QUANTITATIVE TRAIT VARIATION IN LIMBER PINE (*Pinus flexilis*)

by

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Abstract

Limber pine (*Pinus flexilis*) is one of the North American white pines under duress. Pressured by several forces including an introduced fungal pathogen (*Cronartium ribicola*) and changing climate, this is a species of conservation concern in many regions. The ability to adapt to shifting conditions depends on the amount and distribution of standing genetic variation. To evaluate the patterns and extent of variation in limber pine, I examined needle traits with regards to pathogen infection, and the distribution of quantitative traits in the context of climate. Specifically, I measured how leaf traits differ after surviving an inoculation with *C. ribicola* and found that survivors of infection had significant differences in needle size, stomatal density and specific leaf area compared to uninoculated controls. In addition, the variance of each trait shifted modestly, pointing to signs of both phenotypic selection and plasticity. Next, I examined the structure of quantitative genetic variation across 16° of latitude by phenotyping traits in a common garden experiment. This trial revealed that population differences explained between 1-24%, and family between 1-20% of the total phenotypic variance, depending on the trait under inspection. This corresponded to a mean Q_{st} estimate of 0.158 (range 0.02-0.19), with growth traits exhibiting the greatest population differentiation. Precipitation-related climate variables were the strongest predictors of differences among populations. These results suggest that limber pine has relatively low levels of quantitative genetic variation among populations, but an almost equivalent amount within populations. Whether or not it will be sufficient to cope with the many stresses this species contends with remains unclear.

Preface

The motivation for my research in this field derives from the good folks of Burmis, Alberta, who also admire limber pine. The study, however, was designed in cooperation with my committee, Dr's Anna Schoettle, Sally Aitken, and supervisor, Amy Angert. All parts of the research were conducted by myself with the help of a number of assistants. Analysis was performed by myself, in consultation with my supervisor, Dr. Amy Angert and committee member Dr. Anna Schoettle.

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whose infinite support, patience and faith made this possible,

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Chapter One: Introduction

Biodiversity increases ecosystem stability (McCann 2000), yet global biodiversity is in decline (Barnosky *et al.* 2011). Biological systems can be perturbed in many ways, for example, through changes in environmental conditions (IPCC 2014), by pests and competitors (Pauchard *et al.* 2009), or through the impacts of invasive species (Richardson 2011). The multiplicative effects of several interacting factors that disrupt local systems can prompt shifts to new equilibria (Chmura *et al.* 2011). Whether or not individual species can survive these shifting circumstances depends on their ability to tolerate, adjust or adapt to them, which in turn is dependent on the available standing genetic variation and the strength of selection (Orr 2005). Forest trees have relatively high genetic variation, although response to selection may be slow due to longevity and polygenic affects (Savolainen *et al.* 2007). This potential adaptational lag time may mean that forest trees are not responding quickly enough to persist under new conditions. A prudent approach to avoid this consequence is to identify significant adaptive traits and conserve genetic variation.

In conjunction with changing environmental conditions, North American forests have experienced several species introductions that impair or jeopardize their function (Campbell & Schlarbaum 2014). These pests, parasites and pathogens can impose high fitness costs such as reduced fecundity or mortality, thereby acting as strong agents of selection (Kawecki & Dieter 2004). However, it remains to be seen if the afflicted species will survive after these encounters. In some cases, like the American chestnut (*Castanea*

dentata), near complete devastation of the natural populations was the outcome (Hayden *et al.* 2011), although intervention efforts aim to restore this species. In other cases such as white pines, genetic forms of resistance (Kinloch & Dupper 2002) exist in natural populations (Holub 2006, Hoff *et al.* 1980), albeit at low levels.

It is well documented that North American white pines (*Pinus* subsection *Strobus*) face a suite of biotic and abiotic pressures (Tomback *et al.* 2011, Burns *et al.* 2007). Local pests like the mountain pine beetle (*Dendroctonus ponderosae*) and dwarf mistletoe (*Arceuthobium blumeri*), habitat fragmentation (Jorgensen *et al.* 2002), fire-suppression (Coop & Schoettle 2009), and land-use change (Means 2011) have combined with climate change to stress these systems. In combination with these pressures, the exotic invasive rust, *Cronartium ribicola* , has had a large negative impact on white pine forest ecosystems (Cleaver *et al.* 2015). Introduced in the early part of the century, *C. ribicola* causes the white pine blister rust (WPBR) disease (King & Hunt 2004). Harmful effects of this disease range from reducing fecundity to fatality (Burns *et al.* 2008), which has led it to be seen as catastrophic to these ecosystems.

Determining methods to conserve these trees is complex. As the exact mechanisms of resistance are not completely characterized and the trees face challenges on many fronts, various tactics have been used. Methods include pruning, controlled burns, and chemical treatments to help stop the spread of the disease (Geils *et al.* 2010). However, the leading strategy for a long-term solution is to distinguish and protect rust-resistant parent trees (Schoettle & Sniezko 2007, and reviewed by Schwandt *et al.* 2010) and plant their progeny into areas that are forecast to be climatically suitable now and in the future (Sgro

et al. 2011, McLane & Aitken 2012). Identifying trees that are inferred to have resistance is currently done in one of two ways: finding individual trees that appear to have few symptoms in areas where the rust is active at high levels (known as putative resistance), and where possible, artificial inoculation tests of progeny from individual seed trees from which the resistance status of the seed tree can be inferred.

Early research on North American white pines concentrated on timber species such as western white (*Pinus monticola*), eastern white, (*Pinus strobus*) and sugar pine (*Pinus lambertiana*), however, the non-commodity species are now also a focus of concern due to their ecological importance (Burns *et al.* 2008). Dubbed the 'high five' because of their common occurrence at high elevation and with needles in fascicles of five, this includes whitebark (*Pinus albicaulis*), limber (*Pinus flexilis*), southwestern white (*Pinus strobiformis*), foxtail (*Pinus balfouriana*), Rocky Mountain Bristlecone (*Pinus aristata*), and Great Basin bristlecone (*Pinus longaeva*) (Tomback *et al.* 2011). Commonly considered pioneer and foundation species in their systems, many five-needled pines are early seral species that colonize fire-disturbed or otherwise inhospitable and exposed habitat (Tomback *et al.* 2011). While these alpine and montane trees are not valued commercially, they improve soil and act as facilitators for a succession of conifers such as Engelmann spruce (*Picea engelmannii*) and subalpine fir (*Abies lasiocarpa*) (Donnegan & Rebertus 1999). Additionally, they promote biodiversity by supplying direct and indirect shelter for wildlife such as owls and other birds, provide direct food stores for bears, squirrels, and corvids with whom many have a mutualistic relationship, and indirect food stores for predators like lynx (Seamans & Gutierrez 1995, Ayers & Lombardero 2000, McCutchen

1996, Siepielski & Benkman 2008, Lanner & Vander Wall 1980, Schoettle & Sniezko 2007). Moreover, they provide ecosystem services that regulate hydrological flow and promote slope stability (Schoettle 2004). Less tangibly, these trees hold aesthetic and symbolic value as iconic species that persevere in unforgiving edaphic and climatic conditions (Palmer 2013). Unfortunately, the environments to which these trees have adapted are predicted to be vulnerable to climate warming and drought (Hansen & Phillips 2014).

Neither renowned like the ancient Great Basin bristlecone nor as high profile as whitebark, limber pine are nevertheless impacted by similar conditions. A white pine of the subgenus *Strobus*, limber pine has been heavily affected by the WPBR disease across much of its range, particularly in the north, where mortality has reached 52% in some populations (Smith *et al.* 2011). Limber pine is distributed from California in the west to the Dakotas in the east and from Alberta in the north to California and New Mexico in the south, spanning a large elevational range from 800 - 3700m (Zavarin *et al.* 1993). Across political boundaries, however, it goes from widespread to rare. In the northern range margins, it is one of the least common conifers in BC (Pigott & Moody 2012, BC Conservation Data Center 2013), and is provincially listed as endangered under the Alberta Wildlife Act (Government of Alberta 2014). In 2014 limber pine was recommended for federal Species at Risk listing by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC 2014).

As its name implies, limber pine are flexible not only physically (they can withstand even some avalanches – Lanner & Vanderwall 1980), but also ecologically. Limber pine's range encompasses a wide breadth of ecosystems from grasslands and foothills to treeline,

which has led it to be called an ecological generalist (Schoettle & Rochelle 2000, Windmuller-Campione & Long 2016). It has also been named both a keystone and pioneer species (Benkman *et al.* 1984), as well as a fugitive species, due to its opportunistic colonization of both moderate and extreme environments (Knowles & Grant 1983). Its conservative growth strategy and therefore protracted life history enables it to tolerate much variability. For example, in exceptionally dry years it trades off carbon gain with acclimation to drought by restricting its photosynthetically active period to less extreme seasons (Letts *et al.* 2009). Its modest competitive ability and broad physiological tolerances have led to projections that limber pine will shift upslope with climate change (Monahan *et al.* 2013); however its plasticity may make it difficult to detect genetic differences that could influence its success in migrating. This prompts the question: How much genetic differentiation exists within and among populations in limber pine for phenotypic traits associated with adaptation?

Descriptions of spatial genetic variation in limber pine vary. The genetic structure of limber pine may reflect the glacial refugia from which each of the populations expanded (Mitton *et al.* 2000), as well as adaptation to local conditions (Kremer *et al.* 2012). Allozyme data have shown that limber pine has high to intermediate levels of differentiation across its range relative to other conifers (Jorgensen *et al.* 2002), and has relatively high levels of within-population genetic diversity (Schuster & Mitton 1999), although population genetic studies encompassing large portions of the species range are few. However, more studies are underway and should fill current knowledge gaps (Schoettle *et al.* 2011, Sniezko *et al.* 2011).

Limber pine exhibits a WPBR-resistance response that is inherited via a single-gene that follows Mendelian segregation, and this R-gene response may be expressed within needles (Schoettle *et al.* 2014). Considering the pathogen's route of infection via needle stomata, morphological and physiological traits may influence susceptibility to the disease and therefore be used as indicators of resistance, as it has in tomato (Ramos *et al.* 1992). There is evidence that some needle characteristics such as cuticular wax and stomatal size, shape, or density impact resistance in both eastern and western white pine (Smith *et al.* 2006, Woo *et al.* 2001). To date, the sole investigation of limber pine needle traits as they pertain to rust resistance suggests interplay between physiological and resistance characteristics (Vogan & Schoettle 2016).

Following long tradition, this research aims to quantify traits in this understudied species. Contributing to knowledge of the variation retained in populations and how it is distributed across the landscape will help clarify limber pine's potential to respond to ongoing changes in its habitat, key among them WPBR and climate change. As fundamental steps in this process, in Chapter Two I assess morphological traits that have been associated with WPBR resistance in other species (Bennet *et al.* in press, Woo *et al.* 2001) by comparing needle traits of trees that have survived infection by the WPBR with those that were not exposed. Then in Chapter Three I investigate geographic variation of traits by studying multiple limber pine families and populations originating from across a latitudinal gradient, grown in a greenhouse common garden. These data will produce knowledge to support the development and refinement of strategies to better conserve and restore this species.

Chapter Two: Needle Trait Response to Rust Inoculation

2.1 Introduction

Various agents of global change are impacting forest ecosystems and their constituent species. These disturbances and their novel combinations often increase direct and indirect pressures on forest trees. For example, introduced species can not only compete directly with native trees for resources but also alter habitat. Tamarisk (*Tamarix* sp.) introduced to North America from Iran has salinized the soil, which has increased sedimentation thereby indirectly shifting species composition (Zavaleta 2000). Another well-documented example is when uncharacteristic climate regimes disrupt populations. Warmer and drier winter temperatures have direct implications for the physiological processes of forest trees, and they also affect pest species like mountain pine beetle (*D. ponderosae*), increasing their abundance and negative effect on tree hosts (Sidder *et al.* 2016). Collectively, these rapidly changing conditions test the tolerances and ability of forests to acclimate and, ultimately, adapt. Predicting how trees will respond to multiple and interacting perturbations has become a management priority for many jurisdictions (Schoettle 2004, Burns *et al.* 2008, Schwandt *et al.* 2010, Mangold 2011).

North American white pine systems have been under chronic pressure from an introduced pathogen for the past century. The fungus *Cronartium ribicola* causes the disease white pine blister rust (WPBR), which has resulted in considerable damage to many white pine species (Burns *et al.* 2008). WPBR has caused extensive mortality of white

pines, and it also reduces fecundity by affecting cone-bearing branches in the crown of the tree (Schwandt *et al.* 2010). First discovered on important timber species, the rust rapidly spread across much of the continent, to several species of ecological importance, diminishing healthy populations of trees and altering both population and community structure. Once dominant, western white pine (*P. monticola*) in the northern Rocky Mountains are now less abundant, covering just 10% of the area within their range (Jain *et al.* 2004), with Douglas-fir, grand fir, western red cedar and hemlock now succeeding them. For non-timber species that live at high elevation or are otherwise inaccessible or of low wood value, losses are expected to have substantial consequences for local ecosystems. For example, pioneering whitebark pine modify microsites to facilitate the development of sheltered areas known as tree islands that include less hardy species. Their loss could mean that other species don't establish, thereby altering community development. This would render the system less productive, having cascading effects on habitat and resources for wildlife (Tomback *et al.* 2016). However, the extent to which pressure from the rust will ultimately alter host populations or how those changes may interact with other simultaneous threats such as climate change remains unknown.

While it is notoriously difficult to predict future outcomes in complex systems (Kane & Hingham 2015), standing variation is vital for the capacity of a population to respond to change (Orr 2005). Despite not co-evolving with *C. ribicola*, North American white pine species have inherent variation that includes resistance to the WPBR pathogen (Kinloch & Dupper 2002). One class of resistance is characterized by a decrease in the severity or progression of disease symptoms (Kinloch 2004). Another class of resistance is R-gene

resistance, denoted as *Cr* (Kinloch *et al.* 1992) in white pines. This is a single gene resistance that provides a pathogen-specific response protecting an individual from developing disease symptoms. R-gene resistance in white pines is known to occur in four of the North American white pine species: sugar (*P. lambertiana, Cr1*), western white (*P. monticola, Cr2*), southwestern white (*P. strobiformis, Cr3*), and limber (*P. flexilis, Cr4*) (Kinloch *et al.* 1992, Kinloch *et al.* 2003, Kinloch & Dupper 2002, Schoettle *et al.* 2014), although resistance frequency varies amongst species and populations (Hoff & Bingham 1980, Sniezko *et al.* 2011). Resistance of this kind is expressed as distinctive infection on needles (needle spot; a response that produces necrotic tissue around an infection site to contain the infection locally), abscission of needles (needle cast), and, of course, the lack of disease development.

Leaf traits predict whole plant performance in many systems and are often the first visual indicators of distress. Due to their central role in energy capture and as a focal point for infection in the white pine pathosystem, these functional traits have attracted attention as possible indicators of rust resistance (Woo *et al.* 2001, Smith *et al.* 2006, Letts *et al.* 2009). However, when it comes to using leaf traits as indicators of response under pathogen pressure, results have varied. For example, lower stomatal density has been seen to mitigate frequency of hyphal penetration in Brassicas (Sosnowski *et al.* 2001), but has shown no impact in barley (Vaz Patto *et al.* 2003). It positively correlates with signs of WPBR infection (needle spot) in whitebark pine (Bennett *et al.*, in press), but not in western white pine, where increased stomatal size and shape rather than density were

positively associated with susceptibility among accessions (Woo *et al.* 2001). In either case the availability of stomata to WPBR was found to influence infection success.

Comparing differences between known resistant individuals to the population as a whole may help understand how populations change through selective mortality or phenotypic plasticity under a rust-infection setting. I expected selection to remove that portion of the population that was highly susceptible to the rust, thereby decreasing variation while changing the mean value of a trait; whereas plasticity would shift the distribution of phenotypes, maintaining variation but shifting local optima (Figure 2.1). Such genetic and plastic changes driven by pathogen infection could alter how the trees respond to other stressors. For example, stomata are key pathways for both CO_2 diffusion and infection; therefore, rising CO_2 levels known to affect stomatal density (Van de Water 1994) may also affect susceptibility to infection. Establishing connections between R-gene resistance and other physiological or morphological traits could improve forecasts of how rust-response changes interrelate with other concurrent changes (Klein *et al.* 2013, Vogan & Schoettle 2015).

In this study I asked whether exposure and resistance to the WPBR are accompanied by changes in leaf traits in limber pine, and what the implications may be in altered climate scenarios. The limited research studying leaf morphological traits that may relate to resistance in North American white pines has been done on commercial species (*P. monticola, P. strobus*)(Woo *et al.* 2001, Smith *et al.* 2006), but similar work has now begun on non-timber species (*P. albicaulis*, Bennett *et al.* in press.). The goal of this research was to investigate needle traits that accompany resistance in limber pine trees

that have successfully withstood a rust pathogen treatment compared to the original populations that had not been exposed. I capitalized on an experimental screen for rust resistance conducted in a common garden in which seedlings from several populations were inoculated with WPBR and compared to uninoculated controls. I hypothesized that exposure to WPBR would cause selective mortality of susceptible individuals, revealing morphological characteristics that are correlated with *Cr4* resistance. Specifically, I predicted that selective mortality would result in a decrease in trait variance in survivors compared to controls as well as a shift in trait means such as a decrease in stomatal density, smaller leaves, and lower specific leaf area (SLA).

2.2 Methods

2.2.1 Experimental Design

A rust screening experiment was conducted at the Dorena Genetic Resource Center (DGRC; Cottage Grove, Oregon) (Schoettle *et al.* 2011). Briefly, limber pine seed was collected from populations in Colorado (Figure 2.2), ranging in elevation from 2681-3431m (Table 2.1). Seed was sown in spring of 2009 and seedlings grown in greenhouse conditions until inoculation in autumn that same year. Seedlings were subsequently transferred outside in spring of 2010. Trees were randomly separated into two groups per population prior to inoculation. In a paired design of treatment and control groups (heareafter referred to as "inoculated survivors" and "uninoculated controls" respectively), inherent resistance to the WPBR was tested via inoculation screening and disease-response

phenotyping. Further details regarding the screening experiment are available in Vogan & Schoettle (2016); however results from previous trials indicate that resistance is consistent with single gene inheritance (Schoettle *et al.* 2014). The current study used surviving trees from the above-mentioned rust screening to compare needle morphology between the uninoculated controls and inoculated survivors. From each of the 19 out of 21 original populations that had sufficient numbers of surviving seedlings, I sampled up to 10 inoculated survivors and 10 uninoculated controls, although not all populations had 10 survivors per treatment. In total, 176 inoculated survivors and 178 uninoculated controls were phenotyped (see Table 2.1). Based on a post hoc power analysis conducted in the pwr package in R (Champely 2015), a power of 0.80, and an alpha level of 0.05, this sample size is sufficient to detect a small effect size (f=0.15).

2.2.2 Sampling

One shoot from each of the surviving 354 trees was collected in late May and early June of 2015. Samples were placed in plastic bags and kept fresh with freezer packs in a cooler for less than 48 hours before being processed. In accordance with standard protocols (Perez-Harguindeguy, N. *et al.* 2013), I excised one-year old, fully expanded, apparently pathogen and herbivore-damage free needle fascicles from three regions along the shoot. This provided three sample fascicles per tree, and a total of 1062 fascicles. The average length of the needles from each fascicle was measured to the nearest millimeter in order to compare needle length across groups. Needle width was determined via microscopy.

One needle from each of the three fascicles was randomly chosen for digital imaging. Distal and proximal ends of the needles were embedded in rope caulk and mounted to card stock that could be placed on a stage (Figure 2.3). I isolated two ~1mm^2 counting areas from the central portion of one adaxial side of each needle for assessment. As optical microscopes have been found to be as effective a method of measuring stomatal density in pine as scanning electron microscopes (Hultine & Marshall 2001), I micrographed each counting area at ~200x magnification using a digital microscope (Dino-Lite, Pro-AM413ZT) with a variable polarizing filter to reduce glare. I calibrated the microscope to the nearest 0.01 mm and entered individual needle magnification for each image. This allowed for length, width and area calculations of the counting regions to be performed via the DinoCapture digital imaging software. I visually inspected each image and counted stomata manually, quantifying the number of stomata, number of stomatal rows, and the total length of stomatal rows. Stomata that straddled the area boundary were determined to be in the area if more than half of the stoma was encompassed by the boundary. I averaged all estimates from two counting regions per needle and three needles per tree. Tissue segments of one to three cm from each of the three imaged needles were thereafter oven-dried (Perez-Harguindeguy, N. *et al.* 2013) for 48 hours at 60 °C and weighed together (group of three) on a Mettler Toledo AL104 scale for specific leaf area (SLA) calculations.

2.2.3 Analysis

I calculated surface area using Euclidean geometry by assuming needle fascicles approximate a cylinder. While needles do taper at the tips, only a segment from the middle

of each needle is measured and weighed for calculations. White pine species typically have three-sided needles with five needles per fascicle. These are arranged with the ten adaxial sides facing toward the centre and the five abaxial sides forming the outer surface or circumference (Figure 2.4). Therefore, I calculated the surface area of a fascicle segment as the sum of two rectangular adaxial sides and one arc segment (the abaxial side) multiplied by the number of needles in the fascicle and simplified to the following formula:

[1]

$$SA = 2rL(n + \pi)$$

where r = the radius of the cylinder (width of an adaxial side), L= the length of the needle segment, and n= the number of needles in the fascicle, as atypically this number strays from five. I calculated total leaf area (TLA) by standardizing surface area per needle and summing the three needle segments then estimated SLA by dividing this area by the sum of the oven-dried mass of the three needle segments.

Stomatal density analysis can be conducted in more than one way. Conventionally, stomatal density is calculated as the number of stomatal openings per unit area. This metric was developed for determining the stomatal density of broad-leaved species, where stomatal pores are distributed more or less evenly across the planar surface of the leaf. The stomata of coniferous needles are distributed differently, however, with stomata arranged in rows and recessed into the epidermal surface to prevent water loss. Due to this morphology, linear density (calculated as number of stomatal openings per unit length) is an appropriate alternative (Kouwenburg *et al.* 2004), though the area method has been

successfully applied to conifers (Schoettle & Rochelle 2000). In this study, I assessed density by area in order to easily relate it to specific leaf area; however, linear density was also calculated for comparison purposes.

Data were analyzed using R version 3.2.3 (R Core Development Team 2015). I measured stomatal counts, length, width, and mass of needles and calculated the additional parameters of surface area, stomatal density, and SLA from them. Because I was explicitly interested in changes in trait variance, I tested the hypothesis that uninoculated controls would have greater variance than inoculated survivors using Levene's test for homogeneity of variance. For traits that did not fail Levene's test, I compared differences in trait means of the ~10 seedlings per two treatment groups from 19 populations with one-way ANOVA using the following formula:

[2]

$$Y \sim treatment + popID + treatment*popID$$

2.3 Results

2.3.1 Differences in Trait Means Between Treatments

With the exception of mass and the number of stomatal rows, means for the remaining traits were significantly different by treatment (Figure 2.5). No significant differences were found in trait means amongst populations, nor were there interactions between population and treatment (full results given in Table 2.2). Inoculated survivors had a 10% lower mean stomatal density (by area) than uninoculated controls. When

evaluated as linear density, the decrease in stomatal density was 2.6%. The survivors also had, on average, 11% longer and 7% wider needles, however the allometric relationship of needle length and width between the two treatments remained congruent. Mean SLA was 8% higher in inoculated survivors than in uninoculated controls.

2.3.2 Differences in Trait Variance Between Treatments

With the notable exception of needle length ($F_{37,316}$ =1.5, p=0.05), uninoculated control and inoculated survivor groups did not differ significantly in the variance of most needle traits (Levene's tests: stomatal density: $F_{37,316}$ =0.71, p=0.90; needle width: $F_{37,316}$ =0.93, p=0.59; and specific leaf area: $F_{37,316}$ =0.98, p=0.51). This test also indicated that the assumption of homogeneity of variances when testing with one-way ANOVA was met in these traits. Nevertheless, inspection of histograms of individual trait values revealed directional trends consistent with predictions (Figure 2.6). In both stomatal density and needle length (top panel), variation in the inoculated survivors was slightly lower, which is consistent with phenotypic selection. In needle width and specific leaf area, though (lower panel), variation was slightly increased in inoculated survivors, which resembles a plastic response, although a capacity for plasticity could also be adaptive.

2.4 Discussion

Understanding how populations will react to novel and interacting perturbations has become a central concern for natural systems (Johnson 2000), but making predictions

about future trajectories is challenging. To date, the introduction of *C. ribicola* has noticeably reduced survival and fertility of many North American white pine species (Loo 2009), although to what extent it has affected standing variation is unclear. Theoretically, a pathogenic fungal inoculation treatment should impose strong directional selection pressure, thereby narrowing the genetic variation by removing susceptible individuals and shifting the population towards a new optimum. However, such changes could have pleiotropic effects on responses to concurrent changes in other selection pressures such as shifting climate trends. Several pine species are already on the edge of their abiotic tolerances (Mueller *et al.* 2005, Gitlin *et al.* 2006, Sthultz *et al.*2009), together these stressors could either push them past the limit of their ability to respond to individual factors through opposing selection or reinforce their responses if selection is concordant.

Since the point of infection is at the leaf, I asked if a change in leaf traits was associated with survival following a rust inoculation treatment. From theory, those individuals that were exposed to and withstood an inoculation with *C.ribicola* would demonstrate a narrower range of observed variation in leaf traits as well as a change in the average value of those traits. I also predicted that the direction of average changes would correspond to increased physical defense mechanisms. There was a small but significant response to the inoculation treatment. However, only one trait responded in the direction expected (stomatal density). The remaining traits either didn't vary between inoculated survivors and uninoculated controls (needle mass), or responses were in the opposite direction to expectations (needle size, SLA).

In accordance with my prediction, stomatal density of inoculated survivors was lower than in uninoculated control groups (Figure 2.5). This difference, combined with a slight (but non-significant) change in variance reflects that changes might be genetic (due to differential survival of individuals with lower stomatal density) or plastic (stomatal density changed in needles that developed post-infection). Stomatal patterning is under genetic control (Wright *et al.* 2004, McKown *et al.* 2014,) and also influenced by environmental conditions (Cornelissen *et al.* 2003). More sparsely distributed stomata are known to be a response to abiotic conditions such as drier or higher CO_2 environments (Beerling 1983). In this experiment, all individuals experienced similar abiotic conditions; the only difference being exposure to the *C. ribicola* pathogen. Given that leaves from prior years can send signals to developing leaves (Lake *et al.* 2001), and a lower concentration of stomata may be associated with a lower chance of invasion success (Niks & Rubiales 2002), these results suggest that the inoculation treatment may have influenced the development of the subsequent leaf cohort. The change in stomatal density would be consistent with a response to inoculation treatment that trades off higher conductance potential with the need for defense against negative biotic interactions. If this is correct and durable, it would complement expected abiotic changes such as interactions with increased CO_2 levels and survival under water stress (Pearce *et al.* 2006). However, conductance was not measured here and if stomatal size and density are largely coupled (Heatherington & Woodward 2003), then it is possible that the total area for gas exchange remained static.

Contrary to my hypotheses, both average leaf size (length and width) and SLA were greater in inoculated survivors when compared to the uninoculated controls (Figure 2.5).

There was also a trend for slightly increased variation in needle width and SLA (Figure 2.6). Larger leaves can indicate healthy plants and increased growth rate (Grime 1979). Likewise, higher SLA positively correlates with relative growth rate (Poorter & Evans 1998). In this case, the higher SLA of inoculated survivors was driven by higher leaf area rather than lower leaf mass. Taken in conjunction with reduced stomatal structures, it may represent plasticity, genetic differences, or a combination of these two effects. For example, plants that have resistance may have constitutively lower stomatal density or induced response to the rust challenge via enhanced leaf size reflecting compensatory growth. Compensation for the effects of pests and pathogens is a known response that enables a host to recover from a negative interaction and offset the energy-intensive cost of defense (Dietrich *et al.* 2005). In addition, the enhanced resource acquisition that larger leaves would allow coupled with lower stomatal density that prevents water loss may balance necessary acclimation to the warmer temperatures and more variable precipitation predicted for the future (Donat *et al.* 2016). Alternatively, because leaf size has generally been found to decrease in environments with increased biotic or abiotic stressors, and producing smaller leaves mitigates water stress (Meinzer *et al.* 2011), the lower stomatal density seen here could simply be a function of larger leaves designed to recoup the costs of defense. A count of stomata between groups did not reveal significant differences, which lends credence to this interpretation (Figure 2.7).

Interestingly, the effect of treatment group on these trait values was not impacted by population of origin (Table 2.2). This is compelling evidence that despite the small size of the shift in leaf trait values, the changes are due to rust-resistance rather than other

population factors such as elevation which is known to impact some leaf traits. Consistent results across populations may also signify that many of the populations sampled in the southern part of the range encompass the necessary variation to compensate for the pathogen's effects, although to what extent remains unknown. Tempering this inference is that not all populations had sufficient surviving seedlings to measure and therefore two populations were excluded from the comparison. Differences in trait variance were small; having a baseline from all populations prior to the inoculation treatment may have highlighted greater differences between the two groups, and thus more significant differences in variance.

Over their long lifetimes, trees must tolerate numerous stressful interactions and a wide range of fluctuating inter-annual conditions, and they have developed defensive strategies that address both biotic and abiotic pressures. Recent research has inferred that rust resistance in limber pine could be related to other stress tolerances, such as cold- or drought-hardiness (Vogan & Schoettle 2015). Versatile mechanisms that overlap with, or are additive to other defense pathways, would be an efficient use of resources in the severe environment that these trees inhabit, yet may still come at a cost of allocation to plant defense. An added cost of defense has been identified in limber pine in the form of decreased growth increment at the whole-plant level following infection (Vogan & Schoettle 2016). The shift in means and slight changes in variation seen here support the idea that phenotypic leaf traits could be associated with resistance to infection. In particular, it appears as though the inoculated survivors compensate for the challenge of the rust through increased resource acquisition via larger leaves. However, rather than

decreasing water loss through lower stomatal area, increased expansion of leaves is dependent on, and limited by, water (Lockhart 1965), therefore may come at a cost of increased water consumption. If this is so, then this cost may have detrimental effects under natural settings if predicted fluctuations in precipitation lead to more frequent or prolonged periods of drought.

Chapter Three: Variation in Limber Pine Seedlings Along the Species Range

3.1 Introduction

Genetic diversity within a species confers resilience to environmental conditions and provides the raw material for adaptive evolution. The degree of diversity influences the breadth of tolerance and future adaptation, yet the spatial genetic variation is often shaped by underlying ecological elements (Turesson 1923). Restricted genetic variation due to directional selective agents such as climate or pathogens could constrain an individual population's ability to respond to novel conditions. Alternatively, if much of the total variation is within and among families within populations, then the variation necessary to respond to a variety of conditions is present locally. Clarifying the geographic structure of genetic variation and identifying some of its causes are critical components of effective management and conservation strategies (Sgro *et al.* 2011).

Many factors influence the partitioning of adaptive genetic variation within and among populations of temperate conifer species. Founder events or historical isolation by distance associated with glacial refugia may underlie varying levels of genetic diversity at the outset (Kremer 2012). Over time, however, these effects can be moderated by gene flow. Effective dispersal of pollen and seed is promoted by large population sizes and spatial continuity. Thus, species that have high gene flow among populations tend to have higher levels of variation, whereas those with long generation times, low fertility or fecundity, or from non-contiguous stands are more at risk of depleted variation (Aitken *et*

al. 2008). Notwithstanding gene flow that could increase adaptive variation within, yet homogenize populations, selection and adaptation within local environmental conditions stimulates differentiation among populations (Kremer *et al.* 2012). Adaptation in response to varying optima along gradients result in clinal patterns of genetic variation that are prominent in widely distributed tree species. In temperate conifers, typical adaptive clinal responses to environmental heterogeneity are observed in traits such as height, growth rate, phenology (e.g. bud timing), and physiology (e.g. frost hardiness), although teasing apart the basis of these differences is challenging.

Common garden experiments are a classic technique used to partition the genetic and environmental contributions to trait variation among geographically distant populations (Clausen *et al.* 1940, Langlet 1971). When forest trees are grown in common environments, genetic differences among populations typically reflect geographic and climatic gradients (Aitken *et al.* 2008) including north to south, low to high elevation, dry to wet, maritime to inland and more (Aitken & Hannerz 2001). In some cases, environmental and genetic influences act in the same direction (co-gradient variation; Conover *et al.* 2009). For example, more northerly provenances tend to be shorter and to grow more slowly when compared to those from lower latitudes due to the selective pressures of lower temperature and shorter season lengths (Rehfeldt 1992). In other cases, environmental and genetic influences are opposing (counter-gradient variation; Conover & Schultz 1995). For example, depending on nursery conditions, northern provenances might have delayed phenology in nature but accelerated phenology in a common garden

compared to southern provenances (Liang 2015). Investigating these patterns provides insight on population diversity and potential response to disturbance.

Limber pine are confronted by a suite of threats already discussed (Chapter One, page 2); additionally they are disadvantaged by their slow rates of maturity and patchy distribution. However, they have broad physiological tolerances that enable them to occupy a wide ecological niche (Windmuller-Campione & Long 2016). Their realized niche encompasses terrain from grasslands to alpine and breadth extends across two countries from the Black Hills and northern Rockies to the Sierra Nevada and Sangre de Cristo mountain ranges. Whether this extensive range arises from geographically structured variation among populations is not fully resolved. To examine genetically based trait variation, I conducted a greenhouse common garden study of progeny from wild trees originating across 16° of latitude. The objectives of this study were to determine: 1) are there measureable, genetically-based differences in growth, phenological and morphological traits in limber pine? And, if so, 2) how much of the variation in these quantitative traits is due to among- versus within-population variation? 3) Is variation in these traits among populations predicted by climatic differences among their sites of origin? Identifying patterns of population differentiation permits detection and quantification of adaptive clines along environmental gradients. These interpretations can provide insight into the adaptive capacity of the species, which is crucial for making management decisions.

3.2 Methods

3.2.1 Seed Sources

I obtained seeds from Alberta to New Mexico along the Rocky Mountains. This spans the majority of North-South range from 37° to 52 ° in latitude (Figure 3.1). Collections were made from natural populations over a span of sixteen years (1999-2014) by several organizations (USDA Forest Service, the BC Ministry of Forests, Lands and Natural Resources Operations Tree Seed Centre, and the Alberta Tree Improvement and Seed Centre), who graciously donated seeds for this study. The trial was designed with a nested structure using ten seedlings from each of four to five open-pollinated families originating from each of 29 populations along the latitudinal gradient. To increase the likelihood of having ten seedlings per family, I began with a minimum of 30 seeds per maternal family. I weighed seeds in batches of 10 preceding stratification and estimated average seed mass per family.

3.2.2 Stratification

I modified a cold and moist process to stratify seeds based on recommendations from the Alberta Tree Improvement Centre and the US National Park Service (Lindsay Robb and Erin Borgman, personal communication). Starting in January 2015, petri-dishes were filled with 60 g of dried, clean sand (#11-Fine, ASTM 2011). Without pre-treatment, scarification, or X-ray viability testing, I divided families into lots of ten seeds, weighed the lots, and randomized and embedded them in the prepared, numbered petri-dishes; 10 mL

dH_20 was added to each. Petri dishes were cold-stratified at a constant 2 °C for between 70 to 81 days (10 to 12 weeks) and checked weekly for moisture and mould. Mould was common and recurrent. Rather than removing and discarding affected seed, all were treated during the stratification process. Treatment consisted of washing each lot in cold water using a fine steel mesh sieve to rasp off fungus, soaking for 5 minutes in a 1% H_2O_2 solution, rinsing in cold water then returning to sterilized petri dishes. Fresh sand, baked at 200 °C for four hours, lined the sterilized dishes, and the same ratio of 60 g sand:10 mL dH20 reapplied. Barnett (1976) noted that chemical treatments may hasten germination; the <2% of seeds that precociously germinated during stratification were potted before full planting commenced.

3.2.3 Planting, Germination & Culture

Culturing protocols were based on recommendations from the USFS Dorena Genetic Resource Facility, OR (Lee Riley, personal communication). Seed was direct-sown onto media over a two-week period in April 2015. Osmocote controlled release fertilizer 14-14-14 Classic (3-4 mo release rate) plus Osmocote Micromax (Everris NA Inc., Dublin, OH) minor nutrients were admixed with HP Pro-Mix Mycorrhizae growing media (Premier Tech Horticulture, Riviere-du-Loup, PQ) then covered with #8-#16 forestry grit. Seeds were assigned unique, error-detecting code identifiers (Appendix A), planted into bar-coded (Appendix B) Ray Leach SC10 “cone-tainers”, and positioned in their random lot order into 98-cell racks (Stuewe & Sons, OR). Greenhouse conditions were set to 20-25 °C during the

day, 15-18 °C at night with a 16-hour photoperiod. Seeds were top-watered daily until germination. Thereafter, the soil surface was kept moist until the end of June.

Once germination was complete, I arranged ten seedlings each from 145 families in a partially incomplete block design with 44 blocks, and 33 families per incomplete block (Clatworthy 1955). Seedlings from different families were assigned to each block in non-overlapping combinations. Early seedling mortality slightly reduced numbers, leaving a total of 143 families with nine having fewer than 10 individuals for a total of 1407 seedlings (Table 3.1).

To ensure seedling health during the first growing season, I cultured as follows: after three months of growth, a low concentration [80 ppm N] of water-soluble Technigro 15-5-15 Plus CalMg fertilizer (Sun Gro Horticulture, Bellevue, WA) was applied weekly starting in July. Beginning in September until mid-October, this schedule was alternated with [80 ppm] Technigro 6-30-30 Plus. Soil surface was permitted to dry before watering. Growth conditions were maintained as specified until October, when a vernalization process was introduced. This involved regular temperature and day length reductions to prepare the trees for the six-week chilling period required for winter dormancy. For eleven weeks the set temperature decreased by 2 °C and the day length by one hour until by mid-December the temperature set point was between 1-3 °C with no supplemental lighting (see Figure 3.2 for achieved points). Shade cloth was deployed on all exterior walls and I installed black-out cloth to the interior wall to prevent light from leaking in from neighbouring compartments. Shade cloth was removed and temperatures increased in the

spring for the second growing season (Figure 3.2), with fertilization treatments commencing in May 2016 after bud break, so as not to influence flush rates.

3.2.4 Measurements

3.2.4.1 Growth & Phenology

Germination was monitored daily for the first month after sowing in April 2015, and then every other day for the second month. Germination was determined by emergence from soil rather than protrusion from seed coat, so is conservative. Seeds that had not germinated after 60 days were discarded. Following germination, I counted and measured the length of cotyledons at full expansion. Expansion was effectively complete by 60 days of growth, as determined by additional measurements at 120 days of growth.

I examined size and development in the following two growing seasons. In the first growing season (2015), I measured seedling height at 60, 180, and 240 days of growth; in the second growing season (2016), I measured height weekly between February and May until bud flush was 94% complete. I assessed height to the nearest millimeter from the base of the cotyledons to apical meristem to account for differences in planting depth. Stem diameter was evaluated at 1 cm above the root collar. Seedling stems are not necessarily cylindrical; therefore, stem widths were measured with digital calipers in three orientations at 240 days growth (during dormancy). These three widths were averaged to arrive at stem diameter per individual. In addition, I monitored the development of mature, secondary needles over a period of six months during the first season. Surveys were

conducted every other week in June, July and August of 2015 and final census was completed in November the same year.

Growth rate was calculated on an individual basis as the coefficient of height over time [cm/day]. To avoid bias toward taller trees, growth rate was also standardized by using the coefficient of ln(height) over time for relative growth rate [% growth/day]. Secondary needle emergence was assessed as individual status at the date when 50% of the trees had developed secondary needles. Computationally, this was determined from the inflection point of a logistic regression (Chuine *et al.* 2001).

I tracked bud development starting in late autumn of the first growing season and into the spring of the following season. Despite the fact that bud set can be somewhat ambiguous in culture (Landis 2012), it was scored after 240 days growth and converted to a binary factor of set or not. Bud flush was more clear, although also not perfectly distinct. Bud flush was scored based on a 7-level system developed by Anna Schoettle (personal communication) and similar to Ekbert (1984) (Table 3.2), although if buds elongated prior to scales parting, they were scored as flushed. Each individual was assessed as flushed or not at a time of 50% of total flush (Savolainen *et al.* 2007). Measurements are summarized in Table 3.3.

3.2.4.2 Needle Traits

I selected three seedlings from all but two populations for needle sampling in the spring of 2016. To reduce sampling effort, two populations (Kicking Horse and Crowsnest, BC) plus three surplus families were excluded due to other geographically proximal

populations; this resulted in 130 families being sampled. Of those, I excised three primary needles and three secondary fascicles (where available) from the previous year's growth. Four families did not have any secondary needles present, (one each in Laramie, WY, Shirley, WY, GLEES, WY and Mosca Pass, CO). Processing all three primary needles, and one needle per each secondary fascicle immediately upon sampling, I digitally imaged each using the sampling procedure as outlined in the second chapter (2.2.2). Three exceptions to this protocol were: 1) I micrographed the primary needles at ~150x magnification. This is because they are wider than secondary needles, so a reduction was necessary to fit the specimen in the field of view; 2) I calculated surface area of primary needles by assuming overall needle length and width approximates a triangle; and 3) I used custom, automated image-processing software (Appendix C) based on the ImageJ platform (Schindelin *et al.* 2012) to measure and count regions of interest.

3.2.5 Analysis

All data were analyzed using R version 3.2.3 (R Core Development Team 2015). To maintain consistency, the two populations dropped during needle trait data collection, were removed from the analyses. This reduced the total to 27 populations, 130 families, and 1277 individuals. I used the Lme4 package (Bates *et al.* 2015) in model construction, the MuMIN package (Barton 2016) for model averaging and to derive conditional R^2 values, and LmerTest to obtain p-values (Kuznetsova *et al.* 2016).

3.2.5.1 Climate Variable Selection

I downloaded thirty-year normal climate variables for the period between 1981-2010 for each population's geographic origin, from climate WNA v5.30 (Wang *et al.* 2016). This program provides an interpolated data set based on PRISM (Daly *et al.* 2008) data and generates both calculated and derived variables for annual, seasonal, or monthly time periods. Selecting only seasonal variables reduced the set from over 250 available temperature, precipitation and insolation variables to an array of 57. I then employed numerical methods described below to further filter these high-dimensional data.

To explore which climate variables could be dropped based on multicollinearity, I took a penalized maximum likelihood approach. Cross-validation of the data and model fit were tested using the Elastic-net and LASSO (Least Absolute Shrinkage and Selection Operator) regression algorithms in glmnet (Friedman *et al.* 2010). To start the cross-validation, the glmnet package subdivides the data (composed of needle traits as response variables and a series of climate variables as predictors), into *k*–fold randomly chosen subsets. All but one of the subsets are used to 'train' a generalized linear model to the data. That means that each subset is used to predict a model on each of the *k-1* folds. These are then averaged and tested on the remaining subset of data to generate a mean squared error. Depending on which penalization criteria (or mixture of them) is chosen along the continuum between Ridge ($\alpha=0$) and Lasso ($\alpha=1$), an assessment is made on how well the model fits to the test data. This is an iterative process (the regularization path) wherein the process starts again with a new set of predictor variables to be trained and tested until the mean squared error is minimized.

Rather than choosing the parameter for the minimum means square error fit, I chose to use a parameter within two standard errors of the minimum. This foregoes some accuracy (as measured by mean squared error) in favour of simplicity, but does not produce a model with demonstrably dissimilar results (Friedman *et al.* 2007). In addition, while selecting an α=0.5 is often considered to determine the strongest fit, I was specifically looking for a sparse model to reduce the number of climate variables, which LASSO delivers (Tibshirani 1996). Regardless, both models showed considerable overlap in the climate variables recommended as candidate predictors, so I used LASSO (α=0.95) to further reduce the testable variables to a maximum of nine per response variable.

3.2.5.2 Model Selection

As indicated above, response variables included growth, phenology and needle traits (Table 3.3). I output a correlation matrix to compare these traits and determine if they were redundant using a threshold of 0.75; as no two traits were correlated at this threshold (Table 3.4), all were included in further analysis. In addition, I used seed mass as a covariate to account for maternal effects.

Initially, I looked at the partitioning of trait variance among families and populations by extracting variance components with a random effects model of the following form:

[3]

$$Y \sim (1|population) + (1|family) + (1|block)$$

While I did test for interactions between population and block and family and block, the estimated variance in each case was close to zero, so they were dropped. Population- and family-level variance proportions were standardized by the total variance to transform them to percentages. I then compared models with and without each factor with a likelihood ratio test (compared to a χ^2 distribution with one degree of freedom for significance testing). To compare differentiation among populations, I used the original variances to calculate Q_{st} using an adjusted formula to account for seedling relatedness, as outlined by Bower & Aitken (2008):

[4]

$$Q_{st} = \frac{Variance_{pop}}{2 \times 3Variance_{fam} + Variance_{pop}}$$

Next, I examined how traits varied spatially and with environment of origin. For spatial analyses, I used latitude as a predictor variable in models of the form:

[5]

$$Y \sim latitude + needle\ type + maternal\ effect + (1|family) + (1|block)$$

Because latitude and elevation were strongly, negatively related (R^2= 0.80, P<0.001), I report results for latitude only, unless a trait's relationship to elevation was not a direct inverse of its relationship to latitude. For climatic analyses, I used the climate variables from the LASSO regressions as predictors instead of latitude, then used the 'dredge'

function in MuMIN to weight the output, and conducted model averaging on those with a delta AIC of <2.

3.3 Results

3.3.1 Variance Partitioning

Depending on the trait under inspection, populations explained between 1 and 24% of the variance and families between 1% and 21%. Total growth traits had greater variance explained by population and family collectively (30%) than phenology (14%) or needle traits (22%). The proportion of variance was slightly larger among populations than families for growth traits and mixed for needle traits, whereas for bud timing, family variance was much higher than population. For stem, height and growth rate measures, an almost equal amount of variance was partitioned among- and within- populations (11-20%, 8-17% respectively. Mean for each: 15%). Models of bud timing showed between 1-6% (mean 4%) of the variance was allocated among populations compared to 13-14% (mean 13%) between them. There was greater residual error in the remaining models; nonetheless, relatively equal variation was explained among populations (7-24%, mean 12%) and families (1-20%, mean 10%) in needle traits. Q_{st} estimates ranged from 0.02 for bud flush through 0.19 in growth rates. Higher Q_{st} estimates were seen in two needle traits, as the variance among populations was large compared to family. Full results for each trait are given in Table 3.5.

3.3.2 Maternal Effects & Germination

Seeds ranged in length from 5 to11 mm and in mass from 0.03 to 0.19 g. Seed mass is known to indicate maternal provisioning, and those seeds endowed with greater average

weight had greater germination (R^2=0.26, P<0.001). Seed mass was also positively correlated with cotyledon length (R^2= 0.59, P<0.001) and cotyledon number (R^2=0.18, P<0.001). Although Borgman *et al.* (2013), recommends using cotyledon length to account for maternal effects, here the two were correlated but the effects of seed mass were stronger (as compared via AIC), therefore I used seed mass to represent maternal effects in the models. Seedling germination occurred within two weeks of planting (87%), and was almost complete (97%) within three weeks. Germination was seen in all families with an overall germination success rate of 60% (Table 3.1), though there was no correlation between germination success and seed age (measured from date of collection), elevation or latitude (results not shown).

3.3.3 Latitudinal Clines in Growth & Phenology

At the time of growth cessation after the first growing season, seedlings ranged in height from 0.7 to 9.8 cm from the base of the cotyledons to apical meristem, 1.7 to 11.4 cm from soil to the apical meristem, and 1.2 to 4.7 mm in stem diameter. Overall height and stem diameter per individual were correlated (R^2= 0.52, P<0.001), yet showed opposing relationships to latitude (height: *m* = +0.07 cm/° of latitude, R^2= 0.42, P<0.001; stem diameter: *m* = -0.03 mm/° of latitude, R^2= 0.35, P<0.001; Figure 2.3 Panels A & B). While the responses were continuous across latitude, after the first growing season seedlings sampled from the northern half of the range were almost 20% taller and 8% thinner in stem diameter than those from more southern latitudes. Absolute growth rate was faster in the northern accessions than the southern in the first growing season (R^2= 0.60, P<0.001;

Figure 2.3 Panel C), but over a full year of growth (1.5 growing seasons from April 2015-May 2016), relative growth rate showed a negative relationship with latitude (R^2= 0.65, P=0.001; Figure 3.3, Panel D).

With the exception of bud set, phenological traits including bud flush and secondary needle production tended to be advanced in populations from higher latitudes (Figure 3.4). The production of secondary needles commenced earlier in northern trees versus populations from more southern regions that were slower at initiating these more mature needles (R^2= 0.56, P<0.001; Figure 3.4 panel A). Initiation of secondary needles started in the first growing season at three months after germination in some trees and populations. Approximately 50% of individuals had produced at least one fascicle by five months after germination and 80% of individuals developed needle fascicles in the first season (Figure 3.7). Northern individuals both set bud later (R^2= 0.21, P<0.003; Figure 3.4 panel B) and broke bud earlier (R^2= 0.21, P=0.79; Figure 3.4 panel C) than those in the south.

3.3.4 Latitudinal Variation in Needle Traits

In contrast to phenology and growth, needle traits showed fewer relationships with latitude (Table 3.6). Needle size was smaller for northern trees. In either primary or secondary needles originating from higher latitudes, length was shorter and width was narrower than those from lower latitudes (Figure 3.5, Panels A & B). Primary needles showed a weak negative relationship between SLA and latitude (Figure 3.5, Panel D). SLA for secondary needle measurements showed no latitudinal trends, nor did stomatal density of either needle type (Figure 3.5, Panel A). Although comparing primary to secondary

needles is unconventional given their clearly distinct morphologies (Table 3.6), in looking for possible reasons why primary needles may be more susceptible to rust than secondary needles, I did find that primary needles had lower stomatal density, and specific leaf area than secondary needles (Figure 3.6).

3.3.5 Relationships to Climate Variables

Climate trends across the source populations were variable. In some cases, sites had similar climate variable values, in other cases there were marked differences. For example, while mean annual temperature showed a relatively narrow range across the latitudinal gradient of -1.8 to 6.5 °C (R^2= 0.02, P<0.001), there was greater variability in northern locations, with colder lows and higher precipitation and humidity compared to drier locations further south. (Eg's north vs. south. Mean coldest month temperature: -11.3 °C vs. -4.0 °C, R^2= 0.31, P<0.001; mean summer precipitation: 560 mm vs. 180 mm, R^2= 0.13, P<0.001; mean annual relative humidity: 70% vs. 49%, R^2= 0.45, P<0.001).

For those response traits where the LASSO model reduced the set of climate variable predictors to zero (needle traits) or was unable to reduce the set at all (seed mass), results from the glmnet cross-validation of climate variable selection using LASSO indicate that climate variables were not suitable predictors of this phenotypic data. For all other phenotypic data, climate variables concerning moisture and sunlight at seed origin were the best predictors for growth and growth rate. Phenological traits also were also dominated by precipitation and insolation predictors, but stratified to include some temperature variables. Full results shown in Table 3.7.

3.4 Discussion

Many populations show spatial patterns of differentiation in quantitative traits that suggest adaptive clines. For example, much of the phenotypic variance in Douglas-fir and larch is associated with elevation and latitude of origin (St. Clair *et al* 2005, Campbell 1979, Rehfeldt 1995). Because elevation and latitude cannot be agents of selection *per se*, the underlying drivers of clinal differentiation are frequently attributed to climatic gradients associated with latitude and altitude, but pinpointing exactly which variables remains a significant challenge (Prendeville *et al.* 2013). Although one may expect adaptive differentiation in wide-ranging species due to heterogeneous selection and isolation by distance, in many conifers much of the variation seen among populations is also encompassed within populations (Yeh & Layton 1979). For example, whitebark pine exhibits variation in cold hardiness among populations, but also among families within populations (Bower & Aitken 2006). The limber pine grown in this common garden study demonstrate clinal variation in several traits along a gradient of latitude that relate to climate variables and photoperiod, yet like whitebark pine, also harbour quantitative genetic diversity in some traits within populations.

Adaptive population divergence is expected in wide-ranging species that experience varied conditions and whose lineages have originated from distinct glacial refugia (Kremer *et al.* 2012, Mitton & Latta 2000). For populations to persist, however, there must also be sufficient standing variation within each population to respond to environmental

fluctuations or alterations. In this study, for most traits, families within populations explained either an equivalent or slightly smaller percentage of variance than among populations, with the exception of phenology traits which were higher for families (Table 3.5). The proportion of genetic variation among populations is commonly evaluated with Q_{st} estimates to facilitate an understanding of a species potential to respond to change. Higher values suggest that the trait under inspection has been under greater diversifying selection resulting in greater genetic differentiation among populations (Savolainen 2007). Q_{st} is usually used in comparison with an estimate of the proportion of total neutral genetic diversity due to differences among populations (F_{st}). These figures can have relatively high levels of uncertainty, depending on sampling design. The sampling in this study extends over a large part of the limber pine range, and I report Q_{st} values per trait to compare these estimates to other published results. These results for limber pine are slightly lower than a study of European conifers that also have large, fragmented ranges which quote an average $Q_{st} = 0.192$ as approximately twice as large as F_{st} (Alberto *et al.* 2013). The average Q_{st} here is 0.158, and is not quite twice as large as a published average F_{ST} value of 0.094 in limber pine across a similar geographic extent (putative allozyme loci; Jorgensen 2002). Results here are within the ranges for each trait category, with the exception of stomatal density, which is nominally higher than the published range for that feature (Alberto *et al.* 2013). These results are also consistent with both ponderosa pine (Savolainen 2007) and whitebark pine (Bower & Aitken 2008), and imply that, as well as having moderate within-population variation, limber pine populations do differ in adaptive traits across the gradient of latitude tested (Figure 3.8), although less so than many other species.

Spatial patterns were identified in traits frequently used as proxies for fitness, including seedling growth and phenology (Campbell 1979, Liepe *et al.* 2016), as well as to a lesser extent in ecophysiological needle characteristics (Alberto *et al.* 2013). These differences are likely to be genetically based, though the remaining influence of maternal effects cannot be ruled out even after accounting for variation in seed mass. There was a positive relationship between height and stem diameter, yet these two traits showed opposing relationships with latitude (Figure 3.3, Panels A & B). The height:girth ratio increased over latitude (R^2= 0.43, P<0.001), with taller individuals originating from higher latitudes exhibiting a thinner form than individuals from lower latitudes that were comparatively shorter and stouter. This could be due to differential resource allocation in response to climate variables, as for example, trees derived from more southerly populations may allocate resources belowground rather than above, developing strong root systems at the outset to protect against drought. Stem diameter, also, has been shown to have a relationship with latitude and summer drought in Douglas-fir (St. Clair *et al.* 2005).

The positive relationship between height and latitude was contrary to predictions, as one would generally expect trees from lower latitudes to be taller (Moles *et al.* 2009). However, due to the corresponding negative relationship with elevation, this result could be a response to a longer growing season at lower altitude. Alternatively, in environments where growth is limited by the short duration of favourable temperatures for growth as in northern latitudes, these trees may be genetically programed to grow quickly to make sufficient gains and complete development over a season. This interpretation is bolstered

by the earlier production of secondary needles (Figure 3.7) and in absolute growth rate in the first growing season (Figure 3.3, Panel C). As well, bud flush rate in the second growing season was more rapid for populations in northern latitudes (Figure 3.4; panel D), although the relative growth rate showed a negative relationship with latitude over two growing seasons (Figure 3.3, Panel D). The indeterminate nature of first-year seedling growth may well have impacted these rates (Howe *et al.* 2003). Determinate growth in the second and subsequent years is likely to vary among populations and be more indicative of future growth (Chuine *et al.* 2001). If this is so, then the trajectory indicates that trees from the lower latitudes will eventually outgrow those from higher latitudes.

Phenological traits such as bud set and bud flush timing impact growth and are largely cued by abiotic forces. Temperature and photoperiod influence bud set, chilling days and heat sum actuate bud flush (Campbell1979, Cooke *et al.* 2012). In temperate zones, the ability to optimize growth through timing is vital. Appropriate growth initiation and cessation balances maximal resource capture while avoiding damaging cold (Howe et al. 2003). Because temperatures tend to be colder, day length more variable and the period of conditions favourable for growth shorter in higher latitudes, the expectation was that trees from these regions would break and set bud earlier and grow faster to take full advantage of the growing season. Within the common garden, the genetic cline in bud flush fit expectations with seedlings from higher latitudes flushing modestly earlier and elongating faster than those from farther south, however the relationship between bud set and latitude was contrary to expectations (Figure 3.4, Panel B). Surprisingly, seedlings from northern origins set bud later than those from more southerly populations. While this

could reflect counter-gradient variation where trees from higher latitudes are not at their phenotypic optima, it could also be due to greenhouse conditions. Triggering pine seedlings to set bud in the first year under nursery conditions is often challenging due to a propensity for recurrent flushing known as lammas or polycyclic growth (Landis 2012). This involves continuous or consecutive cycles of shoot growth in the same growing period. In the field, it has been associated with a strategy of maximizing light capture (maples – Verdu & Climent 2006) or with recovery from herbivory (Douglas-fir – Roth & Newton 1996), but may be initiated by favourable greenhouse conditions (Figure 3.2). While the mechanism cannot be determined from these data, it is possible that seedlings from southern sources set bud earlier to avoid drought injury. As well, a longer favourable period under greenhouse conditions may have contributed to seedlings from higher latitudes growing taller and allocating biomass to aboveground growth, including transitional needle development earlier during the first growing season.

Shoot growth and ontogeny is of interest in these pines because, while all needles are susceptible, juvenile (primary) needles appear to be especially susceptible to rust infection (Jacobs *et al.* 2009). The timing of secondary needle production was examined to determine when, on average, the transition to mature needles occurs, as well as to compare differences in developmental progress spatially. There was a positive relationship with latitude in the proportion of emergence of secondary needles. In several families from lower latitudes secondary needles were not observed in the first growing season at all. This suggests that seedlings in southern populations that rely on primary needles for a longer duration may be at increased risk of infection via these highly susceptible needles. It is

reasonable to think that the transition to a higher level of maturity may bring with it more complex defense functions, but both primary and secondary needles of *Pinus strobus* produce waxes, phenolics, and resistance proteins (Jacobs *et al.* 2009) often associated with defense. Unsurprisingly, the primary and secondary needles measured here were different in every trait measured (Figure 3.6), although the values were counter-intuitive. In theory, leaves with low SLA, sparse stomatal density and shorter needles would be more robust; however it is the more susceptible primary needles that displayed these characteristics. If primary needles are more susceptible, the only measure that is not misaligned with this concept is in needle width, where the primary needles are markedly wider. Definitive needle-level resistance mechanisms have yet to be elucidated; anecdotally, however, differences in wax were stark. Primary needles had a glaucous wax composition and, visually, appeared to have less frequent occlusion of the stomata by wax than secondary needles. Data on frequency of visual occlusion await analysis, however if primary needles do have incomplete development of wax production due to immaturity as seen in *Pinus halepensis* (Boddi & Bonzi 2002), it may explain their increased vulnerability.

All together, northern trees were taller and slimmer, with smaller needles and accelerated initial growth rates while southern trees were shorter and stouter, with larger needles and slower growth rates in the first year. Many studies have related clinal growth and fitness patterns to climatic drivers. In most temperate forest tree species, temperature explains much of the variation in growth (Aitken & Bemmels 2016), which coincides with phenology as previously discussed. This study found that spatial variation was better explained by climate variables associated with insolation and precipitation than

temperature, although relationships with some temperature variables did emerge (Table 3.7). For example, maximum winter temperature was significantly associated with bud flush, which corresponds nicely with expected chilling requirements for this phenological trait (Bower & Aitken 2006). The timing of secondary needle production correlated negatively with spring insolation and positively with summer temperatures below 18 °C, which may indicate that milder conditions facilitate growth, a common pattern (Alberto *et al.* 2013). The remaining phenotypic traits were best predicted by climate variables related to water; in particular spring and summer precipitation, and autumn evaporation, humidity and moisture deficit indices, and to a lesser degree insolation.

The LASSO approach to identifying important climatic variables allows the data to inform climatic variable selection with little *a priori* bias, producing intriguing results and highlighting contrasts among location of origin. For example, it may be that elevational differences across the latitudinal gradient confine populations to similar temperature regimes, suggesting a relatively narrow realized niche with regards to temperature. Mean annual temperature varied by up to 8.3 °C across the latitudinal gradient; whereas, the range in mean annual precipitation was 730 mm. Failure to detect strong relationships with temperature is not because temperature represents a negligible influence; rather it suggests that with regards to this species, the realized niche for precipitation is wide, and therefore local adaptation to water availability is of critical consequence. In the dry, inland areas where limber pine grows, water-stress intensified by high irradiance may well interact to shape growth patterns, although relationships to underlying environmental gradients may be difficult to fully resolve, as they are likely to be non-linear. Liepe *et al.*

(2016) suggested that multivariate regression trees may be a more appropriate analytical technique to decipher trait interactions and infer adaptive significance.

Moderate to high levels of genetic diversity are relatively common in temperate forest trees, contributing to their ability to respond to stressors. Observed differences in this study suggests that limber pine harbours both geographically structured landscape-level variation, as well as within-population variation, albeit at lower values when compared to many other conifers (Savolainen 2007). These results also indicate that water availability appears to be one of the major drivers structuring adaptive genetic variation in this species. However, projections of future precipitation are uncertain. Both increases and decreases have been recorded over the past sixty years, but current predictions indicate that summer conditions will get wetter (~5-20% increase) in northwestern Canada and drier (~10-30% decrease) in the southwestern U.S. (Fettig *et al.* 2013). *C. ribicola* requires high humidity to move to white pines. These potential differential climate changes over the range of limber pine may buffer opposing pressures. For instance, northern populations could be released from growth restrictions due to temperature, yet humid conditions are more conducive to the pathogen. In contrast, southern populations may gain some relief with regards to pathogen pressure if it's drier, yet experience more acute drought stress (Sturrock *et al.* 2011). These predictions regarding precipitation could be inaccurate. Alternate predictions involve increased temperature and a shortage of water even in areas not currently water-deficient, and at a cost of high mortality to trees (Allen *et al.* 2015). If lineages that exhibit moderate drought tolerance also show rust-resistance, as has been suggested (Vogan & Schoettle 2015), then limber pine may have the necessary variation to

adapt under predicted future climate regimes. Alternatively, if limber pine is on the edge of its physiological abilities, or if rust-resistance imposes too high a cost under the pathogen's pressure (Vogan & Schoettle 2016), then compounded with increased heat and drought, and coincident with a reduction in fecundity, the future survival of this species is far from certain.

Conclusion

Limber pine are a species of growing concern in many jurisdictions. Like all of the North American white pines, they are under pressure from pests and pathogens, land transformation, and climate change. They also demonstrate a remarkable range of tolerance with regards to stress, environment, and location. The physical and physiological flexibility of limber pine has led the species to be deemed an ecological generalist whose apparent broad ecological scope may be beneficial in the light of continuing environmental disturbance (Windmuller-Capione & Long 2016). However, they are also a species with long generation times that is distributed in disjunct patches, and is partially reliant on a mutualist to disperse genetic material. This puts limber pine at risk of reduced adaptive genetic variation and slow reaction times, which combined could very well push these trees over the edge of their capability to tolerate harsh conditions.

This work complements previous research by examining effects of the response of limber pine to infection by *C. ribicola* (Chapter Two) as well as quantifying trait variation among populations and families within a common garden setting (Chapter Three). Identifying variation in adaptive traits encompassing a large portion of the species range has rarely been done in limber pine yet is essential for determining levels and distribution of genetic diversity. Unfortunately, for species of great longevity such as this one, inferences generalized from seedling common garden studies stretching only one or two years need to be viewed with some caution. Although the seedling stage may experience strong natural selection (Kremer *et al.* 2012), many developmental changes occur between

seedling stage and maturity that cannot be accounted for in a short period of time, therefore extrapolating these results to a full range of ages, and in natural settings, is problematic. Fortunately, the continuation of this work is underway. Several of the seedlings were provided for restoration planting in an area hard-pressed by industry and WPBR (Appendix D). The majority of seedlings from this study, however, have been out-planted in a longer-term provenance trial replicated in two locations; one on the northern edge and one in the southern portion of the species range. This extended research will reveal genotype-by-environment interactions and further our knowledge of adaptive variation in limber pine and the climate factors that help shape it, as well as aid in the development of protocols for restoration planting material and seed transfer guidelines for genetic resources managers.

Tables & Figures

Table 2.1: Sampling details for limber pine seed source populations used in inoculation trial.

Population	Latitude	Longitude	Elevation [m]	Surv [n=]	Uninoc [n=]
SWTB	40.03	-105.5	2681	10	10
LILB	40.31	-105.5	2748	10	9
RMBPB	40.4	-105.8	2802	7	9
SDOB	40.03	-105.5	2823	10	8
PVB	39.91	-105.6	2980	10	10
DMB	40.39	-105.6	3023	10	10
RLB	40.01	-105.6	3054	10	9
CPB	40.65	-105.7	3120	6	10
MIRB	39.92	-105.6	3127	10	10
GEB	39.52	-105.7	3155	10	9
PIRB	40.27	-105.6	3179	7	10
HUB	40.23	-105.6	3222	8	10
BLB	40.65	-105.7	3227	10	10
ECB	40.3	-105.6	3253	10	8
TSB	40.3	-105.5	3254	10	10
UTB	40.37	-105.7	3259	10	9
RACB	40.4	-105.7	3296	8	8
JENB	39.93	-105.7	3324	10	9
NRB	40.05	-105.6	3405	10	10

Table 2.2: Results of ANOVA testing between uninoculated controls and inoculated survivors for each trait. Figures are F-statistics, p-values in brackets. Degrees of freedom for: treatment = $df_{1,316}$; population = $df_{18,316}$; treatment x population = $df_{18, 316}$.

Trait	Treatment Effect	Population of Origin	Treatment by Population
Length [cm]	28.6 (**<0.001**)	0.445 (0.98)	1.51 (0.09)
Width [mm]	37.5 (**<0.001**)	0.894 (0.59)	0.721 (0.79)
Stomatal Density [#/mm^2]	36.5 (**<0.001**)	1.20 (0.26)	1.13 (0.32)
Stomatal Density [#/mm]	8.31 (**0.004**)	1.04 (0.42)	1.57 (0.07)
Surface Area [cm^2]	31.5 (**<0.001**)	0.800 (0.71)	0.767 (0.74)
Specific Leaf Area [cm^2/g]	21.0 (**<0.001**)	1.55 (0.071)	0.854 (0.64)
Rows [count]	0.0358 (0.85)	0.704 (0.81)	1.12 (0.33)
Mass [g]	0.0404 (0.84)	1.05 (0.40)	0.716 (0.79)

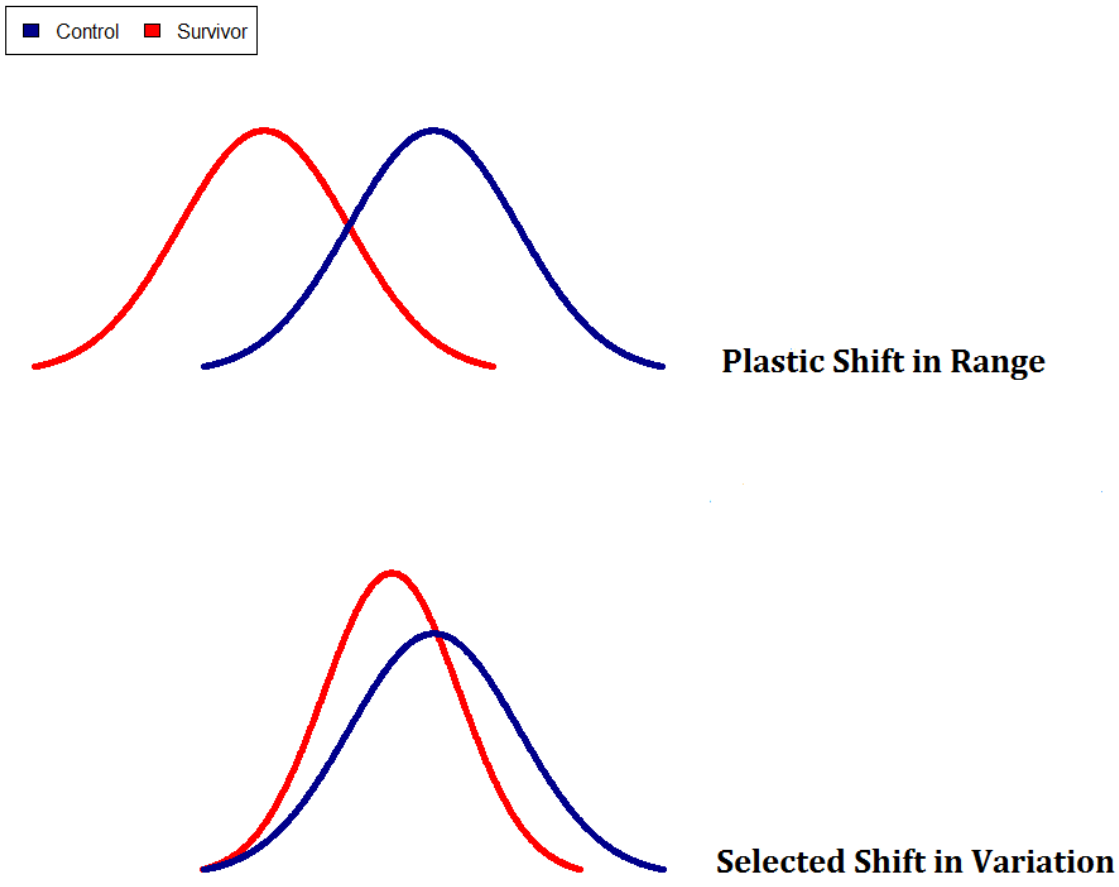

Figure 2.1: Theoretical predictions of the shift in trait values from uninoculated control to inoculated survivors of limber pine within a generation. Changes may be plastic as a response to inoculation or genetic due to differential survival of individuals.

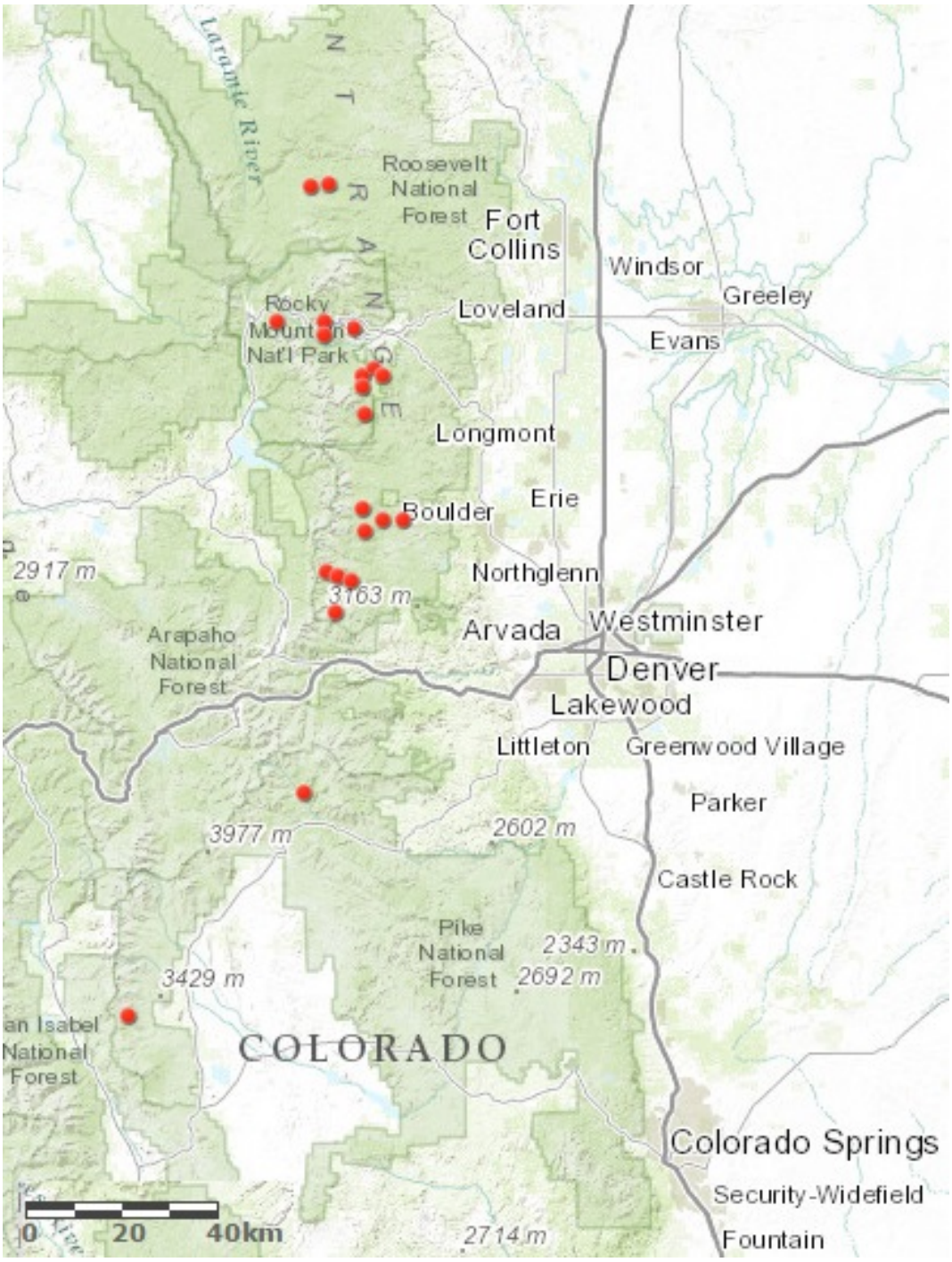

Figure 2.2: Locations of source populations along the Colorado Front Range.

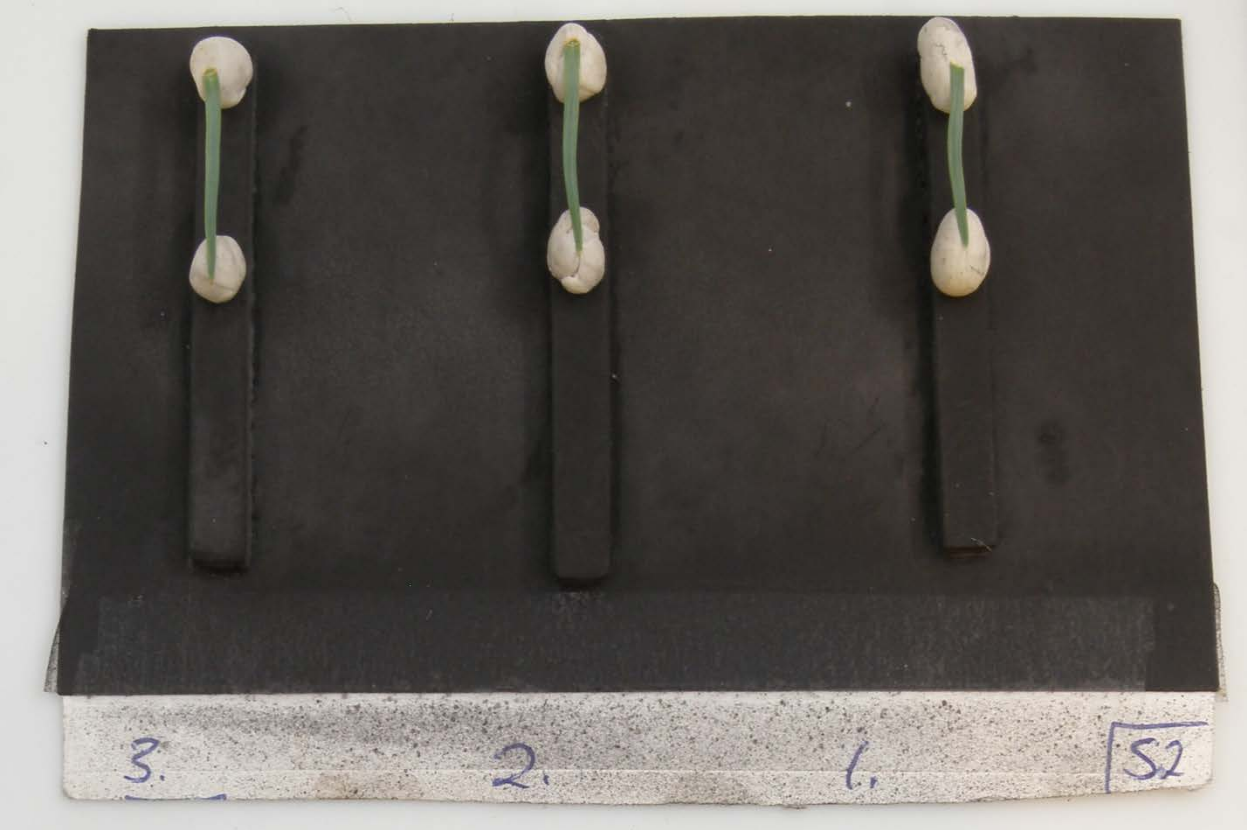

Figure 2.3: Example of primary needles prepared for imaging by mounting to blackened card stock.

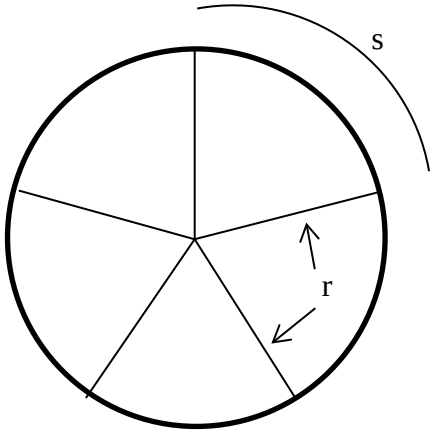

Figure 2.4: Diagram of a fascicle cross-section of a five-needled pine, where ‘s’ is the length of the arc-segment (abaxial side) and ‘r’ is both the radius and the width of an adaxial side (two per line, as each triangular needle section has two).

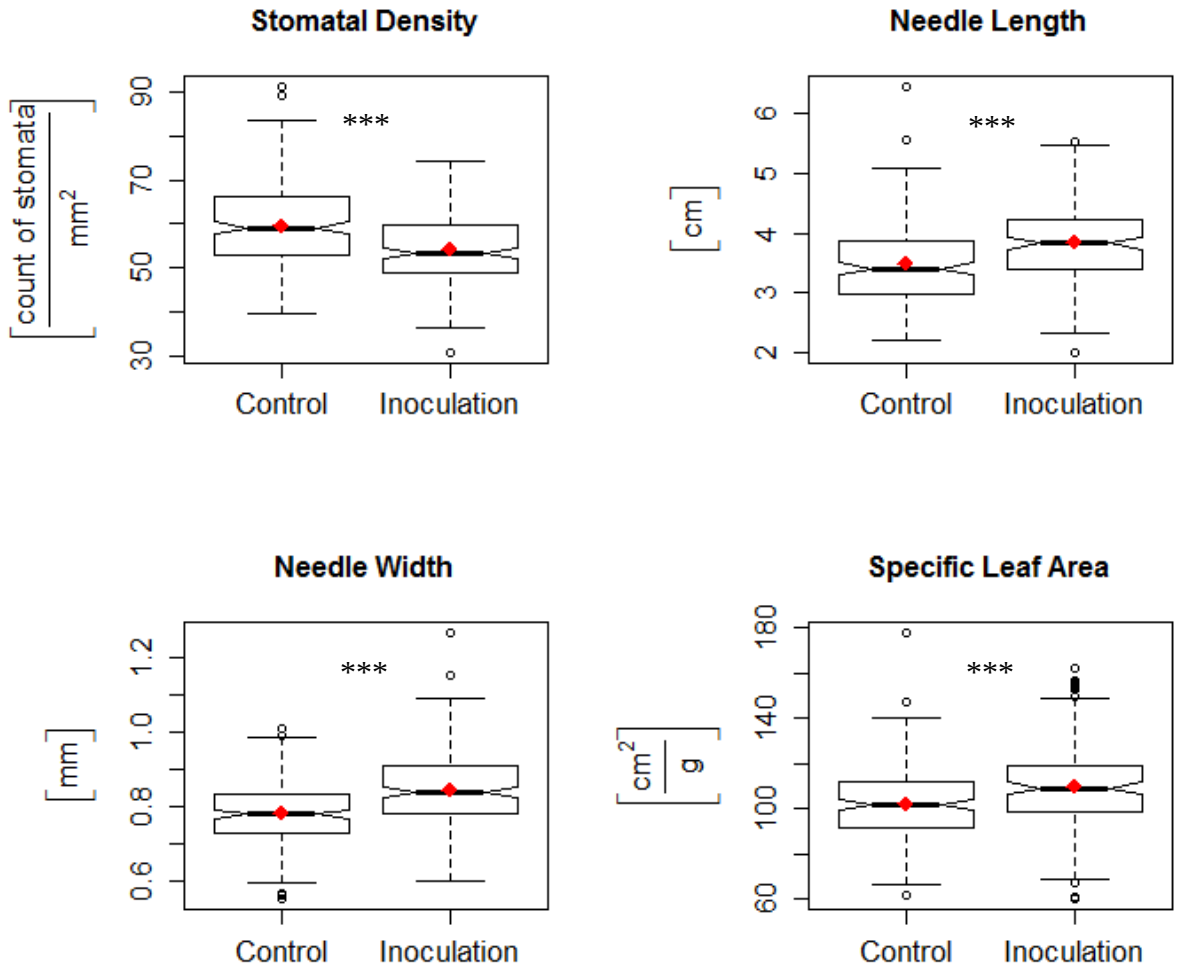

Figure 2.5: Differences between uninoculated controls and inoculated survivors in four traits; stomatal density, needle length, needle width and specific leaf area. Box plots show the interquartile range, red dot indicates mean and the notch is the 95% confidence interval.

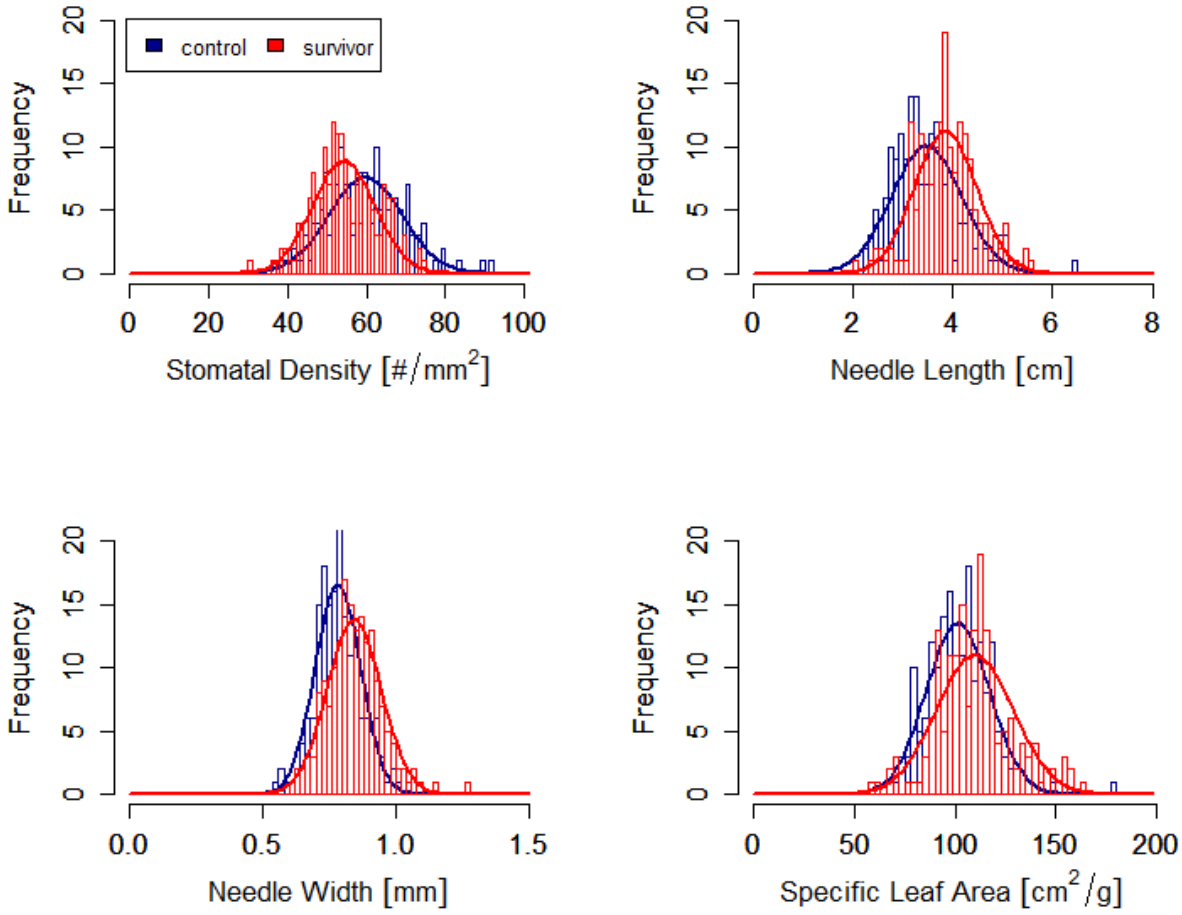

Figure 2.6: The distribution of trait values between uninoculated controls and inoculated survivors in four traits: stomatal density, needle length, needle width, and specific leaf area.

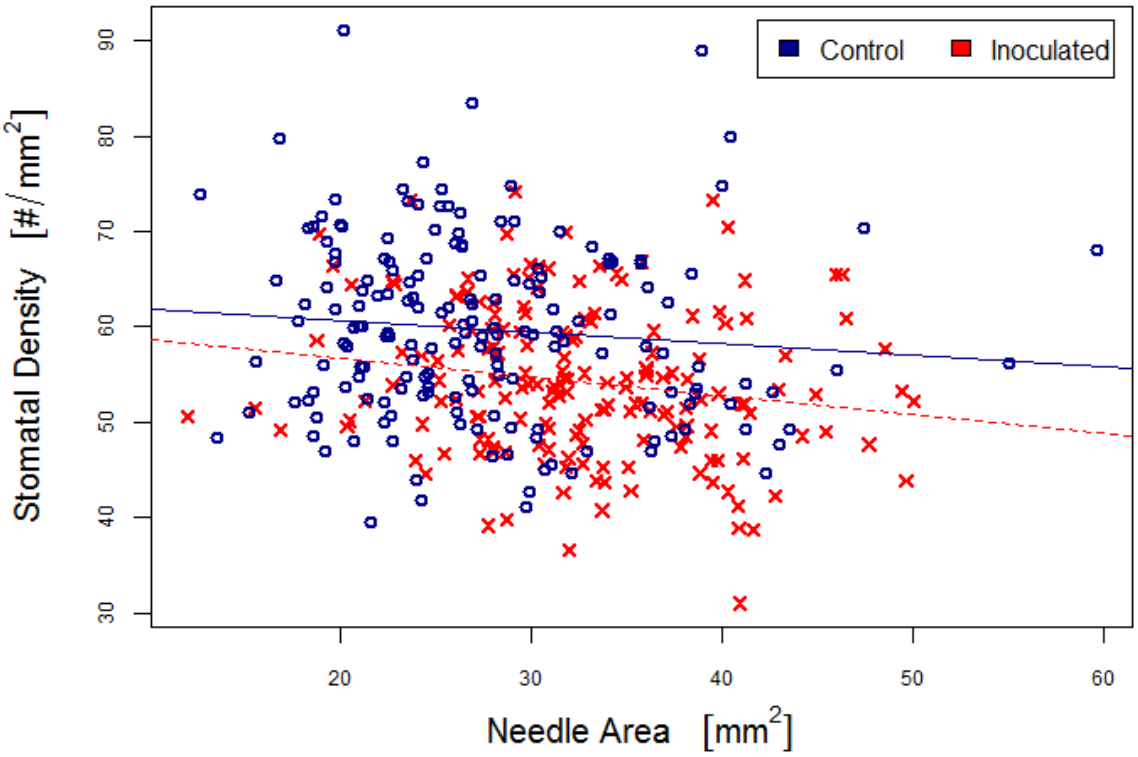

Figure 2.7: Comparison of stomata over the leaf surface between treatment groups.

Table 3.1: Location, number of seedlings per family, and germination success for seed sources in greenhouse common garden trial. Ordered by latitude.

Population	Latitude [°]	Longitude [°]	Elevation [m]	Germination Rate [%]	Seedlings [n=]
Windy Point	52.2549	-116.3962	1373	70	50
Abraham Lake	52.2547	-116.3975	1380	82	20
Panther Corner	51.626	-115.4881	1861	23	35
KickingHorse Canyon	51.3004	-116.8907	961	66	50
Kicking Horse	51.25	-116.8333	1000	47	50
Sentinel Point	50.3926	-114.5846	1566	50	50
Kananaskis	50.3848	-114.6737	1618	95	10
Columbia Lake	50.2342	-115.8263	1012	50	50
Crowsnest Pass	49.6496	-114.6778	1600	60	50
Crowsnest	49.6291	-114.6315	1430	55	46
Andy Good Creek	49.5255	-114.6192	1786	60	50
Prairie Bluff	49.3146	-114.101	1806	60	68
Kings Hill	46.8331	-110.6889	2365	34	28
Stump Town	46.1373	-113.039	1863	78	40
Ruby Valley	45.0725	-112.0208	1912	76	50
Double Springs	44.2162	-113.8433	2438	48	50
Buffalo Basin	43.9977	-108.7369	1792	92	40
Green Mt	42.3575	-107.6293	2253	76	50
Ferris Mt	42.2953	-107.3154	2345	95	50
Laramie Peak	42.2441	-105.6016	2386	86	50
Shirley Mts	42.1618	-106.7129	2270	84	50
GLEES	41.3783	-106.2513	3317	22	33
Jelmview	41.0322	-106.0879	2647	62	50
Browns Lake	40.6476	-105.6978	3228	55	50
Estes Park	40.3278	-105.4043	2600	50	50
Kingston Peak	39.857	-105.6399	3431	53	50
Geneva	39.5176	-105.7127	3161	76	50
Pikes Peak	38.9048	-105.0562	2949	56	47
Horn Peak	38.0707	-105.5881	3241	75	50
Mosca Pass	37.7322	-105.4641	2928	53	50
Upper Shuree	36.7872	-105.1864	2917	68	40

Table 3.2: Bud development stages and scoring.

Score	Description
0	Bud dormant, tight; scales converge to a point
1	Bud scales parting, looks hairy
2	Top surface of bud free of scales, looks knobby but no green tissue
3	Fascicle tips with green needle tissue emerging. Little to no stem extension (<0.5)
4	Fascicle tips with green needle tissue extended beyond sheath and stem extension (0.5-2.0 cm)
5	Fascicle tips with green needle tissue extended beyond sheath and stem extension (>2 cm)
6	Green needle tissue extended well beyond sheath (>1.0 cm) but little stem extension (<0.5 cm)
7	Green needle tissue extended well beyond sheath (>1.0 cm and stem extension (>0.5 cm)

Table 3.3: Traits measured in the common garden with units and timing of measurement. Year 0 is the initial growing season, Year 1 is the subsequent growing season.

Type	Trait	Units	Year 0	Year 1
Growth	Germination	[occurrence date]	spring	
	Height	[cm]	winter	spring
	Stem Diameter	[mm]	winter	
	Growth Rate	[cm/day]	derived	
	Relative Growth Rate	[%/day]	derived	derived
Phenology	Secondary Emergence	[occurrence date]	summer	
	Bud Set	[occurrence date]	winter	
	Bud Flush	[occurrence date]		spring
	Bud Flush Rate	[proportion/day]		derived
Needle Traits	Stomatal Density	[#/mm^2]		spring
	Length	[cm]		spring
	Width	[mm]		spring
	Specific Leaf Area	[cm^2/g]		spring

Table 3.4: Trait correlation matrix. Height (H), stem diameter (SD), growth rate in the initial growing season (GR), relative growth rate over two growing seasons (RGR). For both primary(p) and secondary (s) needles, length (L), width (W), stomatal (Density), and SLA. Then seed mass (Mass), cotyledon length (CL), latitude (Lat), and elevation (Elev).

	H	SD	GR	RGR	pL	pW	pDensity	pSLA	sL	sW	sDensity	sSLA	Mass	CL	lat
SD	0.35	1													
GR	0.52	0.21	1												
RGR	0.06	0.04	0.67	1											
pL	0.27	0.45	0.11	0.04	1										
pW	0.12	0.27	0.14	0.12	0.38	1									
pDensity	-0.12	-0.04	-0.05	-0.02	-0.19	-0.21	1								
pSLA	-0.36	-0.23	-0.13	0.09	-0.24	-0.19	-0.01	1							
sL	0.08	0.23	-0.03	-0.02	0.26	0.16	0.01	-0.12	1						
sW	0.15	0.27	0.07	0.07	0.25	0.33	-0.07	-0.16	0.71	1					
sDensity	-0.03	0.03	0.05	-0.03	-0.03	-0.11	0.35	0.03	-0.36	-0.37	1				
sSLA	-0.24	-0.26	-0.05	0.06	-0.21	-0.16	0.04	0.34	-0.62	-0.47	0.25	1			
Mass	0.25	0.29	0.09	-0.07	0.22	0.03	-0.01	-0.09	0.09	0.13	0.03	-0.17	1		
CL	0.27	0.38	0.11	-0.13	0.33	0.14	-0.04	-0.2	0.09	0.06	0	-0.18	0.59	1	
Lat	0.23	-0.29	0.07	-0.12	-0.23	-0.27	0.05	-0.18	-0.25	-0.26	-0.03	0.06	-0.05	0	1
Elev	-0.35	0.16	-0.12	0.11	0.07	0.22	-0.03	0.21	0.14	0.18	0.04	0.06	-0.19	-0.18	-0.89

Table 3.5: Within- and among- population variance in each trait with corresponding p-values from likelihood ratio tests in brackets. Q_{st} calculated as per Equation 4.

	Population Variance	Family Variance	Block Variance	Q_{st}
Height [cm]	0.199 (< 0.001)	0.166 (< 0.001)	0.0119	0.166
Stem Diameter [mm]	0.154 (< 0.001)	0.136 (< 0.001)	0.00867	0.159
Cotyledon Length [cm]	0.244 (< 0.001)	0.206 (< 0.001)	0	0.165
Secondaries [occurence]	0.029 (0.18)	0.0515 (0.073)	0.0112	0.0858
Bud Set [occurence]	0.0604 (0.026)	0.135 (< 0.001)	0	0.0691
Bud Flush [occurence]	0.0135 (0.48)	0.129 (< 0.001)	0.0614	0.0172
Absolute Growth Rate [cm/day]	0.155 (< 0.001)	0.165 (< 0.001)	0.00868	0.135
Relative Growth Rate [%/day]	0.111 (< 0.001)	0.0771 (< 0.001)	0.0201	0.194
Needle Length [cm]	0.119 (< 0.001)	0.0124 (0.63)	0	0.614
Needle Width [mm]	0.098 (< 0.001)	0.126 (< 0.001)	0.0194	0.115
Stomatal Density [#/mm^2]	0.0656 (0.017)	0.116 (< 0.001)	0.016	0.086
Specific Leaf Area [cm^2/g]	0.0689 (0.003)	0.0437 (0.13)	0.00142	0.208
Mean	0.102	0.11	0.0135	0.158

Table 3.6: Needle trait model results. First two columns are models of different needle types (primary and secondary) in relation to latitude. Last column is the difference between needle types. Figures are R-squared values, p-values in brackets.

Needle Trait	Primary by Latitude	Secondary by Latitude	Primary by Secondary
Length [cm]	0.36 (**<0.001**)	0.12 (**<0.001**)	0.33 (**<0.001**)
Width [mm]	0.35 (**<0.001**)	0.21 (**<0.001**)	0.91 (**<0.001**)
Stomatal Density [#/mm^2]	0.19 (0.435)	0.12 (0.688)	0.63 (**<0.001**)
Specific Leaf Area [cm^2/g]	0.17 (**0.0017**)	0.098 (0.364)	0.40 (**<0.001**)

Table 3.7: Climate predictor variables that explained trait values as selected by LASSO and linear mixed model regression.

Trait	Climate Predictors	R^2	P-value
Height	Spring Precipitation (PPT_sp)	0.417	0.042
	Spring Moisture Deficit (CMD_sp)		<0.001
	Summer Insolation (Rad_sm)		0.012
	Autumn Relative Humidity (RH_at)		<0.001
Stem Diameter	Reference Evaporation (Eref_at)	0.354	<0.001
	Summer Precipitation (PPT_sm)		<0.001
Growth Rate	Spring Precipitation (PPT_sp)	0.272	<0.001
Secondary Development	Spring Insolation (Rad_sp)	0.557	0.009
	Summer Degree days <18°C (DD_18_sm)		0.010
Bud Set	Reference Evaporation (Eref_at)	0.199	0.001
Bud Flush	Winter Maximum Temperature (Tmax_wt)	0.210	0.003
	Summer Precipitation (PPT_sm)		<0.001
	Autumn Moisture Deficit (CMD_at)		0.001

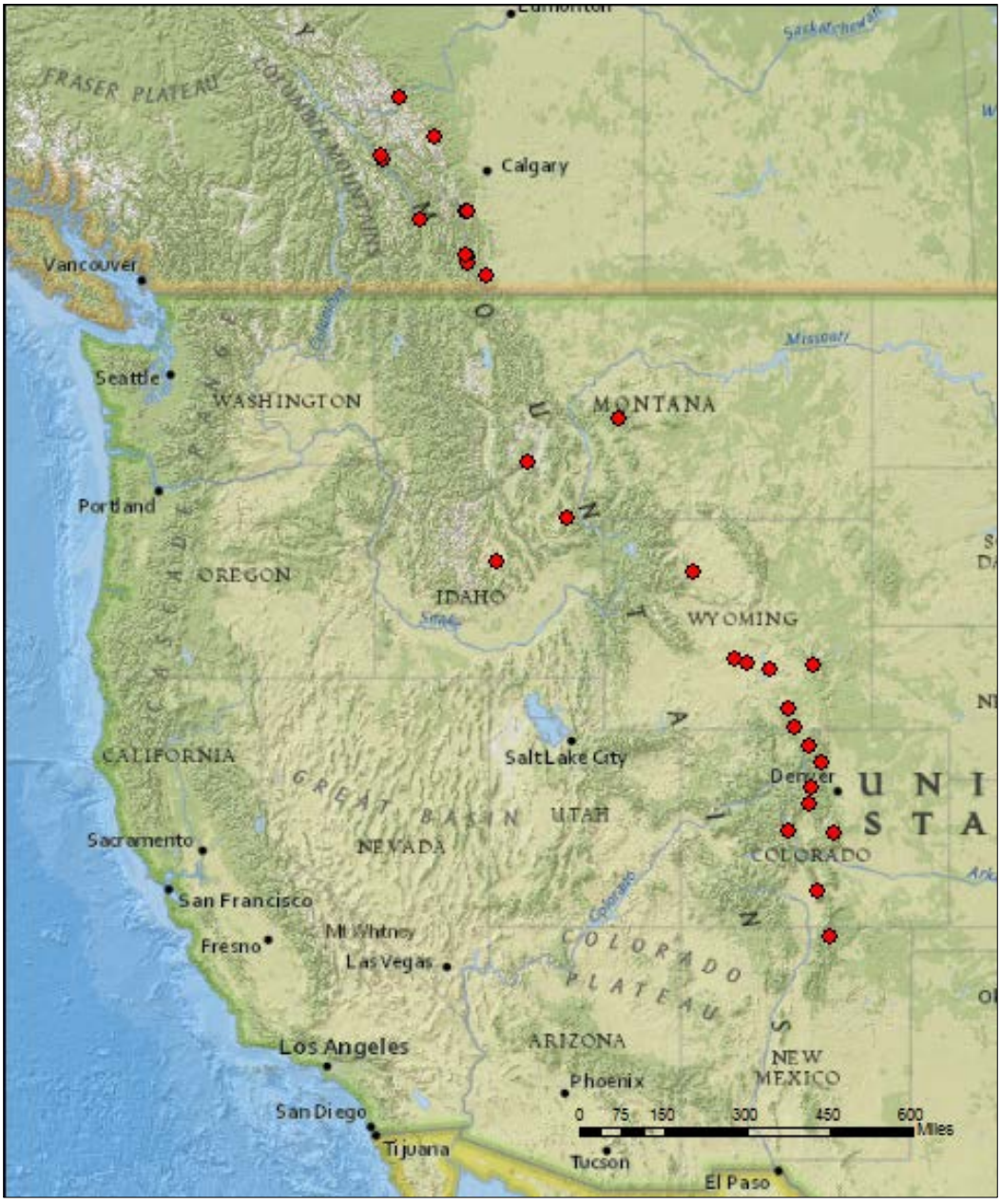

Figure 3.1: Locations of seed source populations for the common garden.

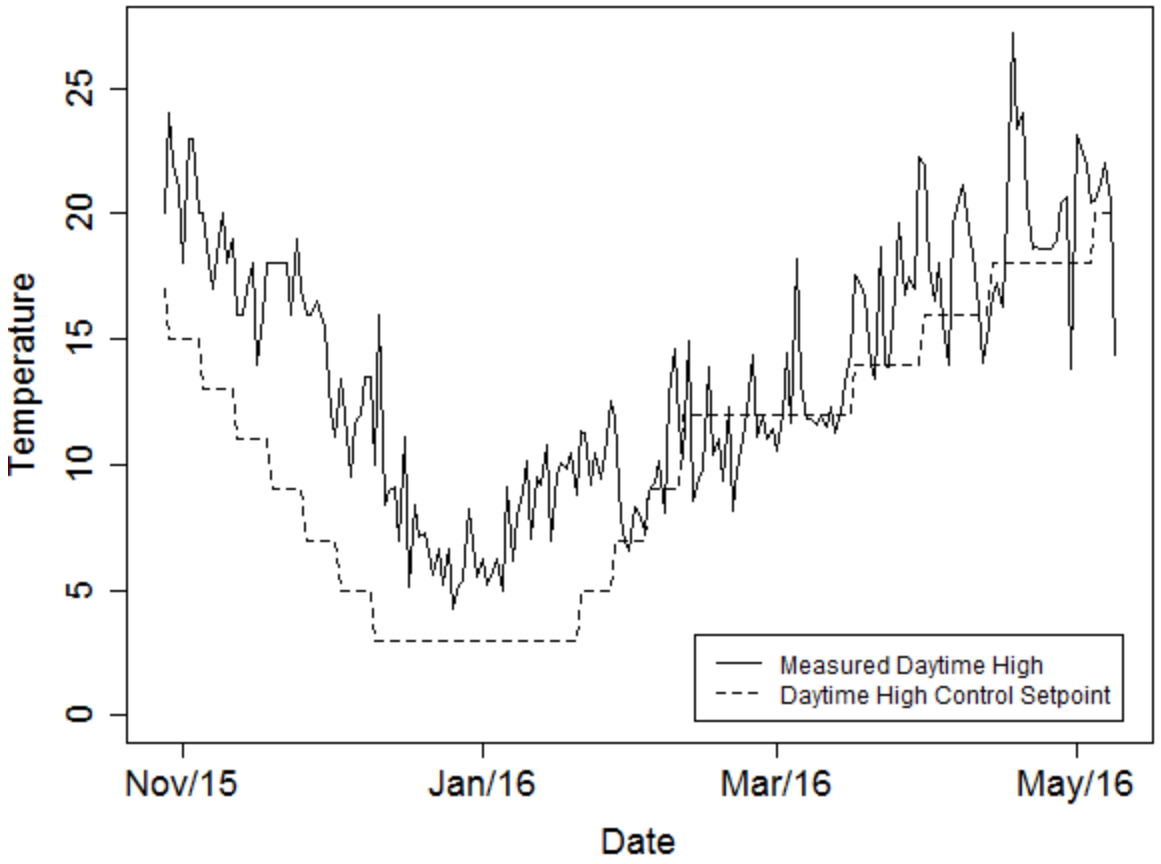

Figure 3.2: Greenhouse temperature conditions over the transition between growing seasons. Plot depicts the difference between set points and achieved temperatures during autumn chilling and spring warming periods.

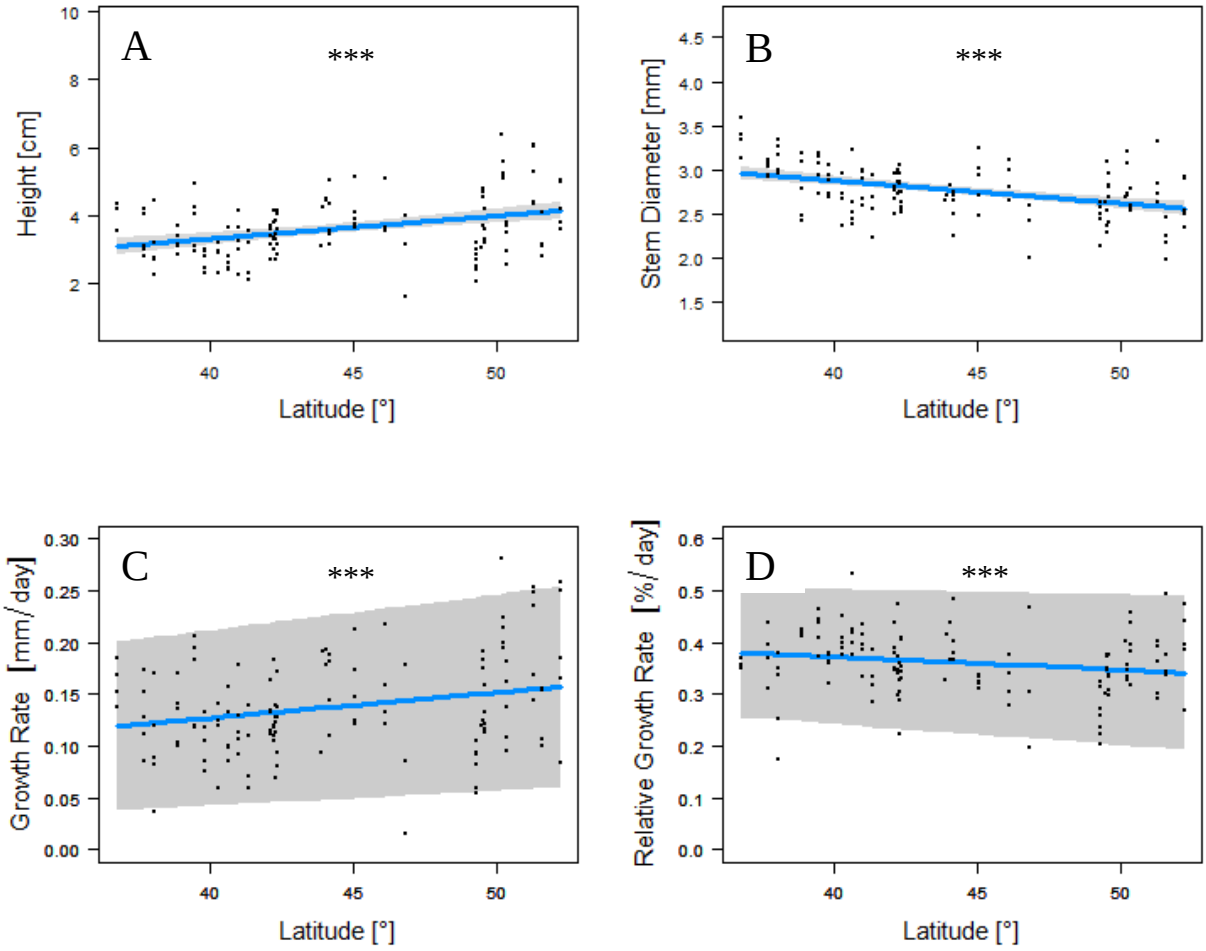

Figure 3.3: Latitudinal variation in quantitative traits related to growth. Panel A) regression of seedling height on latitude of origin. Panel B) regression of seedling stem diameter on latitude of origin. Panel C) absolute growth rate over the first growing season by latitude. Panel D) relative growth rate over two growing seasons by latitude. For clarity, points indicate family-level averages, however best fit line from statistical analyses based on regression models on individuals. * indicate slopes significantly different from zero.**

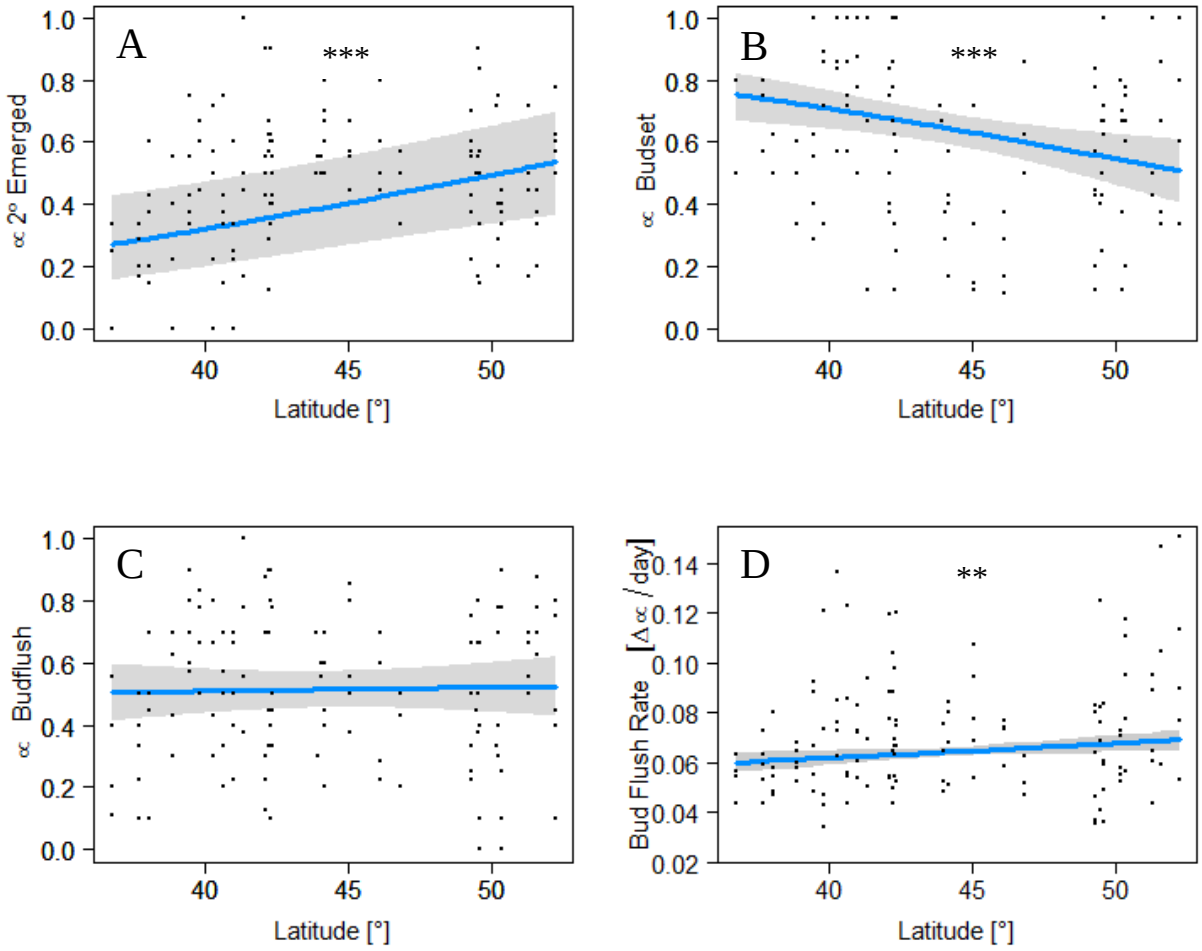

Figure 3.4: Latitudinal variation in quantitative traits related to phenology. Panel A) the proportion of secondary needles emerged as at 50% of total emergence. Panel B) the proportion of individuals that had set bud by the 240th day since germination. Panel C) the proportion of individuals that had flushed as at 50% of total bud flush. Panel D) the proportional rate of bud flush at its maximum. For clarity, points indicate family-level proportions, however best fit line from statistical analyses based on logistic regression models on the individuals. * indicate slopes significantly different from zero.**

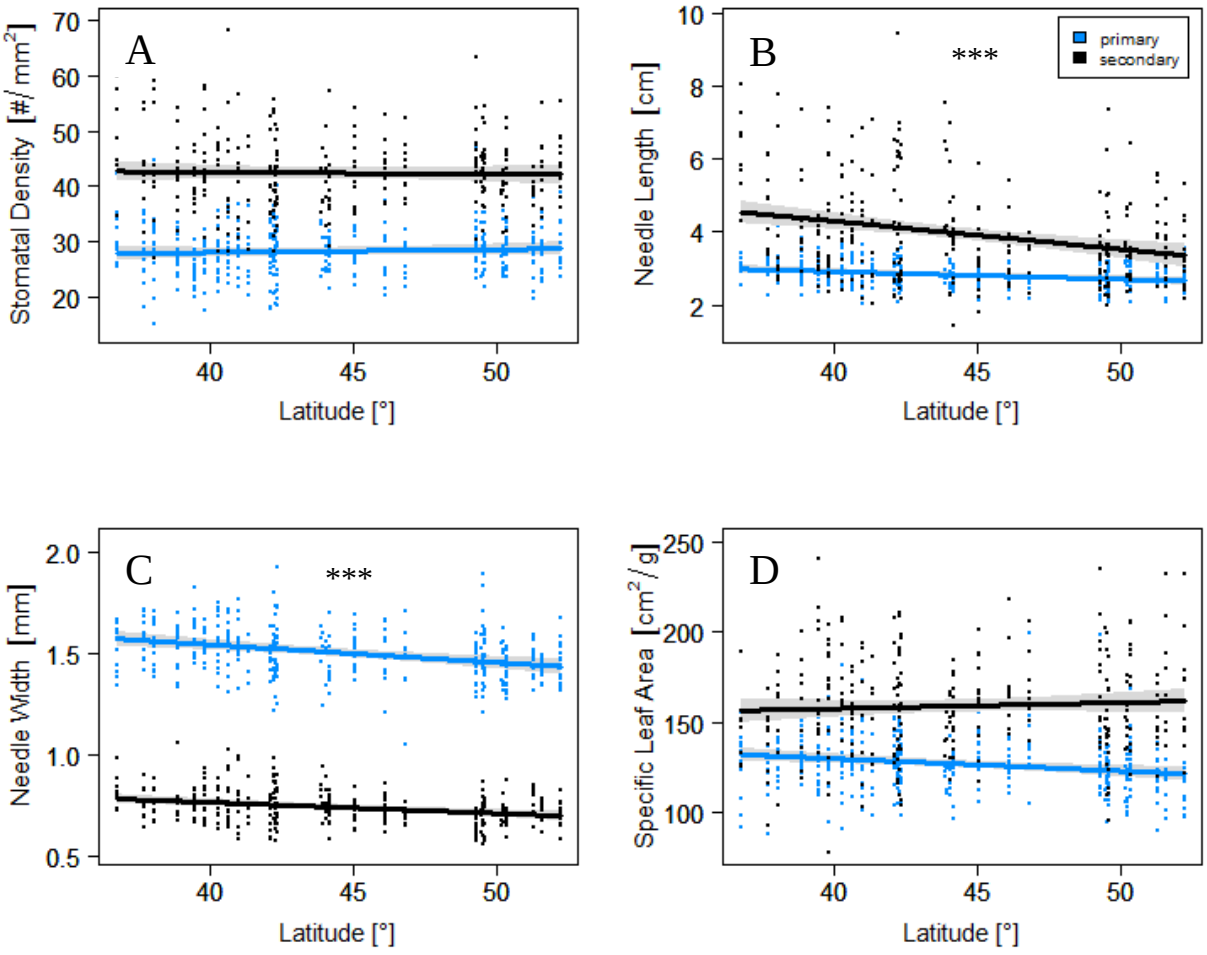

Figure 3.5: Latitudinal variation in needle traits. Primary and secondary needles per individual shown as blue and black, respectively. Panel A) regression of stomatal density on latitude of origin. Panel B) regression of needle length on latitude of origin. Panel C) regression of needle width on latitude of origin. Panel D) regression of specific leaf area on latitude of origin. * indicate slopes significantly different from zero.**

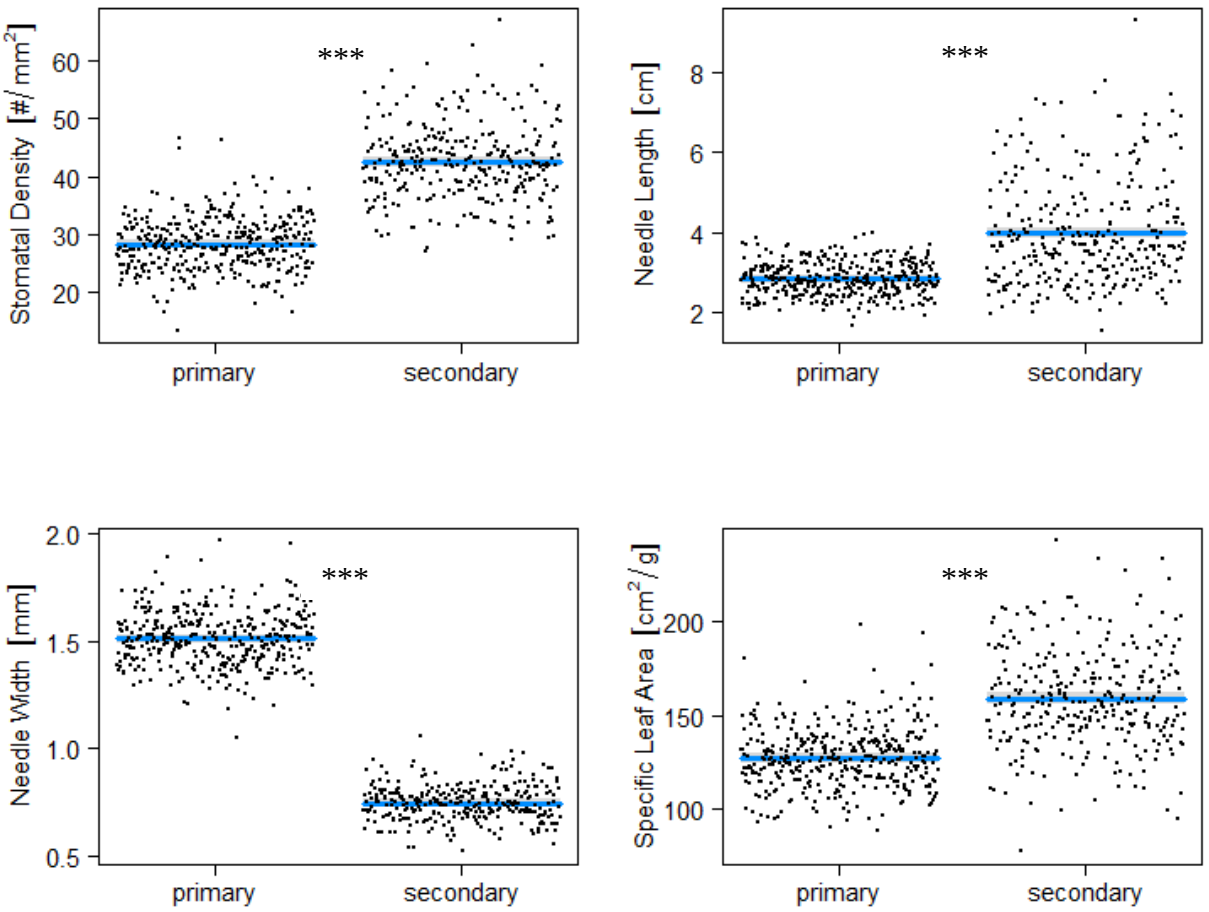

Figure 3.6: Differences between primary and secondary needles per individual. * indicate significance.**

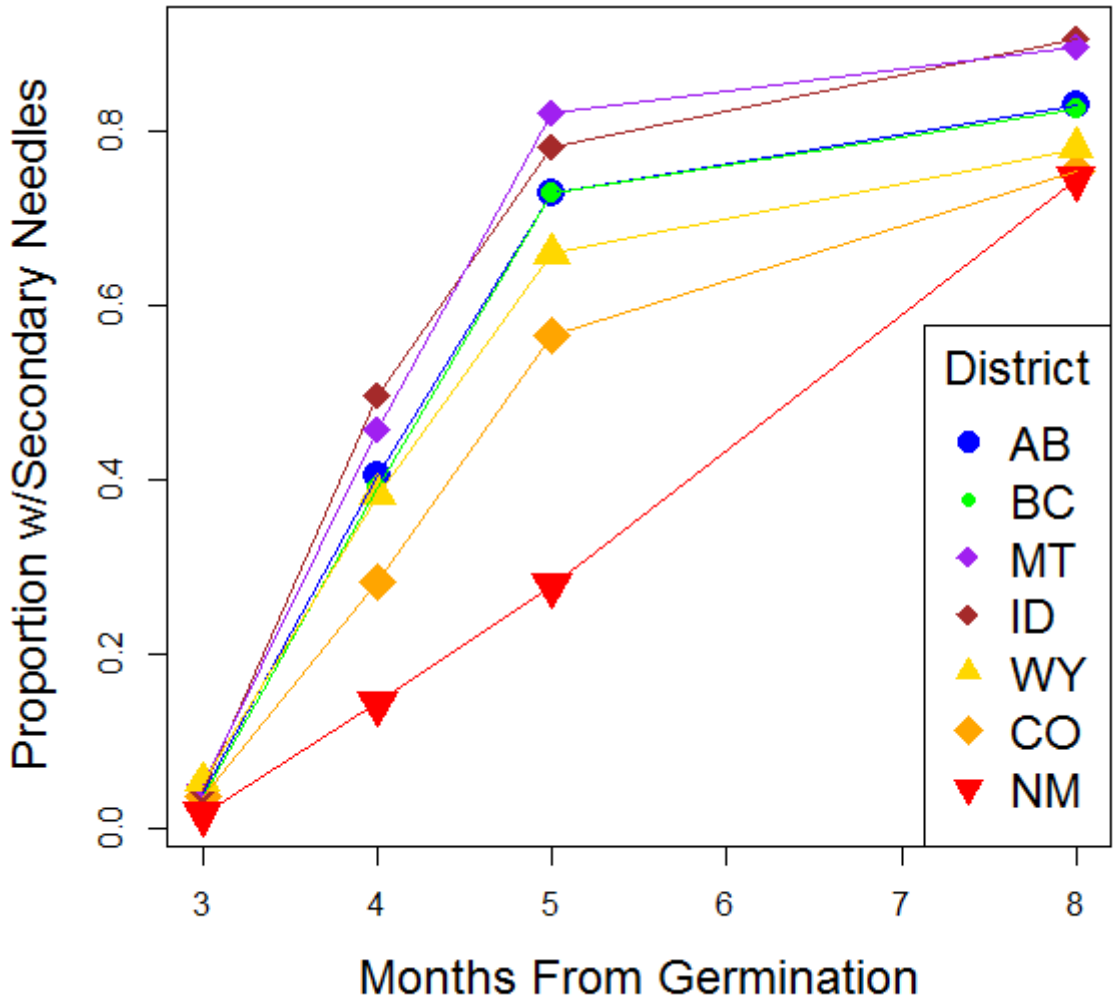

Figure 3.7: Secondary needle development by state or province arranged from north to south.

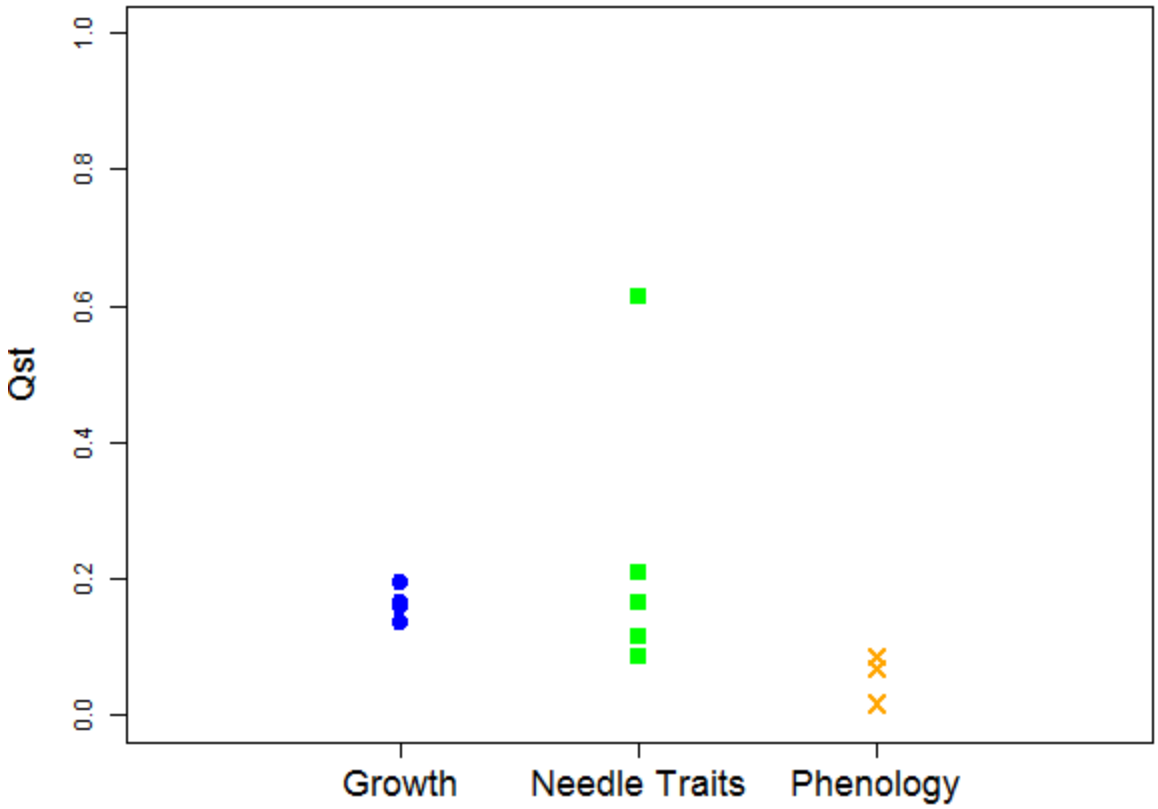

Figure 3.8: Comparative values of Q_{st} for limber pine quantitative traits. Plot based on Figure 4 of Savolainen *et al.* (2007).

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Appendices

Appendix A - Assigning Unique Identifiers using Error-detecting Code

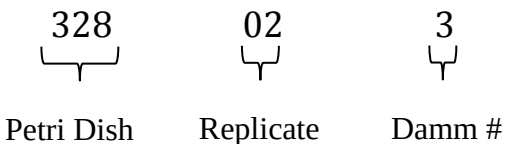
In accordance with good study design, individuals from all of the populations were randomized, however, each tree needed to remain identifiable. To avoid researcher bias from displaying the details of each individual's origin, I introduced a numeric identification system that did not require site location nor family number. These unique identifiers were used to identify trees, record their measurements, and because error-detecting codes were used to create them, provide insurance in case of mistakes.

The ability to detect transcription or scanning errors ensures consistency and accuracy while recording measurements. Error-detecting code allows for the recovery of otherwise lost data when errors are made during the measurement process. Based on the noisy-channel coding theorem in information theory (Shannon 1948), this approach is used in many industries to maintain the reliability of data (Fenwick 2014). Practically, it can be accomplished by the addition of information to data using a specific logic. The additional information allows for compatibility testing to determine if the data is corrupt. In this case, a checksum digit was simply appended to each unique identifier.

A number of algorithms can be used to achieve functional error-detection coding. For example, to verify credit card and social insurance numbers the Luhn (Luhn 1960) or the Verhoeff algorithms (Verhoeff 1969) are often used. In this case, the Damm algorithm (Damm 2004) was employed. The Damm algorithm is designed to detect both single-digit

transcription errors and adjacent transposition errors, both common when writing data down. This method does not require the use of non-numeric characters, leading zeroes do not affect it, and it uses only one lookup table as opposed to several. For all these reasons, it was chosen as the simplest and most effective for this application.

Unique identifying numbers were created for each individual seed. Numbers were selected by random from the range covering from 001 – 999 and assigned to petri dishes. These three digits of the petri dish in which the seed was stratified became the first numbers of the unique ID. Each of the ten seeds in a petri dish were assigned a replicate number between 01 – 10, which became the next two digits, and the check-digit was the last (the Damm number) to make up a six-digit unique code as shown below.



Upon receiving scanned or written data associated with unique identification numbers, the ID numbers were assessed for authenticity. Although errors were few (< 6%), the use of the error detecting Damm numbers enabled quick and easy detection and reparation of data where required. This allowed for confidence that the records accurately reflect the assigned trees.

Appendix B - Barcoding Sampling Units

Keeping track of over 7500 individuals is a big task. Although each seed had been assigned a unique code, the most efficient and accurate way to display this identifier and to monitor ongoing progress was with the use of barcodes. Barcodes are a form of Automatic Data Capture (ADC) methods that facilitate the process of gathering and entering data. There are several different kinds of barcode and choosing an appropriate type for one's application is central for successful implementation.

My project involved working in a greenhouse with trees in narrow, conical pots which fit into racks that placed them close to one another. Therefore, I had to design labelling and barcoding solutions that would be reliable under wet and high ultraviolet conditions and also versatile enough to work in confined spaces. The largest label that was heat-, water-, light-, abrasion-, smudge-, and stain-resistant and fit in the available dimension on the pots was 0.75 x 1.5 inches (Worth Poly Laser Stock; Worth Data, Santa Cruz, CA). Although I only required enough space to encode six digits, the size and aspect ratio of the printable surface impact ADC format choices. The selected barcode had to fit and function on this small, curved (rather than flat) surface. It had to be easily scannable without requiring manual shifting of the pots, and effective even if those pots were dirty, damaged, or deformed. Interleaved 2 of 5 (i2of5) is a linear barcode that meets these requirements and is supported by most scanner hardware.

The i2of5 format produces a low density code which means that it encodes only a small amount of information (strictly numeric characters), but is highly reliable. In my

application, its aspect ratio of close to one means that an i2of5 barcode uses the label area efficiently and contains ample redundancy. This makes for an easy and dependable scan without the need for precise scanner aiming even if on an angle, misaligned, or in cases of feature damage or distortion. This format is easy to encode with guard symbols, data and check digits (in addition to the Damm check digit) and can be generated in MS Word for relatively easy printing.

Once implemented, the use of barcodes on specimens is helpful for data collection and corresponding ease of data entry, however consideration must still be paid to planning. For example, preparation of a data receipt program is required to properly keep track of all the measurements. Similar to creating datasheets with all of the unique identifiers pre-printed, creating a data receipt program on the barcode reader ensures no specimens or variables intended for measurement are overlooked. It also enables direct-entry of measurements or indirect via encoding common values on a sheet of paper that can be scanned as the measurement for the study unit. There is an associated cost of time when phenotyping with a barcode reader rather than paper and pencil, but the overall process is much shorter as data entry is a simple process of uploading.

Appendix C - The Magic Stoma Analyzer

To systematize the measurement of small scale properties on thousands of needle images, protocols for computer-assisted image processing were developed by Don Holtz in the Java programing language as a software application on the ImageJ platform.

Initial execution required careful consideration of image characteristics that could be used to accurately identify the needles and count the stomata. Needle images were photographed on a high contrast background (see Figure 2.3) in order to use a colour separation technique that removed background noise from the foreground image. This process is not unlike chroma key compositing, or 'green-screening' (Gonzalez 1987). The digital micrographs were taken as 1280 pixel x 1024 pixel x 24 bits-per-pixel bitmap images. Scaling from pixel units (px) of length to true units (mm) was done using calibration images and simple optics ratios to compensate for varying magnifications between individual images and the calibration images. This optical setup produced a resolution of ~2 μm/px, which resulted in a typical stomatal size of ~50 μm or 25 pixels and spacing of ~100 μm or 50 pixels.

Once the needle area was extracted from the background, further processing was used to isolate regions of interest. Broadly, this took advantage of geometric and colour features of the pine needles. It involved using stomatal rows for detection of stomatal areas and a principle component analysis on colour for determining which of the hue, saturation, or brightness channels most effectively separated salient features. From that, a sequence of

highly tunable heuristic algorithms for semi-automated counting and measuring of needle area and stomatal counts was established.

The procedure measured foreground image area using thresholding, performed a convolution with an asymmetric blur to highlight stomatal rows, contrast enhancement to standardize and stretch intensity differences between stoma and non-stoma areas, watershed separation to resolve individual stoma, and a particle analysis on the remainder to count them. Image quality was different among images in terms of lighting and contrast. The software process was designed to capture the widest range of images possible, but be supervised so that parameter settings at every step could be adjusted and output checked and modified by the operator. The system outputs a .csv file of measurements that includes image name, magnification, length, width, area, stomatal count, areal and linear density, and stomatal configuration within each counting region.

Appendix D - Restoration Planting

27 Oct 2016

Dear Ms. Gass,

As you know, Shell Canada provides energy responsibly and holds itself to high environmental standards. When projects may affect local biodiversity, we apply stringent mitigation standards to help reduce any impacts our operations may have. We also work with biodiversity partners to restore natural habitats and ecosystems, particularly in critical habitats, which are important to the conservation of endangered species like limber pine (*Pinus flexilis*) in Alberta.

It is with much pleasure that we accept your kind donation of limber pine seedlings for our reclamation project at Waterton 18 (16-20-004-01W5M) and Waterton 19 (4-11-004-01W5M) well sites. As limber pine are recommended for Species at Risk listing in Canada, and the seedlings you have offered are not only the progeny of the trees from the area surrounding these sites, but half the seedlings come from a family line that has been shown to be White Pine Blister Rust resistant, these seedling plugs are very well suited for these two restoration sites.

With this letter, we would like to acknowledge your contribution (as well as that of Dr. Angert and the University of British Columbia) and assure you that the donated trees have been planted at the sites in accordance with our rigorous performance standards, and photos are (attached) showing the seedlings on site at Waterton 18. We have additional photos if required.

Sincerely,

DAR Foreman

Decommissioning/Abandonment/Reclamation

Projects & Technology

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