shown to be a good predictor of WUE (Farquhar et al. 1989). Here, δ^{13}C was used as a metric of WUE for determining the presence of genetic differentiation in these populations and provided insight into the link between biomass allocation, growth, and water stress. See Ehleringer (1990), O'Leary (1988), and Farquhar et al. (1989) for more detailed information on the use of carbon isotope ratios.

To prepare samples for analysis of δ^{13}C, samples were dried in an oven at 60 °C, 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). We analyzed 100 samples representative of both treatments and all families, including 16 random duplicates. A control sample with known isotope composition was also run every 12 samples to document machine precision (0.2 per mille (‰)). Sample analysis was done randomly over 2 days, represented as block 1 and block 2.

As the atmospheric carbon dioxide levels in a greenhouse differ from those outside (–8‰ relative to Pee Dee Belemnite 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 (δ^{13}C) and not as the standardized isotope discrimination (Δ) using the following equation:

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

where R is the ratio of ^{13}C to ^{12}C, and units are in per mille (‰).

Data analyses

Mixed model analyses of variance (ANOVA) was used to evaluate differences in seedling performance traits 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 using proc mixed in SAS version 9.3 (SAS Institute, Cary, North Carolina). Cotyledon length was also included in the model as a covariate to help account for maternal effects (Borgman et al. 2014). The measured traits included total dry mass; root-to-shoot ratio; stem diameter; root length; primary needle length, measured at approximately 60, 90, and 120 days; RGR, based on needle length measured at 90 days (midtreatment) and 120 days (posttreatment); mortality; water potential; and δ^{13}C. Data from each harvest were analyzed separately. A generalized linear mixed

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[1] Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjfr-2014-0399.

model using a binary distribution and a logistic link function was used in the analysis of mortality to account for the binary nature of the data. In cases where there was no significant interaction between region and treatment, a follow-up analysis was conducted using the same model but substituting the continuous variable latitude for the categorical variable region to verify that grouping populations into regions did not obscure continuous variation related to latitude of origin. Assumptions of normality were met in all cases based on the normal and homoscedastic distribution of residuals.

A multivariate recursive partitioning and regression tree analysis was then conducted to link the seedling growth traits measured at the final harvest with geographic and climatic variables. Climatic variables for each seed source were derived from Rehfeldt's (2006) climate surface (http://forest.moscowfsl.wsu.edu/climate/customData/ [accessed 26 July 2012]). Seedling traits or geographic and climatic variables were first checked for correlation; if any two of either group had a Pearson's correlation coefficient, r, greater than 0.9, then one of the two variables was thrown out of the model to reduce collinearity (see Supplementary Tables S1 and S2)[1]. Remaining seedling traits were root biomass, cotyledon length, root length, shoot biomass, stem diameter, root-to-shoot ratio, RGR, and needle length measured at approximately 60, 90, and 120 days. Of 23 geographic and climate variables, those remaining in the model were elevation, growing season precipitation, Julian date of the last freezing date of spring, spring precipitation (April + May), mean annual precipitation, mean annual temperature, mean minimum temperature in the coldest month, degree days over 5 °C (based on mean monthly temperature), summer precipitation (July + August), summer precipitation balance (July + August + September)/(April + May + June), and mean temperature in the coldest month.[1] This analysis was run several times using a different combination of the 23 geographic and climatic variables to ensure that the selection of variables based on the correlation matrix did not have a large influence on model results; it did not. These analyses were done in R version 3.0.2, using the package mvpart (R Foundation for Statistical Computing 2013, available from http://www.r-project.org/).

Results

Prior to the first watering treatment, the pretreatment harvest data revealed that seedlings sourced from the south showed significantly longer primary needles ($p = 0.0391$), longer cotyledons, ($p = 0.0013$), and lower root-to-shoot ratios ($p = 0.0036$) compared with seedlings sourced from the north (Table 3; Fig. 3). In contrast, root length, dry biomass, and stem diameter were not significantly different between source regions prior to the watering treatment.

Midtreatment water potential values in the dry treatment averaged −1.2 MPa and were significantly lower than the well-watered treatment levels, averaging −0.8 MPa ($p = 0.0084$), and the pretreatment levels, averaging −0.7 MPa ($p = 0.0059$), indicating increased levels of water stress for seedlings in the dry treatment (Fig. 4). By the midtreatment harvest, southern source seedlings continued to have longer primary needles and had significantly greater total dry mass and larger stem diameters. Only the RGR was significantly affected by the watering treatment (Table 3). There were no significant region × treatment interactions.

By the final posttreatment harvest after the 2-month drought treatment, significant growth differences based on source origin were found for root length, needle length, diameter, and total dry mass, and southern seedlings were generally larger (Fig. 5; Table 3). Additionally, seedlings in the wet treatment significantly outperformed those in the dry treatment for all growth traits ($p < 0.05$), regardless of seed source. At this time, 17.6% of all seedlings were dead or declining in vigor. Significantly more seedlings died in the dry treatment than in the wet treatment ($p = 0.0015$). The highly significant effect ($p < 0.0001$) of the covariate cotyledon length in the mortality analysis suggests that initial plant size greatly influenced mortality (Table 3).

The effect of watering treatment was highly significant for the carbon isotope ratios ($p < 0.0001$). Seedlings grown under the dry treatment had a 1.6845‰ higher ratio than those in the wet treatment. There was no significant interaction between treatment and seed source region ($p = 0.40$); however, when region was replaced with the continuous variable latitude in the model, an interaction between treatment and seed source region was marginally significant ($p = 0.0778$; Table 3; Fig. 6). In that case, for well-watered seedlings, southern seed sources had a slightly higher $\delta^{13}C$ than northern seed sources (Fig. 6); however, within the dry treatment, little latitudinal variation was evident.

Although the nested random factors of site within region and family within site were not of primary interest in this study, ANOVA revealed that they often made significant contributions to explaining trait variation (Table 3). Family nested within site and region was a significant source of variation for most seedling traits measured before the treatment, for RGR measured during the treatment, and for needle length, root-to-shoot ratio, and mortality measured after the treatment. Site nested within region was a significant source of variation for needle length before the treatment and for stem diameter, dry mass, mortality, and $\delta^{13}C$ after the treatment (Table 3).

The regression tree from the multivariate partitioning analysis split on precipitation metrics of seed source location, first on summer precipitation at 135.5 mm, then on the ratio of summer to spring precipitation at 1.195, and finally again on summer precipitation at 93.5 mm. Seedling growth characteristics were generally higher with increasing precipitation (Fig. 7). Variation in seedling growth traits was mostly linear, with 94.87% of variation in one dimension (Fig. 7).

Discussion

The goals of this study were to determine if there was genetic differentiation among limber pine populations in the Southern Rockies, particularly local adaptation to variation in midsummer water availability, to guide planting choices and to improve planting success for management in the face of WPBR. We conducted a greenhouse common garden study to evaluate the growth responses to different watering treatments of seedlings from the northern and southern regions of the Southern Rockies. These treatments reflected the wetter conditions of the south and the drier conditions of the north. We found significant differentiation in growth traits between southern and northern populations and suggestive evidence for local adaptation to water availability among seed sources along the latitudinal gradient.

Differentiation of some growth traits between seed source regions was obvious as early as 2 months after germination. Southern seedlings had 36.1% longer needles, 21.4% longer cotyledons, and 18% lower root-to-shoot ratio than northern seedlings (Table 3). By the midtreatment harvest 3 months after germination, growth differences between northern and southern seedlings were also observed in total dry mass, and southern seedlings were an estimated 57.6% larger and had stem diameters 12.8% larger than northern seedlings. By the final posttreatment harvest 4 months after germination, there were significant differences between northern and southern seedlings in root length, stem diameter, needle length, and total dry mass, giving evidence for genetic differentiation of these traits. Minimum water potentials of seedlings from the drought treatment were comparable with the moderate stress experienced in the field in the Southern Rockies (Moyes et al. 2013). Growth differences among sources were insensitive to water treatment level, suggesting that they are not plastic to different water availabilities, although they may have adaptive significance to performance under environmental conditions or competitive interactions at the source locations.

Table 3. Summary of ANOVA for harvest data showing fixed effects of seed source region, treatment, and cotyledon (covariate to account for maternal effects) and random effects of site nested within region, family nested within site and region, and block.

		Degrees of freedom (df)			
Trait	Effect	Numerator	Denominator	F or Z value	p value
Pretreatment					
Root length	Region	1	37	0.11	0.7378
	Site(region)			—	—
	Family(site × region)			4.06	<0.0001
Needle length	Region	1	12	5.33	0.0391
	Site(region)			1.68	0.0469
	Family(site × region)			3.45	0.0003
Stem diameter	Region	1	37	0.67	0.4171
	Site(region)			—	—
	Family(site × region)			0.12	0.4522
Total dry mass	Region	1	12	3.16	0.1013
	Site(region)			0.90	0.1838
	Family(site × region)			3.56	0.0002
Cotyledon length	Region	1	38	12.16	0.0013
	Site(region)			—	—
	Family(site × region)			4.17	<0.0001
Root-to-shoot ratio	Region	1	37	9.66	0.0036
	Site(region)			—	—
	Family(site × region)			0.14	0.4455
Midtreatment					
Needle length	Region × treatment	1	342	0.25	0.6152
	Region	1	14	13.94	0.0022
	Treatment	1	8	3.93	0.0826
	Cotyledon	1	275	9.79	0.0019
	Site(region)			1.55	0.0605
	Family(site × region)			0.85	0.1977
	Block			1.36	0.0967
Stem diameter	Region × treatment	1	335	0.57	0.4512
	Region	1	15	4.8	0.0445
	Treatment	1	8	0.62	0.4549
	Cotyledon	1	214	26.45	<0.0001
	Site(region)			1.00	0.1577
	Family(site × region)			0.95	0.1713
	Block			1.74	0.0411
Total dry mass	Region × treatment	1	336	1.53	0.2163
	Region	1	15	9.77	0.0070
	Treatment	1	8	4.39	0.0699
	Cotyledon	1	241	40	<0.0001
	Site(region)			1.20	0.1143
	Family(site × region)			1.02	0.1532
	Block			1.49	0.0680
RGR between pre- and mid-treatment harvests	Region × treatment	1	343	2.13	0.1449
	Region	1	15	0.27	0.6139
	Treatment	1	26	29.46	<0.0001
	Cotyledon	1	559	3.62	0.0578
	Site(region)			0.87	0.1933
	Family(site × region)			2.49	0.0064
	Block			—	—
Posttreatment					
Root length	Region × treatment	1	431	0.19	0.6599
	Region	1	15	8.4	0.0108
	Treatment	1	12	35.26	<0.0001
	Cotyledon	1	184	8.01	0.0052
	Site(region)			1.01	0.1567
	Family(site × region)			—	—
	Block			1.50	0.0672
Stem diameter	Region × treatment	1	425	0.05	0.8180
	Region	1	15	6.36	0.0231
	Treatment	1	11	27.24	0.0003
	Cotyledon	1	331	22.92	<0.0001
	Site(region)			1.71	0.0434
	Family(site × region)			—	—
	Block			1.07	0.1419

Table 3 (*concluded*).

		Degrees of freedom (df)			
Trait	Effect	Numerator	Denominator	*F* or *Z* value	*p* value
Needle length	Region × treatment	1	440	0.46	0.4982
	Region	1	49	16.11	0.0002
	Treatment	1	12	56.07	<0.0001
	Cotyledon	1	364	10.04	0.0017
	Site(region)			—	—
	Family(site × region)			2.43	0.0075
	Block			0.85	0.1975
Total dry mass	Region × treatment	1	412	1.75	0.1865
	Region	1	15	10.16	0.0062
	Treatment	1	12	29.21	0.0002
	Cotyledon	1	304	51.49	<0.0001
	Site(region)			1.66	0.0489
	Family(site × region)			0.47	0.3178
	Block			1.33	0.0917
Root-to-shoot ratio	Region × treatment	1	407	0.18	0.6678
	Region	1	15	0.04	0.8386
	Treatment	1	13	27.22	0.0002
	Cotyledon	1	258	0.27	0.6066
	Site(region)			0.25	0.4008
	Family(site × region)			2.17	0.0149
	Block			1.08	0.1407
RGR between mid- and post-treatment harvests	Region × treatment	1	376	0.66	0.4160
	Region	1	15	1.78	0.2014
	Treatment	1	12	81.15	<0.0001
	Cotyledon	1	228	0.87	0.3521
	Site(region)			1.11	0.1331
	Family(site × region)			0.28	0.3914
	Block			0.22	0.4119
Mortality	Region × treatment	1	1020	0.32	0.5717
	Region	1	12	0.11	0.7489
	Treatment	1	966	10.18	0.0015
	Cotyledon	1	966	25.09	<0.0001
	Site(region)			49.41*	<0.0001
	Family(site × region)			–31.20*	<0.0001
	Block			31.22*	<0.0001
$\delta^{13}C$ (categorical)	Region × treatment	1	64	0.72	0.4006
	Region	1	14	0.98	0.3384
	Treatment	1	65	138.94	<0.0001
	Site(region)			1.66	0.0487
	Family(site × region)			0.36	0.3612
	Block			—	—
$\delta^{13}C$ (continuous)	Latitude × treatment	1	64	3.21	0.0778
	Latitude	1	20	1.93	0.1799
	Treatment	1	63	1.73	0.1930
	Site			1.60	0.0547
	Family(site)			0.46	0.3212
	Block			—	—

Note: No interactions between treatment and region were significant except for $\delta^{13}C$ ($p = 0.0778$), which was analyzed using latitude (continuous) in place of region (categorical). If the variance estimate was too small to measure, the *F*, *Z*, or *p* values are indicated by "—". RGR, relative growth rate.

*χ^2 test statistic for likelihood ratio test.

Carbon isotope composition revealed that seedlings from southern sources in the well-watered treatment had less ^{13}C discrimination (higher $\delta^{13}C$) than seedlings from northern sources, suggesting greater WUE in southern sources. This result is counter to our expectations, as summer monsoonal precipitation patterns suggest that southern sources are less water limited than northern sources; we offer two possible explanations for this result. First, positive correlations between $\delta^{13}C$ and dry mass can result from a higher photosynthetic capacity rather than lower stomatal conductance (Hubick et al. 1986; Huxman et al. 2008; Ehleringer 1990; Silim et al. 2001). Second, 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 (less negative $\delta^{13}C$) 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 less negative $\delta^{13}C$ under drought stress can be explained by stomata closing with decreased moisture availability to prevent water loss (Pataki et al. 1998).

The robust growth of seedlings from populations near the southern edge of the limber pine range was surprising; they were considerably larger and healthier than seedlings from northern populations. There is evidence of hybridization with *Pinus*

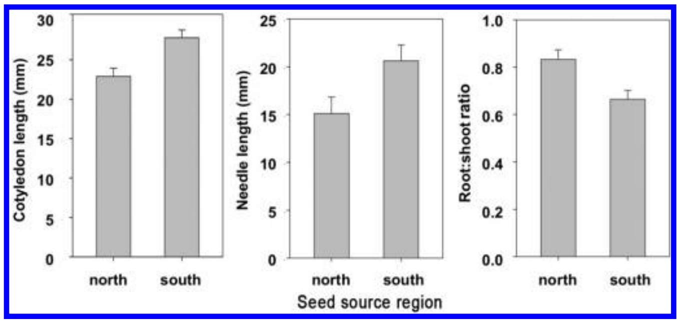


Fig. 3. Pretreatment means with one standard error for cotyledon length (mm), needle length (mm), and root-to-shoot ratio for seedlings sourced from northern and southern regions. All differences are significant at $p < 0.05$.

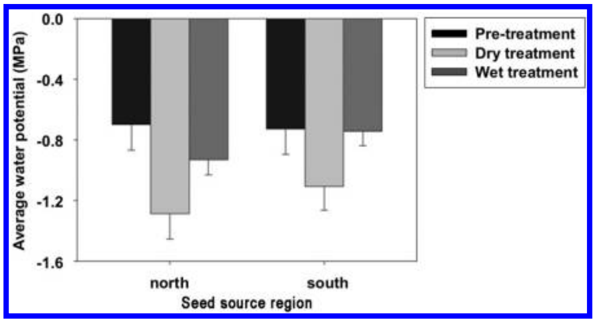


Fig. 4. Mean water potentials (MPa) with standard error before and during water treatment for seedlings from northern and southern seed source regions. Larger negative values indicate increasing levels of water stress. Seedlings in the dry treatment (light grey) were significantly ($p < 0.05$) more stressed than seedlings measured before the treatment began (black) and those in the wet treatment (dark grey). Target water potential was –1.4 MPa, a level reflecting moderately stressed limber pines in the field (Moyes et al. 2013).

strobiformis Engelm. (southwestern white pine) at the southern edge of the range (Steinhoff et al. 1971), which could perhaps explain this vigor, although 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. Alternately, perhaps the increased moisture availability during the growing season at the southern part of the range allows for a greater diversity of species, leading to more competition. Such an environment may select for larger seedlings. There is also a well described trade-off between growth and cold hardiness (Loehle 1998) that could also account for larger southern seedlings.

The imposition of two watering treatments provided the opportunity to evaluate whether populations differed in response to soil moisture, and if so, whether differences indicated adaptation to the local environment. Local adaptation is demonstrated when an interaction is detected in which genotypes exhibit greater fitness in their home environment than genotypes sourced from different environments (Kawecki and Ebert 2004). In this case, we examined early components of fitness (early seedling survival and growth) and simulated one key aspect of the native environment (soil moisture). Though significant trait variation was found among seed sources in our study, evidence for specific local adaptation to midsummer water availability was limited to δ^{13}C. Factors other than summer precipitation among our populations may be imposing selection upon traits and possibly leading to local adaptation; such adaptation could be detected with reciprocal transplant studies in the field. This study was also short in duration in relation to the life span of the tree species. It is possible that seedling, sapling, and

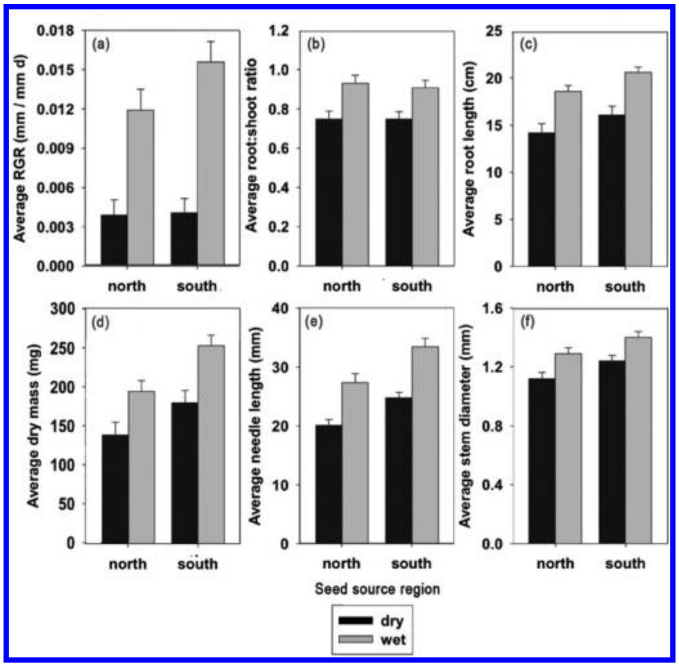


Fig. 5. The following six seedling growth traits, measured at the final posttreatment harvest, are shown for northern and southern seed sources with means and standard errors given for the dry and wet treatments: (*a*) relative growth rate (RGR; $\text{mm}\cdot\text{mm}^{-1}\cdot\text{day}^{-1}$); (*b*) root-to-shoot ratio; (*c*) root length (cm); (*d*) seedling dry mass (mg); (*e*) primary needle length (mm); and (*f*) stem diameter (mm). All traits showed a significant treatment effect. Root length, dry mass, needle length, and stem diameter had significant region and treatment effects ($p < 0.05$).

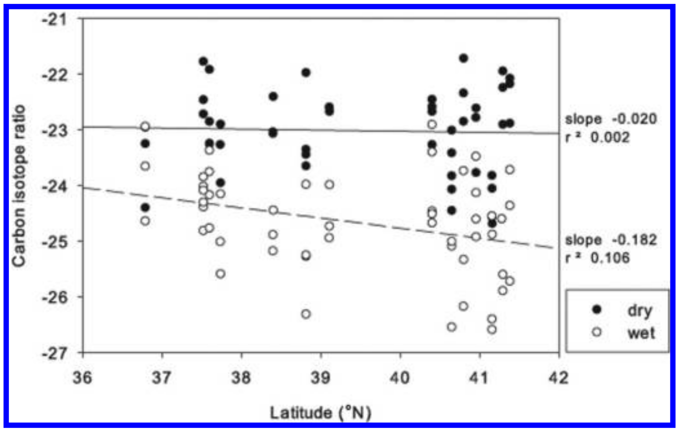
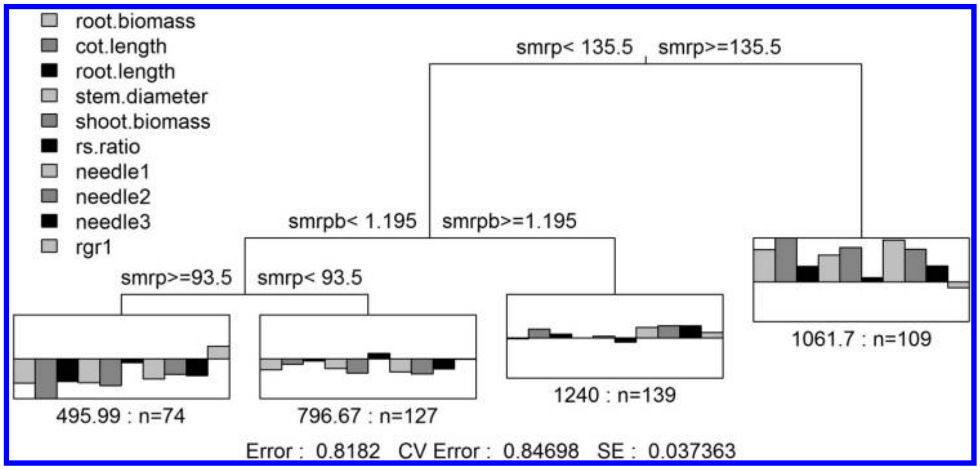


Fig. 6. The carbon isotope ratios are shown for each seedling in the dry (filled circles) and wet (unfilled circles) treatments across latitude (°N). Trend lines were calculated from the least square means for both dry (solid line) and wet (dotted line) treatments. More negative values indicate more discrimination against ^{13}C and suggest lower water-use efficiency. Each circle represents the progeny of a single seed tree. The interaction of treatment and latitude was marginally significant ($p = 0.0778$).

tree growth and adult fecundity would have exhibited local adaptation if the study had continued. We timed the treatment to coincide with the period of extensive limber pine seedling growth (Borgman et al. 2014) to ensure the greatest opportunity of observing significant trait differentiation. Their young age also provides insight into their potential response to drought pressure during establishment.

Fig. 7. Splits from the multivariate recursive partitioning and regression tree analysis were based on precipitation metrics at seed source location, first at summer precipitation (July + August; "smrp") of 135.5 mm, then at the ratio of summer to spring precipitation ((July + August + September)/(April + May + June); "smrpb") of 1.195, and then at summer precipitation of 93.5 mm. Seedling growth traits at the final posttreatment harvest in the model include root biomass (root.biomass), cotyledon length (cot.length), root length (root.length), stem diameter (stem.diameter), shoot biomass (shoot.biomass), root-to-shoot ratio (rs.ratio), needle length before treatment (needle1), needle length during treatment (needle2), needle length after treatment (needle3), and relative growth rate between pre- and mid-treatment (rgr1). The bar graphs at each partition show the average seedling growth trait in that group in the order of listed traits in the upper left corner.



Multivariate analysis provided further information on how seedling growth traits relate to environmental variables (Campbell 1986; Sorensen 1992; Rehfeldt 1994). The regression tree split on variables related to precipitation, suggesting that moisture patterns at the seed sources are largely responsible for differences in seedling growth traits (Fig. 7). Comparing environmental conditions such as precipitation and related variables between seed source and planting target locations may lead to better adapted seedling stock than seed zones alone. These early seedling results suggest that matching climatic variables of seed sources with planting location is a reasonable strategy in the reforestation efforts of limber pine.

Conclusions and implications

We predicted that (*i*) there would be a differential response of seedling growth based on region of seed origin and (*ii*) the response of seedlings to water treatment would depend on their source region, indicating local adaptation to their native midsummer precipitation regime. Most seedling growth traits and patterns of seedling mortality demonstrated differential growth based on region of seed origin. However, seedling growth response between watering treatments did not depend on seed source region (i.e., there was no significant interaction), except for carbon isotope ratios when using latitude as a fixed effect instead of region. Results from this study support the hypothesis that there is genetic differentiation in traits among seed sources from northern and southern areas of the Southern Rockies, with possible mild local adaptation at the seedling stage related to precipitation patterns.

Planting disease-resistant seedlings to increase the frequency of resistance to WPBR in populations before pathogen invasion is recommended for the sustained health and function of ecosystems dominated by limber pine (Schoettle and Sniezko 2007; Burns et al. 2008). Ideally, heritable resistance can be identified within each seed zone to minimize the need to move disease-resistant stock long distances for planting. Studies to identify and quantify resistance to WPBR are underway (Schoettle et al. 2011*b*); however, early results reveal that genetic resistance to WPBR is not distributed uniformly and at least one resistance mechanism is more common in northern limber pine populations of the Southern Rockies seed zone than in southern populations (Schoettle et al. 2014; A.W. Schoettle and R.A. Sniezko, personal communication, 2014). The common garden study presented here suggests that supplementing southern populations with seedlings having heritable resistance to WPBR from the north within this regional zone should be done with caution due to the potential for maladaptation to stresses at the planting location. Further intensive research is needed to examine the physiological and growth traits of northern and southern populations over a longer time period. Additional work is also needed in the southern populations to identify seed trees with heritable resistance to WPBR for use as seed sources for proactive plantings to increase the resilience of these populations to the spreading non-native pathogen. This research suggests that the pattern of precipitation between seed source area and planting location should be matched as best as possible for limber pine in this geographic area.

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