IDENTIFYING CANDIDATE LOCI FOR DROUGHT TOLERANCE IN DOUGLAS-FIR

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A Thesis

Submitted in Partial Fulfillment

of the Requirements for the Degree of

Master of Science

in Forestry

Northern Arizona University

December 2023

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ABSTRACT

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Douglas-fir (*Pseudotsuga menziesii*) is an economically and ecologically important conifer species native to North America that is currently experiencing an increase in drought conditions due to climate change. Two varieties of douglas-fir are widely recognized: coastal (var. *menziesii*) and interior (var. *glauca*). These two varieties hybridize to produce coastal x interior inter-varietal hybrids. This work aims to utilize newly developed genetic resources to analyze population structure and understand the genetic basis of drought tolerance in natural populations of douglas-fir. This study uses an annotated reference genome, genome-wide single nucleotide polymorphisms (SNPs) molecular markers, and physiological measurements in greenhouse seedlings to gain insight into population structure, and to test for associations between phenotypic and environmental data. This work explores the genetic basis of drought tolerance by identifying candidate genes that are significantly associated with water-use efficiency (WUE) (inferred from carbon isotope ratios), photosynthetic rates (inferred from % nitrogen), osmolality, stomatal density, stomatal rows, and heights at 4, 25, 37 and 48 months. Further, population structure was analyzed to confirm genetic varieties and to understand how candidate genes are distributed across the species' geographic range. Lastly, a drought experiment was implemented on douglas-fir seedlings to understand the physiological response to drought stress and expand upon previous work that was only conducted on well-watered seedlings. The results of our population structure analysis indicate four genetic clusters: coastal, hybrid, interior north, and interior south. Our results identified 402 unique SNPs associated with drought tolerance that

map to 337 unique genes. Due to the absence of a chromosome-scale reference genome and linkage map in douglas-fir, candidate loci were located only in scaffolds and not in chromosomes. A trade-off was found between tree height at 48 months old and percent nitrogen in well-watered conditions. Percent nitrogen is indicative of photosynthetic capacity; therefore, our results indicate a trade-off between tree height and photosynthetic capacity. This likely arises from the nitrogen budget where plants can either use their nitrogren for the production of cell walls or for photosynthesis. Results from the drought experiment indicates increased WUE in hybrids compared to the pure varieties when placed under drought stress due to an increase in $\delta^{13}C$. Plants will often avoid drought stress by closing their stomata to avoid transpiration. Hybrid individuals, having an increased $\delta^{13}C$ under drought conditions, are likely closing their stomata and dealing with the ^{13}C that accumulates as they use ^{12}C for photosynthesis. Drought stress and the use of ^{13}C for photosynthesis can produce reactive oxygen species (ROS). Therefore, hybrid individuals may have a better molecular and physiological response for dealing with drought conditions and ROS. Further, plants will store solutes as a method of holding onto the available water within their cells, this is refered to as osmotic potential. However, there was no significant difference in osmotic potential under well-watered or drought conditions in this study. Future studies should increase the sample size of osmotic potential under well-watered and drought conditions to further understand physiological and genetic responses to drought.

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Chapter 1

Thesis Introduction

Douglas-fir (*Pseudotsuga menziesii*) is an important conifer species in North America with a wide distribution. Natural populations grow from Mexico up to British Columbia and all along the western United States. It is also an important commercial species with plantations existing in North America and Europe. Until recently it lacked a reference genome, making genomic studies more difficult. The completion of a reference genome and advances in genomic technologies has made it possible to further our understanding of genomics and genetics in douglas-fir (Neale et al., 2017). Conifers have notoriously large genomes ranging from 6.5 – 37 Gbp with large amounts of repetitive elements (Ahuja & Neale, 2005; Zonneveld, 2012) making genomic studies difficult and expensive. Single nucleotide polymorphism (SNP) data is a powerful method to generate sequence data from large genomes (Ganal et al., 2012). Essentially, SNP markers can be sequenced across the genome to provide a snapshot of the entire genome. They can also be targeted to coding regions to avoid the large number of repetitive elements. Here, we use SNP markers developed in douglas-fir to identify candidate loci associated with drought tolerance related traits.

Drought tolerance is an important mechanism in plants with a complex cascade of genetic, physiological, and environmental variables (Overmyer et al., 2018). Understanding the mechanisms that contribute to drought tolerance in conifers is important for restoration and management practices. Predicted increases in drought due to climate change makes studies in drought tolerance important. Conifers have long life spans and slow evolution rates which limits potential for adaptation to the quickly changing climate. This highlights the importance of understanding the genetic variance of douglas-fir in naturally occurring populations and how that variation contributes to drought tolerance.

Here, we aim to further our understanding of drought tolerance in douglas-fir. In chapter two, we present a genome-wide association study (GWAS) and genome by environment association study (GEA) to test for associations between drought related traits, genotypes and environmental variables. Chapter three provides management implications based on our findings in chapter two.

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Chapter 2

Genome-wide association study of drought tolerance and growth-related traits in douglas-fir (*Pseudotsuga menziesii*) varieties and naturally occurring hybrids

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Number of words (main text):

Number of Tables: 3

Number of Figures: 11

Summary

The genetic basis of drought resistance is poorly understood in conifer species. Genomic association studies are a powerful tool to link single nucleotide polymorphism (SNP) data to phenotypes. A total of 735 SNPs associated with drought related phenotypic traits or environmental variables were identified in this study. Candidate SNPs were mapped to a variety of different genes with differing functions, suggesting that drought resistance and water-use efficiency (WUE) are under polygenic control. Candidate genes were found to be involved in antioxidative defense, amino acid development, and DNA replication/repair. This indicates that there is a complex genetic and physiological drought response in douglas-fir. This study advances our understanding of drought resistance in long-lived conifer species and can contribute to management and restoration efforts under climate change.

Significance Statement

The genomic basis of drought resistance is difficult to study because of its polygenic and complex nature. Incorporating genome-wide association studies (GWAS) can help parse apart the relationship between traits and regions of the genome. This study reports new candidate genes associated with drought tolerance.

Introduction

Understanding the genetic basis of phenotypic trait variation in naturally occurring populations is of great biological interest. Natural populations of widely distributed species experience a wide variety of climatic conditions, experiencing strong differential selection pressures and differing environment conditions. These strong selective pressures may counteract the effects of gene flow and drift in local adaptation (Ghalambor et al., 2007; Le Corre & Kremer, 2012; Savolainen et al., 2007). In populations of forest trees, the polygenic nature of trait and climate adaptation, phenotypic plasticity, and the large amount of genetic and environmental variation across the species' range make it difficult to study local adaptation (Neale & Savolainen, 2004; Newton-Cheh & Hirschhorn, 2005). Understanding genomic-wide genetic variation across the species range and how it is linked to ecologically important traits is critical to gaining a comprehensive understanding of species' potential for adaptation.

Hybridization is an important mechanism in widely distributed plant species that experience a wide range of climatic conditions. Understanding the mechanisms of maintenance of plant hybrid zones is important to understanding fitness in the species. Naturally occurring hybrids may exhibit higher degrees of fitness in the environmental niches that they occupy compared to the parental varieties (Campbell & Waser, 2007). In the bounded hybrid superiority model, hybrids tend to occupy ecotones between parental varieties or species (Moore, 1977). Mosaic models assume that hybrids can be interspersed in the parental environments and that selection can be variable depending on the environmental conditions (Harrison, 1986). Hybrids under this model may be more or less fit than parental genotypes. Several mechanisms may confer increased fitness in hybrids: adaptive introgression, heterosis, and transgressive segregation. Adaptive introgression occurs when selection maintains introgressed favorable alleles in hybrids, generating increased genetic variation (Slarkin, 1985). Introgressed alleles may result in favorable gene combinations in hybrids (Barton 2008). Adaptive introgression has been well documented in several species. For example, hybrids between *Helianthus annuus annuus* and *Helianthus debilis* known as *Helianthus annuus texanus* gained increased biotic resistance from the *Helianthus debilis* parent (Whitney et al., 2006). Heterosis occurs when first-generation (F1) hybrids are more fit than the parents and is commonly documented in crop species (Hochholdinger & Baldauf, 2018). *Zea mays* is a well-studied crop species that has well documented cases of heterosis where F1 hybrids exhibit higher yields than parental varieties (Duvick, 2001). Transgressive segregation is thought to be an important mechanism that contributes to plant adaptation. This mechanism produces extreme or novel phenotypes in segregating hybrids that are not present in parental lines (Rieseberg et al., 2003). Transgressive segregation usually occurs after several generations of hybridization and introgression and it is commonly documented in crop species such as wheat. Breeding exploits transgressive segregation in wheat to confer resistance to biotic factors such as yellow rust (Wallwork & Johnson, 1984) or to increase crop yields (Yadav et al., 1998).

Tree species, specifically, are under increasing threat due to climate change. Predicted shifts in climate may be too fast to be countered by plasticity, migration, or adaptation (Aitken et al., 2008; Alberto et al., 2013; Schoville et al., 2012). Future climate predictions infer increases in drought on a broad scale. Conifers, being long-lived tree species, have slower evolutionary rates and are exposed to a vast range of climatic conditions (De La Torre et al., 2017). Therefore, there is less potential for selective pressures to lead to local adaptation within natural populations of conifers.

Plants utilize complex signaling pathways to regulate responses of defense and stress related genes to environmental stress (Overmyer et al., 2018). Therefore, gene expression is an important aspect of linking genotypes to phenotypes. RNA expression analysis can be an important aspect to dissect phenotypic plasticity in plants. Phenotypic trait variation can be observed at two different levels: the genetic basis and the expression level. Combining SNP data with RNA expression data allows for a more complete picture of how a genotype affects a phenotype. Individuals may share a similar or identical genotype, but different expression levels, resulting in different phenotypes despite the same genetic basis. Drought-related and water use efficiency related traits are often plastic and under polygenic control, making expression analyses more important to link polygenic traits to genotypes. Plasticity and differences in expression may result from a variety of different mechanisms. Epigenetic modifications may increase or decrease expression levels and can be heritable. These modifications can change the structure of DNA using DNA methylation, change histone packaging through acetylation, and changes in miRNA expression, all of which can affect the transcriptional availability without changes in DNA sequence (Iwasaki & Paszkowski, 2014; Sato et al., 2011; Wolffe, 1998).

Water use efficiency and drought tolerance are ecologically important traits in plants with complex physiological and genetic cascades. Drought stress can be defined as drier than normal soil conditions that inhibit the potential for plant growth (Farooq et al., 2012). Upon initiation of drought stress, plants will close their stomates and increase the production of osmolites to prevent water loss (Jogawat, 2019; Martin-StPaul et al., 2017; Zivcak et al., 2016). The ability to use water efficiently and survive low water conditions is critical to plant survival and resilience to climate change (Fang & Xiong, 2015). Populations and individuals that exhibit higher water use efficiency and resilience to low water conditions may be better suited to withstand drought stress which is predicted to increase due to climate change. Hybridization between species or varieties can be an important mechanism in plants to confer resistance to abiotic stressors.

Douglas fir (*Pseudotsuga menziesii*) is an economically and ecologically important conifer species in North America. Douglas fir's natural range spans a large variety of climatic conditions as natural populations are found in low to high elevation zones in Mexico up to British Columbia, Canada (Fig. 1). Two varieties have been identified: coastal (*menziesii*) and interior (*glauca*), but plastid DNA suggests that three varieties may be present instead (Coastal, Interior, and Mexican) (Gugger et al., 2010). Naturally occurring hybrids between coastal and interior exist in British Columbia and the Washington Cascades. Previous studies have indicated that warming temperatures and increased drought conditions may inhibit growth in douglas-fir (Restaino et al., 2016). Furthermore, previous common garden experiments in which plants are grown in a greeenhouse under the same conditions have revealed that individual cold tolerance is correlated with colder temperatures at the seed source (Bansal et al., 2016; De La Torre et al., 2021), and high-elevation seed sources had increased water-use efficiency and greater heat tolerance than low-elevation sources (Compton et al., 2023). Genome wide studies in conifers has been challenging because of their large genome sizes (>10 Gbp) However, with recent advances in next generation sequencing it is becoming easier and cheaper to sequence large amounts of DNA, making tree genomics a viable study system (Neale et al., 2017). Douglas fir is a diploid species with 13 chromosomes and a genome size of about 16 Gbp. The completion of a douglas-fir reference genome has made genomic studies in this species more viable (Neale et al., 2017). Functional annotation of the douglas-fir reference genome is also becoming more robust, increasing the viability and applicability of genomic studies (Velasco et al., 2023). Previous studies have identified single nucleotide polymorphisms (SNPs) that are associated with environmental data and some phenotypic data (Compton et al., 2023; De La Torre et al., 2021; Howe et al., 2020). Sequence data is an important starting point for genomic analyses of large genomes, such as conifer species. Here, we hope to expand upon previous research to further identify and explore genetic regions associated with climate adaptation and drought tolerance. Samples were collected from across the species range for DNA extractions. Genotypes were established using a custom-designed gene-based Illumina Infinium SNP array.

Previous studies found evidence of adaptive introgression conferring increased water use efficiency in douglas-fir hybrids (Compton et al., 2023). Hybrids were also found to be more resistant to cold compared to coastal individuals (De La Torre et al., 2021). However, these studies were limited because they did not include interior populations which may limit understanding of the mechanism of increased hybrid fitness. We aim to further dissect hybridization in douglas-fir by including interior populations. Interior populations are inferred to have higher water-use efficiency and greater resilience to drought conditions.

Here, we present three main objectives: what evidence of increased hybrid fitness do we see in naturally occurring inter-variety hybrid populations? What associations between genotypes and drought tolerance-related traits do we observe across natural populations of douglas-fir? How do these candidate genes contribute to potentially adaptive traits under climate change?

Materials and Methods

Greenhouse experimental design

Seeds from 629 open-pollinated, unrelated individuals from 29 populations comprising interior, coastal, and putative hybrids were planted in a completely randomized design at the Northern Arizona University (NAU) greenhouse facility (Flagstaff, Arizona) in August 2018 (Fig. 1). Seed was collected throughout the species' natural distribution, ensuring a wide environmental variation in the sample collection. Before planting, seeds were cold stratified at 4ºC for 1 month. They were later planted in SC10 containers (Ray Leach Cone-tainers-SC10 Super, Stuewe & Sons, Inc., Tangent, OR, USA). Soil mix contained equal parts of sphagnum peat moss, coarse vermiculite, and horticultural perlite. Seeds were watered three times a week before germination, and daily after germination. Fertilization took place weekly during the months of April through July, using a balanced water-soluble fertilizer at a concentration of 60 ppm. The greenhouse temperatures were maintained between 15-23° C during growing season, from April to September. To follow the natural process of dormancy in the species, dormancy was mimicked by placing trees in a cool greenhouse from October to March. During this period, the greenhouse temperatures varied between 7-15° C.

Survival and growth measurements

Germination and seedling survival were evaluated at four time periods: 4 months, 25 months, 37 months, and 48 months old. The height of the terminal bud measured from the stem base was recorded for each seedling using a standard meter stick. As of October 2023, 405 seedlings remain alive.

Carbon isotope and leaf nitrogen analyses

Newly developed needles from 310 26-month-old seedlings were collected for carbon and nitrogen isotope analyses ($\delta^{13}C$, $\delta\ ^{15}N$) in May 2021. $\delta^{13}C$ is a measure of intrinsic water-use efficiency (iWUE). Individuals that have greater $\delta^{13}C$ values (less Carbon isotope discrimination $\Delta^{13}C$) suggest higher iWUE and drought resistance (Ehleringer et al., 1991; Kerr et al., 2015; Marias et al., 2016). At least 6 needles were collected from each of 310 individuals. Needles were placed into paper coin envelopes and dried in a SHEL LAB Forced Air Oven at 65°C for 48 hours. Dried needles were placed in 2mL tubes with 1 metal grinding ball and ground for 2 minutes in a SPEX SamplePrep 1600 MiniG mill until reduced to a fine tissue powder. After an additional 24-hour drying period, ground needles were then rolled into 4x6mm tin capsules with a target sample weight of 2.00mg +/-0.100mg. Samples were placed in 96-well plates and submitted for carbon isotope analyses. Ratios were measured using elemental analyzer-continuous flow isotope ratio mass spectrometry with an Erba NC 2100 EA (Carlo) interfaced to a Finnigan Delta Plus XL IRMS (Thermoquest, San Jose, Calif.) at the Colorado Plateau Stable Isotope Laboratory located at NAU.

Significant differences in traits between varieties were tested using a one-way ANOVA analysis in R (Table 1). Residuals approximated a normal distribution. Pairwise comparisons between varieties were calculated using the 'emmeans' R package (Lenth, 2023). This approach calculates estimated marginal means (EMMs) for factors in a linear model. This compares group means by variety using contrasts and calculates significant differences between means. All data analyses were carried out in R studio version 2023.06.0+421 (Posit team, 2023) with the emmeans package.

Osmotic potential

A total of 138 individuals were tested for osmotic potential which was measured by first taking a subsection of a non-essential lateral branch for each individual and soaking the branch in DI water overnight to get the branch to full turgor. The following morning the branch was patted dry, wrapped in aluminum foil and stored at -80°C. Following storage, the branch was allowed to thaw for 30 minutes to 1 hour. Ten needles were then plucked from each branch and put into a leaf press. Using the leaf press, liquid was squeezed out of the needles through a stainless-steel filter and onto a paper disc. Osmotic potential was then measured by putting the saturated paper disc into a Wesco Vapor Pressure Osmotic Pressure machine (VAPRO).

Stomatal density

Needles from 91 seedlings (39 coastal, 21 hybrids and 31 interior) were collected from the NAU greenhouse during the Spring of 2021. Three biological replicates were included per seedling. Needles were placed onto glass slides using clear nail polish. Needles were imaged using a microscope (Research Grade Upright Microscope, Fisherbrand, Massachusetts, USA) and a camera (C-Mount Digital Camera, Fisherbrand, Massachusetts, USA), and the number of rows and stomata were counted using the software ImageJ (Rueden et al., 2017). All stomata images had an area of 0.36cm^2. Stomatal density was found by creating an average of the density of three needles per tree, creating a number which could represent the variety of stomata in each individual seedling.

Sample collection and DNA extraction

DNA was obtained from fresh needle tissue collected from seedlings growing at the NAU greenhouse. Seedling seed sources were distributed across the natural distribution of douglas-fir (Fig. 1). Tissue was flash frozen with liquid nitrogen and ground in a bead miller (1600 MiniG, SPEX Sample Prep, New Jersey, USA) for 5 minutes at 1500 rpm. DNA was extracted using a modified CTAB method (Fishman 2020) or the MPBio (MPBiomedicals LLC, Ohio, USA) RapidPure DNA Plant kit. The CTAB method was modified to include a wash step of homogenized tissue with CTAB buffer prior to 65°C incubation. DNA concentration and quality were evaluated with a Qubit 4.0 Fluorometer (Invitrogen Massachusetts, USA) and agarose gels using an E-Gel Power Snap system (Invitrogen Massachusetts, USA). High-quality DNA samples with a concentration above 10 ng/uL were kept for genotyping.

SNP genotyping

A total of 169 seedlings were genotyped using a custom-designed gene-based Illumina Infinium SNP array containing 20,397 single nucleotide polymorphisms (SNPs) at the University of California-Davis Genome Center. This array was previously described in De La Torre et al. 2021 and Compton et al. 2023. Genotypes were called using Genome Studio Genotyping Module v2.0.4 (Illumina California, USA). Filtering criteria included a SNP call frequency >0.80, individual call rate > 0.8 and a minor allele frequency (MAF) >0.01. After filtering, 13070 SNPs were kept for further analysis. PLINK (Purcell et al. 2007) was used to format SNP files for analyses. SNP functional annotations were obtained using the annotation of the Douglas fir's genome assembly version Psme.1.0 (https://treegenesdb.org/FTP/Genomes/Psme/v1.0) and aligning against the full NCBI non-redundant protein sequences database (nr) using BLASTP (e value < e^{-10}).

Population structure

Genetic clusters were determined using a principal component analysis (PCA) in the "adegenet" package in R (Jombart T, 2008). A PCA plot was generated in R with the "ggfortify" (Horikoshi M & Tang Y, 2018; Tang et al., 2016) and "ggplot" (Hadley Wickham, 2016) packages. Further population structure analyses were conducted using the Bayesian program fastStructure (Raj et al., 2014). Ten runs for K values between one and ten were conducted. The chooseK.py python script in fastStructure was used to determine which value of K better explains the population structure of the dataset.

Genome-wide association study of growth and drought-related traits

Carbon isotope ($\delta^{13}C$), nitrogen isotope ($\delta^{15}N$), %C, % nitrogen, C:N, height at four time points (4 months, 25 months, 37 months, and 48 months), stomatal density, and osmotic potential data from seedlings under well-watered conditions were inputted into univariate genome-wide association studies using a general linear model (GLM) and a mixed linear model (MLM) in Tassel v.5. (Bradbury et al., 2007). The first two principal components from a PCA were used to control for population structure and a kinship matrix was used to account for relatedness in the MLM. The proportion of phenotypic variance explained by the SNP (R^2), and the dominance and additive effects were also calculated with TASSEL v.5. Bonferroni correction for multiple testing was used to control the false discovery rate. P-values less than $3.846e^{-6}$ were deemed significant. Further analyses used the R package 'pcadapt' was used to detect SNP outliers based on PCA results (Privé et al., 2020). Fst outliers were analyzed using OutFLANK in R (Whitlock & Lotterhos, 2017).

Genotype-Environment Association Analyses

Climate data was obtained using ClimateNA (Wang et al., 2016). ClimateNA downscales PRISM data (Daly et al., 1994) to scale-free point data, allowing to more accurately predict maternal tree climate variables. All climate data are based off the averages for the years 1991–2020. Climate variables included monthly, seasonal, and annual averages for temperature, precipitation, daily temperature fluctuation, and seasonal ranges in temperature and precipitation. Variables were tested for correlation in R and a heatmap was produced (Fig. 2; Fig. 7; Fig. 8). Univariate genome-wide environmental association (GEA) analyses were conducted in TASSEL using each environmental variable as observations and using SNP markers, population structure, and kinship as fixed effects in a MLM model.

A redundancy analysis (RDA) was conducted in the vegan R package (Oksanen et al., 2019) to estimate the amount of genetic variation explained by environmental variables. RDA aims to quantify the amount of variation in one set of variables that is explained by the variation in another set of variables. Environmental variables were removed if their correlation coefficient (r) with any other included variable was > 0.8 (Fig. 7). Most of the variables were highly correlated with one another, so the variables included in the RDA analysis were summer heat moisture index (SHM), continentality (TD), precipitation as snow (PAS), mean annual temperature (MAT), mean annual precipitation (MAP), and relative humidity (RH) (Fig. 8). Significant SNPs were selected if they fell outside of 2.5 standard deviations of the mean loading score for either of the first two axes.

Drought experiment

A drought experiment was carried out in 4-year-old douglas-fir seedlings grown at the NAU greenhouse. Five individuals per each variety, including a coastal (California-CA), an interior (El Rito), and hybrid (Queen) varieties were selected to be subjected to drought conditions. The experiment ran for a total of six weeks. Before the start of the experiment, seedlings were routinely watered to 100% soil water saturation twice a week, on Mondays and Fridays. Soil water was dropped to 60 – 75% soil water saturation in between watering days. During the first three weeks of the drought experiment, seedlings received decreased amounts of water. Seedlings were watered to 100% soil water saturation only on Fridays to allow soil to dry out more than it normally would. During the following three weeks, seedlings received no water. During the course of the drought experiment, fresh needle tissue was collected to measure osmotic potential and carbon isotope, following the same protocols described above. Samples were collected at four distinct times: well-watered conditions (T0), 3 weeks after the start of drought conditions (T1), 4.5 weeks after (T2), and 6 weeks after (T3).

Enrichment Analysis and Protein Interaction

We conducted an enrichment analysis on gene orthologs of *Arabidopsis thaliana* of our candidate genes identified by GEA and GWAS using PANTHER (Thomas et al. 2021). Enrichment analysis in PANTHER uses gene ontology (GO) terms to identify terms that are over or under represented in a set of genes (p-value < 0.05). Orthologs were obtained by aligning against the araport11 protein database within The Arabidopsis Information Resource (TAIR) using the PBLAST tool (e value $< e^{-10}$) (Berardini et al. 2015). We determined protein-protein interaction by creating a network of *A. thaliana* orthologs using the GeneMANIA app in Cytoscape (Shannon et al. 2003; Warde-Farley et al. 2010). GO terms were analyzed for shared protein domains.

Results

Differences in Environmental Variables Across Genetic Varieties

This study identified significant differences in important climate variables among the varieties' natural geographic distributions. Hybrids, on average, experienced a lower mean annual temperature (MAT) compared to the pure varieties with a mean of 3.5095 °C. Interiors had a mean of 6.6064 °C and coastals had the highest mean annual temperature with a mean of 9.2571 °C. All varieties experienced significantly different MAT from one another (p-value < 0.0001). Coastals experience the most mean annual precipitation (MAP) with a mean of 1910.286 mm. This is significantly different (p-value < 0.05) than hybrid and interior means: 678.857 mm and 775.680 mm respectively. Hybrids and interior are not significantly different from one another in terms of MAP (p-value > 0.05). Hybrids also experienced significantly more precipitation as snow (PAS) compared to the pure varieties (p-value < 0.05) with a mean of 254.57 cm. Interior and coastal individuals had means of 181.25 cm and 137.91 cm respectively. The interior mean was likely inflated due to one outlier that had a value greater than 800 cm. However, interior and coastal means were not significantly different from each other (p-value > 0.05). All three varieties experienced significantly different relative humidity (RH, p-value < 0.05) with hybrids being in between coastal and interior with a mean of 61.4%. Coastal individuals had the highest RH with a mean of 73.5% and interior had the lowest with a mean of 56.7%. Lastly, all three varieties experienced significantly different (p-value < 0.0001) elevational ranges from each other with coastal being the lowest and interior the highest. Mean elevations were 351.57m, 1049.48 m, and 1892.18 m for coastal, hybrid, and interior respectively.

Differences in survival, growth, and drought-related traits under well-watered conditions

Phenotypic traits were measured from 405 individual seedlings. Distribution of seedlings were 168 from coastal populations, 43 were hybrids, and 194 were from interior populations. Nine individuals never germinated, and 98 individuals died between 4-25 months after planting. A total of 112 individuals were dead by 48 months after planting for a total mortality rate of 27.586%. Of the dead individuals, 58 were coastal, 7 were hybrids, and 47 were interior. The mortality rate was the highest for coastal individuals (34.523%), followed by interior (24.227%) and hybrids (16.279%).

Significant differences (p-value < 0.05) were observed between varieties for % nitrogen (Fig. 2), %C, nitrogen isotope, C:N, and seedling height at 4, 25, 37 and 48 months. Carbon isotope was not significantly explained by variety under well-watered conditions. A higher % nitrogen was observed in hybrids than in the pure varieties which corresponds with higher photosynthetic capacity (Evans, 1989; Evans & Clarke, 2019). The mean % nitrogen for pure varieties (coastal and interior) were not significantly different from one another (p-value = 0.14608) with a mean of 0.77% for coastal and 0.82% for interior. Hybrids had a mean of 1.01% which was significantly higher than the pure varieties (p-value = 0.00105) (Fig. 2). Hybrids had the highest % carbon with a mean of 49.32%, however, this was not significantly different than the interior mean of 49.22%. Hybrids and interior seedlings had significantly different means of %C compared to the coastal varieties mean of 48.34% with p-values of 0.0127 and $1.52e^{-05}$ respectively. Nitrogen isotope was observed to be similar to % nitrogen. Hybrids were significantly different than the pure varieties (p-value = 0.00124) with a mean of -0.4305. Coastal seedlings had a mean 0.5474 and interior seedlings' mean was 0.376, however, they were not significantly different from each other. Carbon-nitrogen ratio (C:N) also reflected the patterns observed in the previous nitrogen and carbon traits. Hybrids were significantly different from the pure varieties with a mean of 52.544. Interior and coastal were not significantly different from each other with means of 67.955 and 64.92 respectively.

Results of measuring seedling heights at 4, 25, 37, and 48 months suggested that coastal seedlings were taller on average than hybrid and interior seedlings (Fig. 10). Coastal height at 48 months had a mean of 34.242 cm. Interior had a mean height at 48 months of 31.243 cm which was significantly different than the coastal variety (p-value=0.04127). Hybrids were the shortest on average, with a mean height at 48 months of 25.194 cm (p-value = 0.00155). Heights at 25, 37, and 48 months were strongly correlated with one another (r > 0.7). Height at 4 months was not correlated with the other three height measurements (r 0.7).

No significant differences were observed in osmotic potential between the varieties under well-watered conditions. Coastal had a mean of 634 mmol/kg, interior had a mean of 655.95 mmol/kg, and hybrids had a mean of 520.22 mmol/kg. None of these means were significantly different from one another (p-value > 0.05). This may be because individuals were measured under well-watered conditions or because our sample size was not larger enough to observe significant differences between varieties.

Stomatal measurements showed significant differences between the varieties. Hybrids had the least number of rows with a mean of 8 which was significantly different from the pure varieties (p-value = 0.00538). Interior and coastal were not significantly different from one another with mean number of rows 9.3077 and 8.375 respectively. Coastal individuals had the most amount of stomates with a mean stomatal density of 120.692. Hybrids were not significantly different from coastal with a mean of 107.091 (p-value > 0.05). Interior had the least amount of stomates with a density of 98.125 which was significantly different from coastal (p-value = 0.0127).

Genome-wide association study

A distinct separation was observed between coastal and interior individuals from fastStructure analysis (K=2). The PCA suggests that four genetic clusters exist across the species range: interior south, interior north, hybrid, and coastal (Fig. 9). However, fastStructure suggests that the K value that maximizes marginal likelihood and best explains structure in the data is K=3 which removes the hybrid individuals as separate group. We also observed no differences between our two PCA plots: one with all SNPs and one without the candidate SNPs.

A total of 735 significant associations (after correction for multiple testing) were found among 355 SNPs and 10 phenotypic traits from a GLM. From these SNPs, 82 were nonsynonymous, 227 matched genes, and were distributed in 188 scaffolds in the Douglas fir's genome assembly version 1.0. Results of the GLM analyses showed that candidate SNPs were associated with Nitrogen isotope: 15, %C: 16, % nitrogen: 94, C:N: 62, seedling height at 4 months: 15, height at 25 months: 237, height at 37 months: 129, height at 48 months: 154, osmotic potential: 10. MLM identified 9 of these SNPs, which were coincident with GLM results. Individual traits were associated with a variable number of SNPs ranging from 1 to 157, with 179 SNPs associated with more than one trait. SNP markers had R^2 values between 0.183 and 0.589.

Genotype-Environment Association

Furthermore, a total of 32 SNPs were found to be associated with environmental variables using a MLM. PAS (precipitation as snow): 21, SHM (summer heat moisture index): 6, MAP (mean annual precipitation): 1, CMI (Hogg's climate moisture index): 1, MSP (Mean summer precipitation): 1, bFFP (day of the year on which the frost-free period begins). SNP markers had R^2 values between 0.122 and 0.250. The multivariate (RDA) analysis identified a total of 370 significant SNPs, including 45 for precipitation as snow, 48 for summer heat moisture index, 127 for continentality, 46 for mean annual precipitation, 78 for mean annual temperature, and 26 for relative humidity (Fig. 4). A total of 204 unique SNPs from RDA mapped to a gene in the douglas-fir genome assembly version 1.0 (Supplementary Table 6). 19 genes had more than one significant SNP mapped to it.

A total of 337 unique genes were identified across our GEA and GWAS methods; these genes were distributed across 323 unique scaffolds. In total, 43 unique genes were identified with both GWAS and GEA analyses, 12 of which were identified as outliers using pcadapt (Table 3; Fig. 4).

Differences in survival, and drought-related traits under low-watered conditions

Significant differences in carbon isotope discrimination were observed during the course of our drought experiment. Hybrids did not have significantly higher carbon isotope ratios in well-watered conditions. However, under drought conditions, we found significant differences between hybrids and the pure varieties (p-value = 0.022). Hybrids had significantly higher carbon isotope ratios with a mean of -31.5 across all sampling times. Interior and coastal individuals were not significantly different from each other (p-value > 0.05) and a mean of -32.1 for both varieties. No mortality was observed during the course of the drought experiment. Continuation of the study would have likely resulted in mortality. Under severe (T3) drought conditions we observed that hybrids appeared to have an increase in osmolality (mmol/kg) compared to pure varieties, but this was not significant (p-value > 0.05). In terms of osmotic adjustment, we observed that, on average, interior individuals had the most change between T2 and T3 with a mean of 210.4, hybrids also had a positive adjustment between T2 and T3 with a mean of 69.2, and coastal individuals had a negative adjustment between T2 and T3 with a mean

-77.4 (Fig. 11). None of these means were significantly different from one another (P-value > 0.05) likely due to the small sample size (n = 15).

Enrichment Analysis and Protein Interaction

Our enrichment analysis identified 8 biological processes that are overrepresented in our dataset (p-value < 4e-04). Processes were largely involved in general cellular metabolic and catabolic pathways such as the biosynthesis of phenylpropanoids and lignin metabolism and biosynthesis. The processes with the most number of hits were general catabolic processes (n = 12), organic substance catabolic processes (n = 11), and cellular catabolic processes (n = 8) (Fig. 12). Protein import into the nucleus had the least number of hits (n = 2) (Fig. 12). We identified 3 major clusters of genes (with 5 or more unique genes) with shared protein domains using a protein network in Cytoscape (Fig. 13). Our network indicates that 6 genes are involved in metabolic pathways, 6 genes were involved in the production of late embryogenesis abundant (LEA) proteins, and 5 genes are involved in DNA replication and repair (endonucleases).

Discussion

Population structure mirrors varieties' distinct ecological niches

This study reveals high levels of population structure across the natural range of douglas-fir, in which individuals are clustered into four genetic groups: coastal, hybrid, interior north, and interior south. This confirms genetic differentiation between varieties and within our sample set, and expands upon previous studies that were lacking the interior variety (Compton et al., 2023). This study groups interior north and interior south into the “Interior” variety.

Natural populations of douglas-fir exist in several ecological niches with different environmental conditions that mirror the population structure observed in this study. Individuals that cluster together based on genetic data have very similar environmental conditions. We identified three distinct environmental niches that correspond to each of the varieties: interior, coastal, and hybrid. Hybrids experience the harshest environmental conditions with the least precipitation, the most preciption as snow, the lowest temperatures, and the shortest frost-free period. Hybrid individuals cluster tightly based on genetic data which may suggest local adaptation to these specific environmental conditons. Similarly, the other two varieties have different combinations of environmental variables that define their environmental niche. Interior populations have intermediate environmental conditions with some overlap with hybrids such as precipitation as snow. Coastal populations had the most favorable conditons with the most precipitation and warmer mean annual temperatures. These niches are also coincident with genetic clusters. There is some overlap between these environmental variables such as the northern interior populations and hybrid populations have similar values of precipitation as snow or elevation but this is still consistent with genetic data. Specific SNP markers may not reflect what is expected based on PCA clustering. This may be due to local environmental conditions favoring specific alleles depending on the location. The complexity of drought stress responses in plants and unique combinations of environmental conditions can generate specific genetic packages observed in the varieties. These are likely to be adapted to the local environmental conditions that the parent trees experience.

Evergreen conifers are notorious for their remarkable ability to withstand winter conditions. Cold acclimation is a complex mechanism, driven by several environmental conditions such as photoperiod and temperature (Chang et al., 2021). This cues a physiological response indicating conifers to begin the molecular and physiological processes required for cold acclimation. Freezing temperatures can lead to several mechanisms of damage in conifer tissues such as dehydration from the freezing of extracellular water and solute leakage from the desiccation of cell walls (Sakai & Larcher, 1987; Steponkus, 1984; Sutinen et al., 2001). Water loss is observed in conifers exposed to temperatures lower than their tolerance levels (Steponkus, 1984). Hybrid douglas-fir experience the harshest winter conditions with the most precipitation as snow, lowest mean annual temperatures, and shortest frost-free period. This indicates that hybrid douglas-fir may have improved abiotic stress responses to cellular water loss as tolerance to cold and drought are intrinsically linked in the species (Bansal et al., 2016; De La Torre et al., 2021).

Hybrids are more drought tolerant than pure varieties

Induced drought stress in douglas-fir seedlings revealed responses in $\delta^{13}C$ and osmolality compared to well-watered conditions (Fig. 5). Hybrids exhibited a significant increase (p-value $<$ 0.05) in $\delta^{13}C$ when under drought stress with the most discrimination occurring when under moderate to severe drought stress. This shift in $\delta^{13}C$ may indicate a physiological (stomate closure) or genetic (initiation of stress response cascades) response to drought. Hybrids seem to accumulate ^{13}C under drought conditions as their carbon isotope ratio becomes more similar to that of air. This may indicate that hybrid individuals are closing their stomata to limit water loss through their stomata and accumulating but dealing with the buildup of ^{13}C as their reserves of

^{12}C are depleted from photosynthesis. This result indicates that hybrids are more water-use efficient under drought stress than the pure varieties, which is coincident with previous studies in the species (Compton et al.2023).

Hybrid seedlings from the naturally occurring hybrid zone in the Washington Cascades (US) exhibited higher water-use efficiency and heat tolerance than the pure varieties under well-watered conditions in a common garden experiment (Compton et al., 2023). A higher water-use efficiency was also observed in this study, even though all hybrids were from British Columbia (Canada). This suggests a common response to drought in all advanced-generation, inter-varietal hybrids, in spite of seed source or population of origin.

While the results from our osmotic potential analysis were not significant, we did observe trends in osmolality over the course of the drought experiment. We cannot draw significant conclusions from this experiment, but our results indicate a physiological response to drought in terms of osmolality. This is referred to as osmotic adjustment in which plants accumulate solutes to increase their osmotic potential. Trends from this study indicate that hybrids may have higher levels of osmolality under severe drought stress compared to pure varieties despite starting at the lowest osmolality. Future work should carry out a drought experiment for a longer period of time while expanding upon our sample size and including individuals that are more representative of the entire species range.

Hybrids having increased drought tolerance can arise from several mechanisms: heterosis, adaptive introgression, and transgressive segregation. We would expect to observe heterosis in F1 generation hybrids which are rarely found in naturally occurring populations of hybrid douglas-fir. Furthermore, none of the hybrids in this study are F1 generation hybrids so we are unable to draw any conclusions about an effect of heterosis. It is unlikely that the effect we are observing in our hybrid populations is due to heterosis. Adaptive introgression could generate similar patterns to what we see in our data, but it rarely leads to higher hybrid fitness compared to the parents. Transgressive segregation seems to be the most likely cause of the increase in drought tolerance in drought-stressed hybrids. Hybrids combine the genotypes of pure varieties and can result in unique combinations that can increase water use efficiency. This may be the case in naturally occurring douglas-fir hybrids as water-use efficiency and drought tolerance are polygenic traits and unique combinations of these genes may generate further genetic variation for selection to act upon.

Polygenic basis of water-use efficiency in douglas-fir

Both our GWAS and GEA results suggest a polygenic basis of water-use efficiency, drought tolerance and growth. We believe that our candidate genes are widely distributed across the genome. However, due to the absence of a chromosome scale reference genome we can only identify genes at the scaffold level. It is possible that candidate SNPs are in linkage disequilibrium or located on the same chromosome, but without a complete linkage map we are unable to identify specific regions of the genome that may be associated with water-use efficiency. Previous studies indicate that complex stress responses such as drought and heat tolerance are highly polygenic (Bansal et al., 2016; Fang & Xiong, 2015; Shinozaki & Yamaguchi-Shinozaki, 1996), so we suspect to observe similar results in douglas-fir when a chromosome scale reference is available.

Trade-offs between growth and drought

Previous studies indicate trade-offs between growth and drought, heat (Bradley St Clair & Howe, 2007), and cold tolerance (De La Torre et al., 2021) where slower growing individuals tend to be more resistant to stressors. Our findings indicate that hybrids are shorter on average than the pure varieties. This is coincident with previous findings that coastal individuals grew faster than hybrids (Compton et al., 2023). Here, we expand upon previous studies as they lacked measurements from interior populations and only used hybrid individuals from the Washington Cascades. The hybrids used in this study are from British Columbia and are coincident with Washington hybrids (Compton et al., 2023). Individuals from interior seed sources appear to grow faster on average than hybrids (p-value < 0.05), but shorter than coastal individuals. However, interior individuals were not significantly shorter than coastal individuals (p-value > 0.05). Future studies should include individuals from more interior populations to further our understanding of growth of douglas-fir varieties. Overall, our data indicates that hybrids may be more suited to withstanding drought conditions as they are the slowest growing and most water-use efficient individuals. This suggests that they may be better adapted to future climatic conditions given that high temperatures and drought conditions are predicted to increase in severity (Marias et al., 2016; Philip et al., 2022). Future work should focus on prolonged drought studies with reciprocal transplant experiments to better understand the relationship between growth and WUE.

Pleiotropic effects

Our results indicate that hybrids have significantly higher % nitrogen values on average than coastal and interior individuals. This corresponds with an increased photosynthetic capacity. There is a tradeoff between using nitrogen for photosynthesis or for the production of cell walls (Evans

& Clarke, 2019). The same genes associated with high % nitrogen were also associated with lower stem height as observed in our genotype by trait boxplots (Fig. 3), which might suggest the presence of antagonistic pleiotropy. Higher photosynthetic rate is advantageous but lower stem height would not allow the individual to compete for light in dense natural forests. It is not possible, however, to conclude whether the patterns observed at the seedling stage in this study could predict growth at later development stages, especially considering that higher hybrid growth in mature individuals has been previously reported in the species (Rehfeldt, 1977). In markers that are associated with both height and % nitrogen, we observed homozygous genotypes that are associated with higher % nitrogen values and shorter, slower growing plants and vice versa. Heterozygotes were most commonly observed in interior north populations. Interior south individuals tended to share homozygous genotypes with coastal individuals. Therefore, individuals in higher elevation northern populations tended to have genotypes associated with higher photosynthetic capacity but shorter heights. However, tree height is not the only indicator of tree growth. Growth depends on several factors such as whole-tree leaf area, leaf longevity, and tissue respiration. Using other indicators of growth such as whole-tree biomass, root:shoot ratio, and root length may provide further insights into the relationship between photosynthetic capacity and growth.

Observing pleiotropic effects between growth and photosynthetic capacity in our study can also be indicative of transgressive segregation as hybrids could contain unique groups of alleles contributing to growth, photosynthetic capacity, and drought tolerance. Transgressive segregation may be bolstered by the antagonistic pleiotropic effects observed in our study as the antagonistic relationship could lead to more extreme phenotypes in hybrids as unique combinations of genes are generated (Rieseberg et al., 2003). This process is random and could generate deleterious combinations of genes but that allows grounds for selection to occur. The seeds used in our study were collected from mature individuals which selection has likely already acted upon which is why we observe similar phenotypes in the hybrids in our study. Furthermore, transgressive segregation is more likely to be observed in advanced generation hybrids

Environmental variables play a large role in the production of phenotypes in plants. Several mechanisms such as phenotypic plasticity respond to shifts in the environment to produce a phenotype. Common garden studies are a classic method for studying local adapation in plants and are a popular method of controlling for phenotypic plasticity (De Villemereuil et al., 2016). Common garden studies include plants from different populations and environmental niches and grow them in the same environmental conditions. When plants are grown under the same environmental conditions, we can infer that any differences in phenotype are due to genetic factors. In this study, we implement a genome-wide association study to test for assocations between environmental variables from the parent populations and genotype which is a common approach to infer local adaptation (Faske et al., 2021). To study the effects of environment on phenotype in a common garden study the environmental conditions must be manipulated. We purposely drought stressed douglas-fir seedlings and observed differences in $\delta^{13}C$. By manipulating water availability, we can infer that the observed shift in $\delta^{13}C$ increases WUE in hybrids under severe drought stress. This may be due to local adaptation to the environmental conditions that the parents experienced. Future studies should expand upon the sample size and distribution of seedlings in a drought study to gain a better understanding of how local adaptation can impact WUE.

Functional annotations of candidate genes

Trees can respond to environmental stress in numerous ways such as physiological, morphological, and genetic changes (Kijowska-Oberc et al., 2020). In this study, we identified 43 unique candidate genes associated with water-use efficiency and growth. Candidate genes mapped to several biological processes such as antioxidative defense (peroxidases), amino acid and carbohydrate metabolism, DNA replication and repair, methylation, and DNA binding (histone binding sites), and several other processes.

Our results identified a histone-lysine N-methyltransferase family member SUVH9 candidate gene (PSME_30964) associated with height, % nitrogen, and mean annual temperature (MAT). A previous study in our group identified that this candidate gene is associated with growth, autumn degree days below 0 (DD_0_at), and continentality (TD) (De La Torre et al. 2021). Methyltransferases are important enzymes involved in methylation as they transport methyl groups to DNA for binding. DNA methylation might provide an important stress response in plant species and can occur due to a variety of abiotic or biotic stressors. It can be used to regulate the expression of genes and can be a plastic stress response that is sometimes passed on to future generations. The level of DNA methylation in douglas-fir is poorly understood and genome scale studies are missing in the species (Yakovlev et al., 2012). Other studies in *Populus trichocarpa* have indicated that methylation can play a key role in drought stress response (Liang et al., 2014). This study suggests a potential role of genes involved in DNA methylation in the variation of photosynthetic rate, and growth associated with warmer environments in douglas-fir, although more studies are needed to reach conclusive evidence about this matter.

Our results identified a 4-aminobutyrate aminotransferase-like candidate gene (PSME_04717) that is associated with mean annual temperature (MAT) and % nitrogen. Previous studies have indicated that this gene is also associated with growth, cold hardiness, and continentality (TD) (Compton et al., 2023; De La Torre et al., 2021). 4-aminobutyrate aminotransferase is an important enzyme that accumulates from abiotic and biotic stressors (Shelp et al., 2012). Expression of this gene can improve stress responses through improved photosynthesis, inhibiting reactive oxygen species (ROS), and regulation of stomatal closure (L. Li et al., 2021).

Candidate gene PSME_31058 was identified as a crossover junction endonuclease MUS81 isoform X2 and is associated with relative humidity (RH) and stomatal density (Fig. 3). Endonucleases are enzymes used to cleave DNA for processes such as DNA repair and ligation. Some studies indicate a link between DNA repair and stress signaling in plants. In *Arabidopsis*, there appears to be a link with biotic stress, stomatal closure, and a nuclear DNA mismatch repair (MMR) gene (Ramos & Spampinato, 2023). Our candidate gene, PSME_31058, is not an MMR gene, but it is possible that it is involved in a larger, complex cascade of genes associated with a similar response that involves stomatal closure in response to drought stress. Drought stress can create reactive oxygen species (ROS) that cause DNA damage and this gene may be part of the stress response cascade and may be a factor in DNA repair in response to ROS (Cruz De Carvalho, 2008; Qi et al., 2018).

PSME_39362 is a candidate gene associated with RH and height that maps to cellulose synthase. A previous study in our group identified that this candidate gene is associated with growth, cold hardiness, extreme maximum temperature over 30 years (EXT), Hargreaves reference evaporation (Eref), and continentality (TD) (De la Torre et al. 2021). Cellulose synthase is the main enzyme responsible for the production of cellulose in plant cell walls, which is an important part of plant growth. Coastal individuals are associated with the highest RH, stomatal density and height of all varieties, which suggests that high stomatal density probably evolved as a response to high levels of humidity (RH) in douglas-fir coastal environments, allowing individuals to grow faster and taller during longer and warmer growing seasons. Stomatal density plays a role in stomatal closure which is an important drought response (Martin-StPaul et al., 2017). Given that coastal individuals experience the highest RH and have the highest stomatal density, they are likely able to keep their stomata open more often, which could lead to a reduced drought response compared to the other varieties.

Our enrichment analysis identified several biological processes that our candidate genes are involved in such as metabolic and catabolic processes. These ranged from genes mapping to general processes that are likely contributing to several metabolic or catabolic processes to more specific such as the metabolism of phenylpropanoids. Phenylpropanoids are important molecules in plant defense as they can serve as physical or chemical barriers to stressors and are involved in the production of many key metabolites (Dixon et al. 2002; Fraser and Chapple 2011). A previous study in *Pinus taeda* have identified phenylpropanoids as being involved in drought stress response pathways, specifically the precursor to phenylpropanoids: phenylalanine (Phe) (Frelin et al. 2017). Our protein network identified 6 genes involved with the production of late embryogenesis abundant (LEA) proteins. Previous work in *A. thaliana* has indicated that the overexpression of LEA proteins improves stress resistance to heat, drought, and salinity (Hundertmark and Hincha 2008).

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Tables

	Coastal - Hybrid		Coastal - Interior		Hybrid - Interior	
Trait	T Value	P value	T Value	P value	T Value	P value
% C	-2.509	0.0339	-4.408	< 0.001	0.244	0.9677
% N	-3.314	0.003	-1.458	0.313	2.621	0.025
C:N	3.518	0.0015	1.345	0.3714	-2.889	0.0116
$\delta^{13}C$	-1.95	0.1268	-0.745	0.7369	1.602	0.2466
d15N	3.266	0.0035	1.111	0.5079	-2.754	0.0172
Height 4 Months	1.666	0.2196	4.533	< 0.001	0.532	0.8557
Height 25 Months	3.311	0.003	1.563	0.2636	-2.556	0.0299
Height 37 Months	3.143	0.0053	1.97	0.1219	-2.154	0.0813
Height 48 Months	3.2	0.0044	2.051	0.1023	-2.196	0.0738
Osmolality	1.073	0.533	-0.266	0.9617	-1.271	0.4149
Stomatal Rows	3.009	0.0144	1.956	0.1413	-0.761	0.7297
Stomatal Density	1.756	0.2021	2.657	0.033	1.021	0.57

Table 1: Results from the ANOVA analyses testing significant differences in growth and drought-related traits among douglas-fir varieties (p-value < 0.05). Trait measurements were taken in seedlings growing in well-watered conditions in a common garden experiment. All statistical analyses were conducted in R with the 'emmeans' package.

	Coastal - Hybrid		Coastal - Interior		Hybrid - Interior	
Trait	T Value	P value	T Value	P value	T Value	P value
$\delta^{13}C$	3.72	0.023	0.046	0.9988	3.766	0.0218
d15N	0.351	0.9344	-1.007	0.5753	-1.376	0.3602
%N	-1.986	0.1251	-1.441	0.3274	0.553	0.8456
%C	-0.046	0.9988	0.018	0.9998	0.066	0.9976
Osmolality	0.289	0.9553	1.307	0.4421	1.018	0.5937
Osmotic Adjustment	0.632	0.8036	0.006	1	0.65	0.7937

Table 2: Results from ANOVA analysis testing for significant differences (p-value < 0.05) in $\delta^{13}C$, d15N, %N, %C, osmolality (osmotic potential), and osmotic adjustment across all times. Trait measurements were taken across 3 separate time points across drought conditions in a common garden experiment. All statsistical analyses were conducted in R with the 'emmeans' package.

Gene	# of SNPs	Synonymous	Outlier	GWAS	RDA	Annotation	Previous Studies
PSME_00785	1	.	.	Height_48months, Height_37months, N, Height_25months	MAT	hypothetical protein: RING finger domain and U-box domain superfamily	
PSME_01244	1	.	Y	Height_25months	MAT	RING-H2 finger ATL78-like	
PSME_01847	1	nonsyn	.	Height_25months	PAS	late embryogenesis abundant	
PSME_02059	1	.	.	Height_25months, Height_37months, Height_48months	TD	serine/threonine-protein kinase fray2-like	
PSME_02293	1	.	.	Height_48months, Height_25months, Height_37months	MAT	transcription elongation factor	
PSME_02826	1	.	.	Height_25months	PAS	E3 ubiquitin ligase SCF complex subunit SKP1/ASK1 family protein	
PSME_03594	1	.	.	Height_48months, Height_25months	MAT	probable methyltransferase	

PSME_04717	1	.	.	N	MAT	4-aminobutyrate 51minotransferase-like	growth, cold hardiness, TD, PC1/ height (Compton)
PSME_09027	1	.	.	Height_25months	TD	cytochrome b-c1 complex subunit 7-like	
PSME_15791	1	.	.	Height_25months, N, Height_37months, CN, Height_48months	MAT	C-terminal binding AN, Erythronate-4-phosphate dehydrogenase family protein	
PSME_16470	1	nonsyn	Y	Height_37months, Height_25months, Height_48months	MAT, PAS	Hypothetical protein: unknown function	
PSME_17369	1	.	.	Height_25months, Height_37months, Height_48months, N	PAS	PREDICTED: LOB domain-containing protein 19-like	growth, phenology, DD_0, TD
PSME_20407	1	.	.	N, Height_37months, Height_48months, d15N, Height_25months	MAT	NA	
PSME_20457	1	.	.	Height_48months, Height_25months	PAS	importin subunit alpha	
PSME_24297	1	.	Y	Height_25months	MAT	NA	
PSME_29905	2	.,.	Y	Height_48months, N, Height_25months	MAT, SHM	inositol oxygenase 1-like	

PSME_30964	1	.	Y	Height_37months, Height_25months, N, Height_48months, CN	MAT	Histone-lysine N-methyltransferase family member SUVH9	growth, DD_0_at, TD
PSME_31058	2	nonsyn	.	stomatal_density	RH	crossover junction endonuclease MUS81 isoform X2	growth, MSP, TD
PSME_31264	1	.	.	CN, N, Height_25months, Height_37months, Height_48months	PAS	Phosphatidylinositol 4-kinase beta 1	
PSME_32799	1	.	.	N, CN, Height_25months, Height_48months, Height_37months	MAT	putative UPF0481 protein At3g02645	
PSME_33487	1	.	.	Height_25months	PAS, MAT	Phospholipase D alpha 1-like	
PSME_34149	1	.	.	Height_25months, Height_48months	MAT	catalase-1/2	
PSME_34325	1	.	.	Height_25months, Height_48months, CN, Height_37months, N	MAT	SCF ubiquitin ligase	
PSME_34434	1	.	.	Height_25months	TD	Lipid II flippase	

PSME_34515	1	.	Y	Height_25months	TD	rust resistance kinase Lr10-like	
PSME_38027	1	.	.	Height_48months, N, Height_25months	PAS	GAMETE EXPRESSED 1	growth, cold hardiness, TD
PSME_38855	1	.	Y	N, CN	MAT	unknown protein	
PSME_39362	2	.	.	Height_25months	RH	cellulose synthase	growth, cold hardiness, EXT, Eref, TD
PSME_39868	1	nonsyn	Y	Height_37months	MAT, PAS	alcohol dehydrogenase-like 4	cold hardiness, PAS, TD
PSME_39947	1	.	.	Height_25months, N	MAT	mannan endo-1,4-β-mannosidase 7-like	growth, phenology, DD_0,TD
PSME_40562	1	nonsyn	.	Height_48months, Height_25months	MAT	extensin	
PSME_41710	1	.	.	Height_4months	RH	hypothetical protein: RING finger domain and U-box domain superfamily	
PSME_42027	1	.	Y	N, CN, N	MAT	RING-H2 finger ATL78-like	
PSME_42058	1	nonsyn	Y	Height_25months	MAT	late embryogenesis abundant	

PSME_42936	2	.	Y	Height_37months, CN, Height_25months, N, Height_48months	RH, TD	serine/threonine-protein kinase fray2-like	
PSME_42944	1	nonsyn	.	Height_25months, CN	MAT	transcription elongation factor	
PSME_43045	1	.	.	Height_48months, Height_25months, Height_37months	MAT	E3 ubiquitin ligase SCF complex subunit SKP1/ASK1 family protein	
PSME_46413	1	.	.	Height_48months, Height_37months, CN, N, Height_25months	MAT, PAS	probable methyltransferase	growth, phenology,PAS, TD
PSME_46857	1	.	.	Height_48months, Height_37months, Height_25months	MAT	4-aminobutyrate 54minotransferase-like	
PSME_47657	1	.	.	Height_37months, Height_48months, CN, Height_25months	MAT	cytochrome b-c1 complex subunit 7-like	
PSME_50135	1			Height_37months, Height_25months, Height_48months	MAT	C-terminal binding AN, Erythronate-4-phosphate dehydrogenase family protein	

Table 3: Combined results from GWAS and GEA. Genes in this table were identified by both GWAS and GEA. Table includes number of SNPs mapped to the gene, associated trait(s), and associated environmental variable. Processes, pathways, and functional annotations were obtained using the PSME v1.0 reference genome aligned against the full NCBI non-redundant protein sequences using BLASTP.

Figures

Figure 1: Map of douglas-fir seedling seed sources. Seeds were collected from mature trees at these locations and planted in a common garden within a greenhouse. Multiple individuals were planted per seed source. Genetic groups are based on PCA scores and clustering.

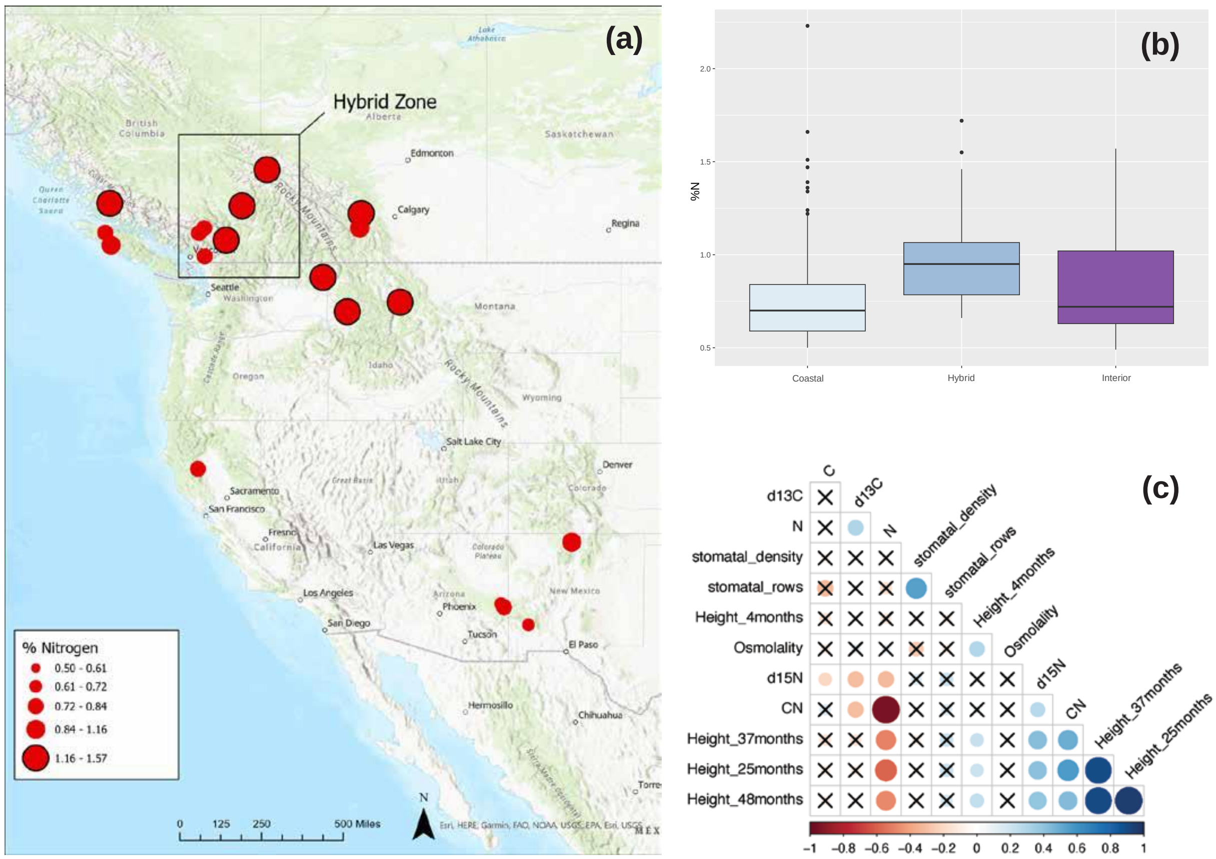

Figure 2: Results from phenotypic trait analysis of douglas-fir seedlings. (a) Map of seedling seed sources with corresponding % Nitrogen values. The hybrid zone in northern populations is highlighted. (b) Boxplot of % Nitrogen values by variety. Hybrids have significantly higher % nitrogen values compared to the pure varieties (p-value > 0.05). (c) Correlation heat map of traits developed with the r package 'rstatix.' Non-significant correlations (p-value > 0.05) between variables have been crossed off. Correlation values are represented using the Pearson correlation coefficient (r).

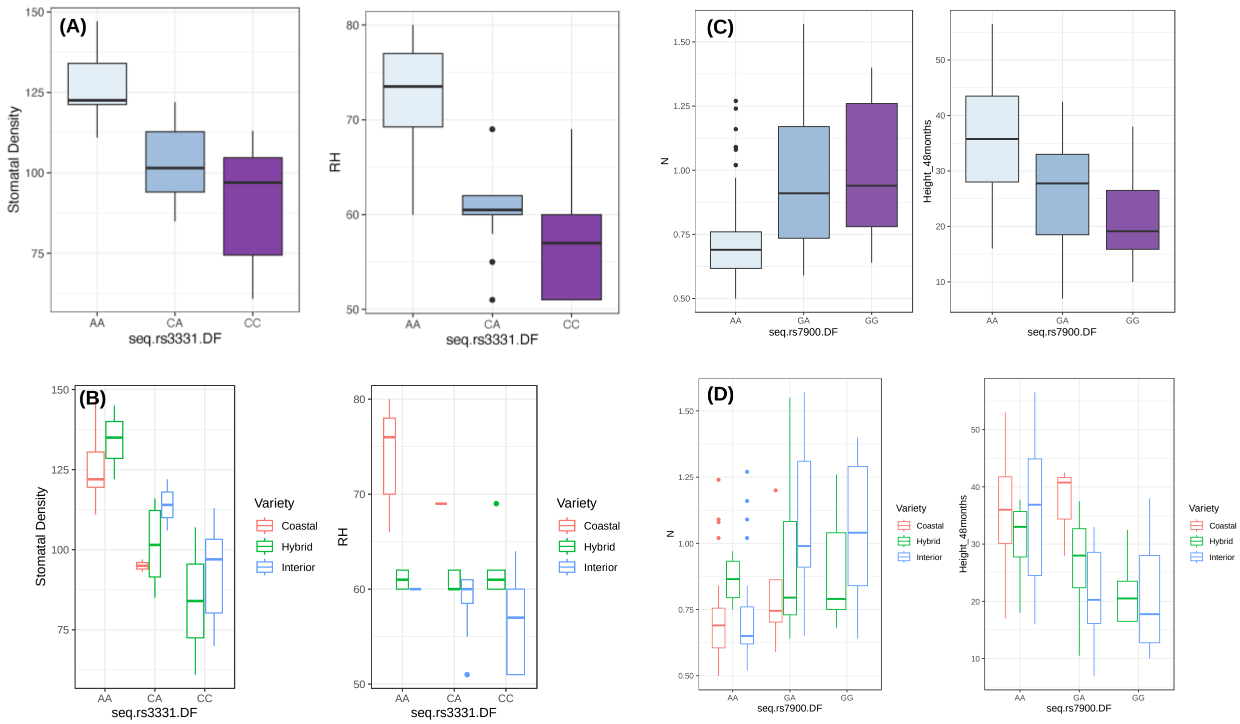

Figure 3: Visualization of GWAS results for seq.rs3331.DF and seq.rs7900.DF plotted with RH and stomatal density, and height and % nitrogen respectively. (A) Seq.rs3331.DF is located on the candidate gene PSME_31058 and is associated with RH and stomatal density. PSME_31058 maps to a crossover junction endonuclease MUS81 isoform X2 which is involved in DNA replication and repair. The boxplot depicts a positive relationship between RH and stomatal density with the genotype "AA" having the highest of both values on average. (B) The boxplots split by variety depict a minor cline across the varieties. (C) Seq.rs7900.DF is located on the candidate gene PSME_15791 and is associated with height and % nitrogen. The boxplots depict a negative relationship between height and % nitrogen. Hybrids and interior individuals contained all genotypes whereas coastal individuals were only homozygous for "AA" or heterozygotes. The "AA" genotype is correlated with lower % nitrogen but higher height values.

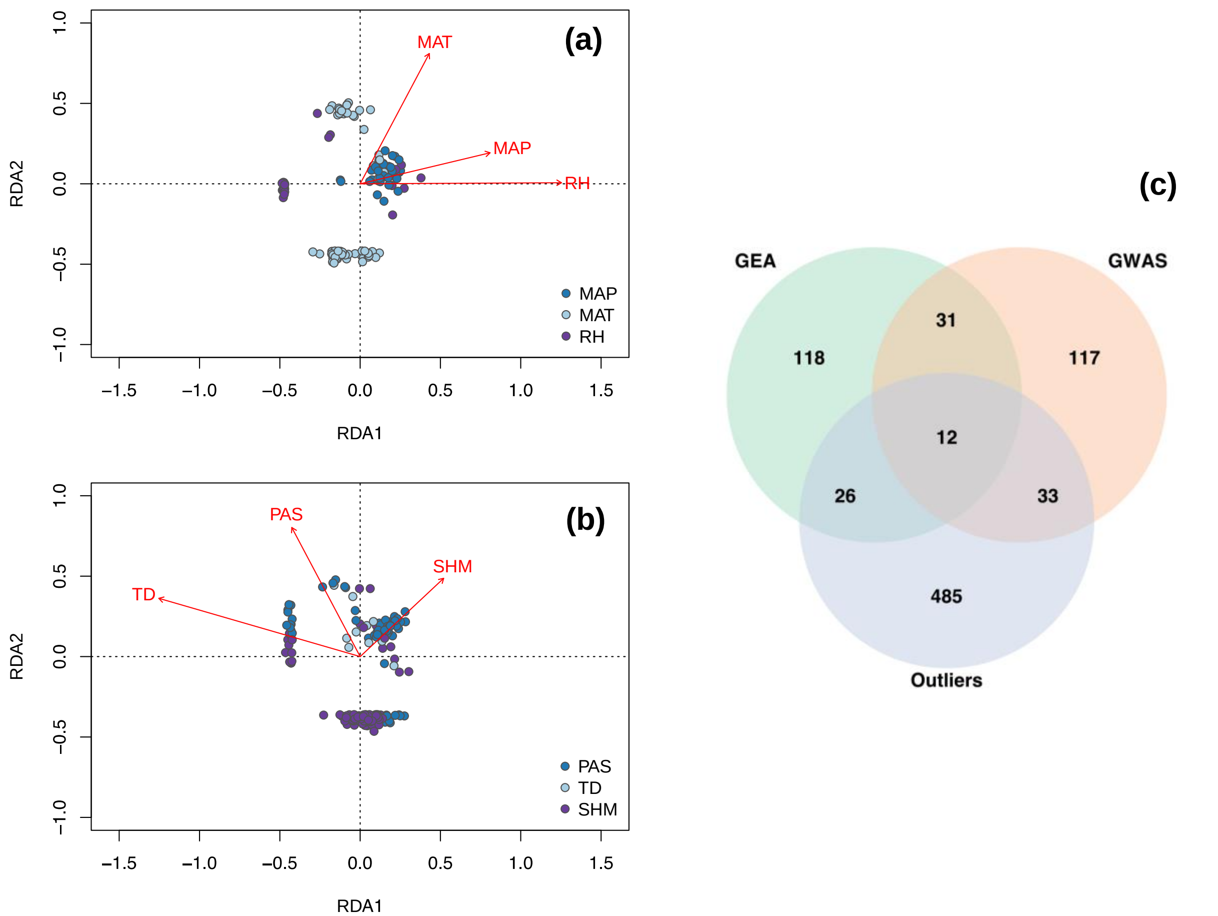

Figure 4: Results of the multivariate RDA analysis by SNP markers or significant correlations with environmental variables such as (a) mean annual temperature (MAT), mean annual precipitation (MAP), relative humidity (RH) and (b) continentality (TD), precipitation as snow (PAS), and summer heat moisture index (SHM) based on 13,070 SNP markers for douglas-fir. (c) Venn diagram for identification of unique genes based on SNP annotations. Groups show the number of candidate genes identified through genome-wide association between genotypes and growth and drought tolerance traits with GLM and MLM models (GWAS); genome-wide association between genotypes and environmental variables using MLM and RDA (GEA), and pcadapt (Outliers). Each unique gene may have several significant SNPs mapped to it.

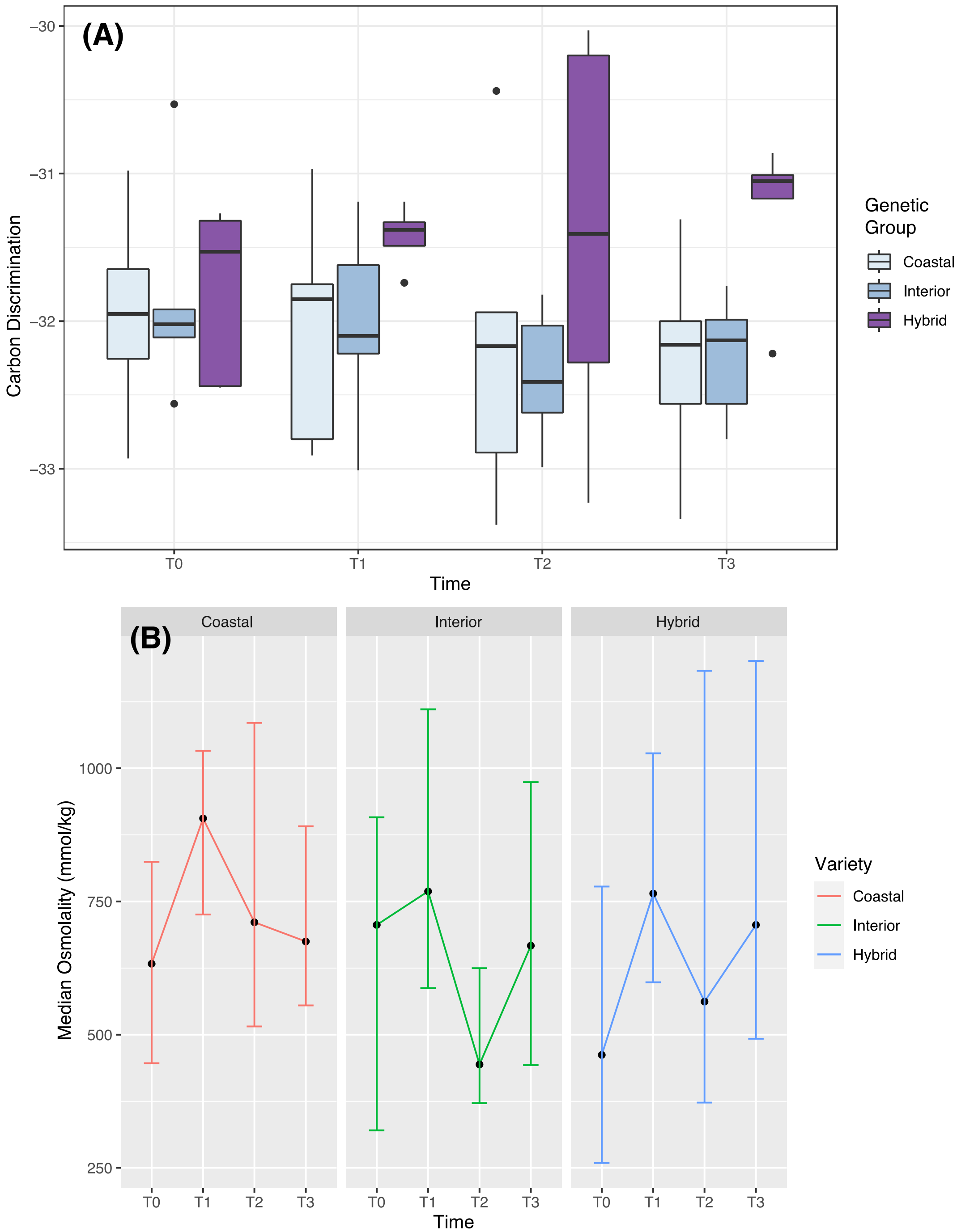

Figure 5: Results from the drought experiment. T0: well-watered conditions. T1:3 weeks of receiving less water. One water per week instead of two. T2: 1.5 weeks of no water (4.5 weeks in total). T3: 3 weeks of no water (6 weeks in total). (A) Boxplot representing $\delta^{13}C$ measurements across all sampling times. Hybrids had significantly higher (less negative) values of $\delta^{13}C$ across all times compared to pure varieties (p-value < 0.05). (B) Line plot of osmolality across all sampling times. Points represent medians of the varieties at that time. Medians were not significantly different from one another.

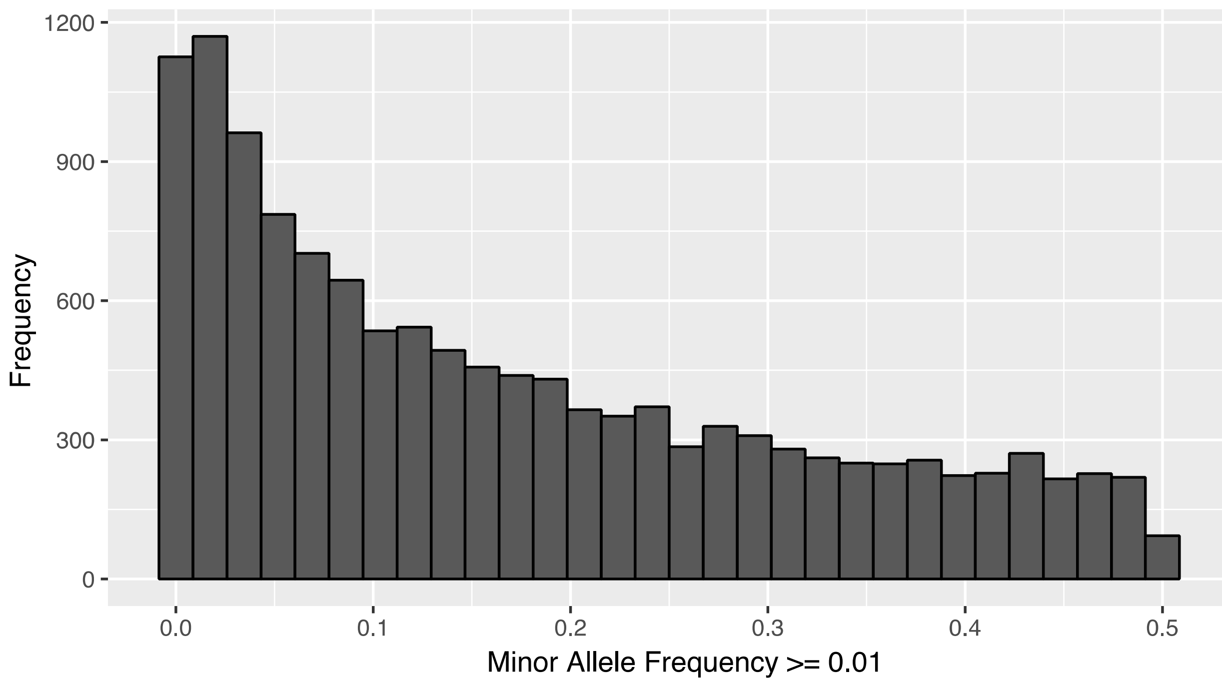

Figure 6 (Supplementary): Distribution of minor allele frequency for the 13,070 SNPs included in this study.

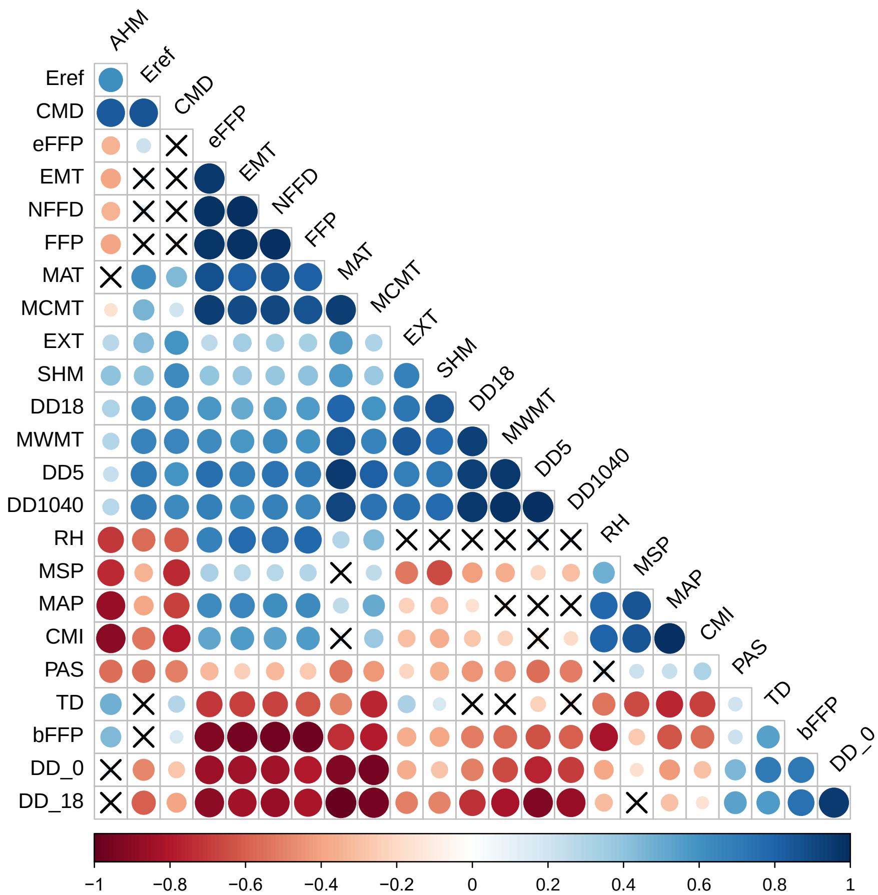

Figure 7 (Supplementary): Correlations among environmental variables obtained from ClimateNA. A heatmap is shown with a gradient between red (negative correlation coefficient) and blue (positive correlation coefficient) with white representing a correlation coefficient of zero. Non-significant correlations are denoted by an "×" in each box.

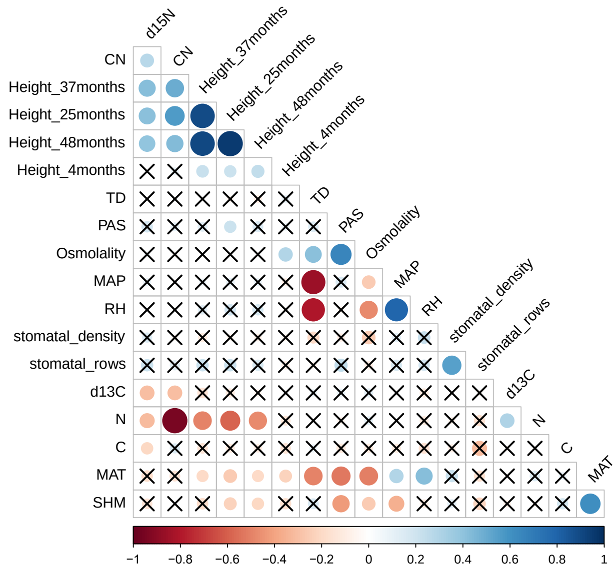

Figure 8 (Supplementary): Correlations among environmental variables and phenotypes. A heatmap is shown with a gradient between red (negative correlation coefficient) and blue (positive correlation coefficient) with white representing a correlation coefficient of zero. Non-significant correlations are denoted by an “×” in each box.

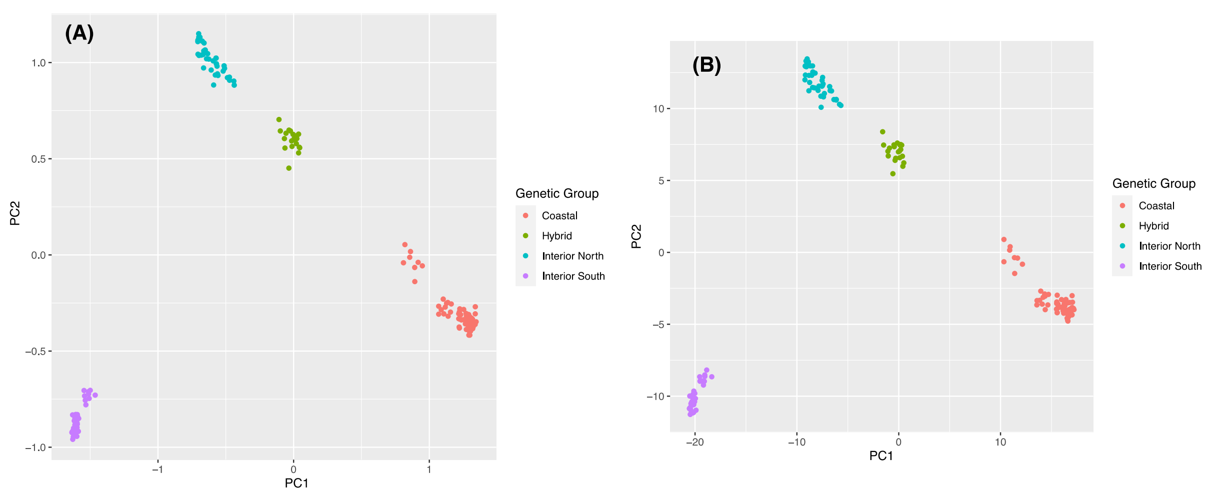

Figure 9 (Supplementary): Principal component analysis (PCA) obtained from SNP data. (A) PCA containing all 13,070 SNPs. Individuals were assigned to the following genetic groups: coastal, hybrid, interior north, and interior south based on PCA clustering and location. (B) PCA with candidate SNPs removed (n = 11,668).

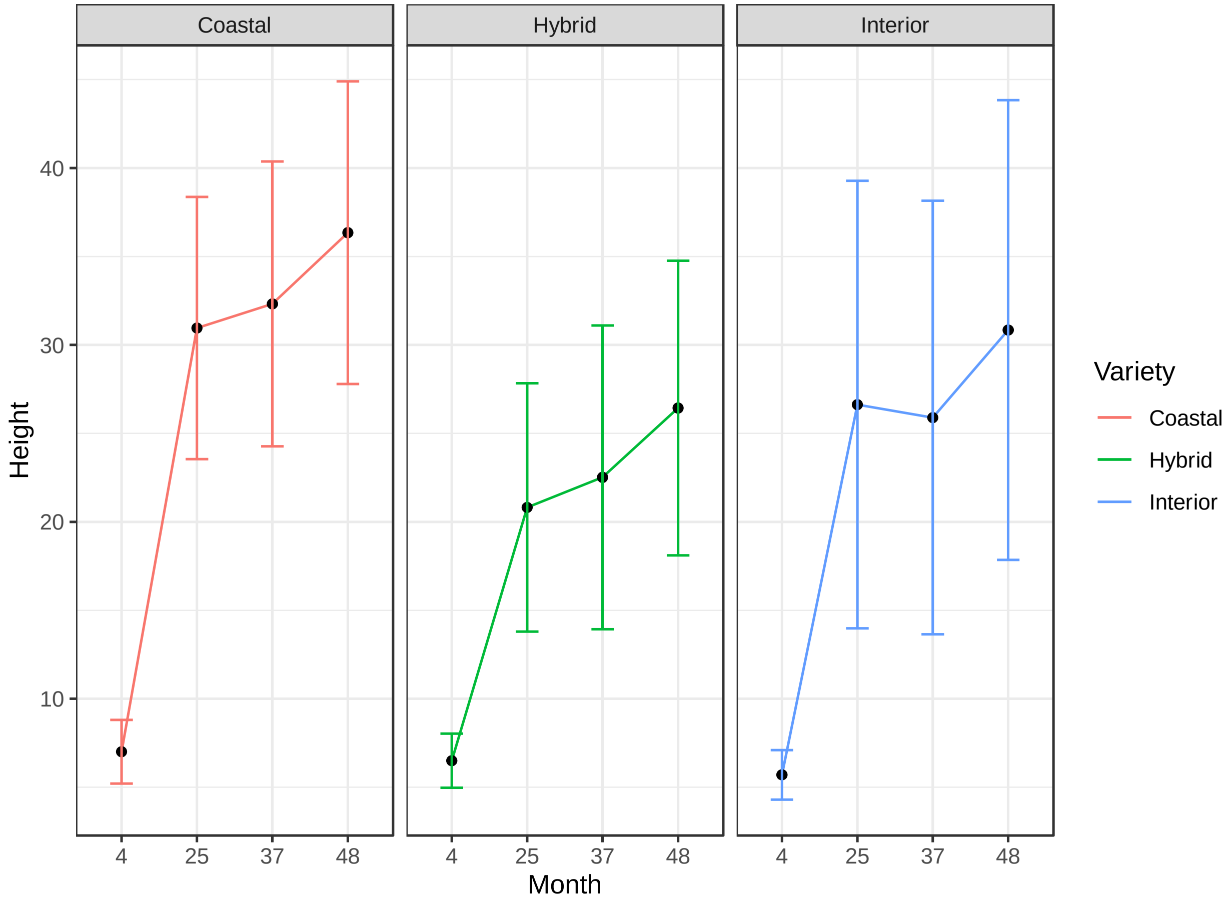

Figure 10 (Supplementary): Line plot of seedling heights at 4, 25, 37, and 48 months. All measurements were taken in a common garden under well-watered conditions. Heights at 25, 37, and 48 months are all strongly correlated with one another ($r > 0.7$). Varieties were significantly different from one another at 48 months (p-value < 0.05). Varieties were not significantly different at 4 months.

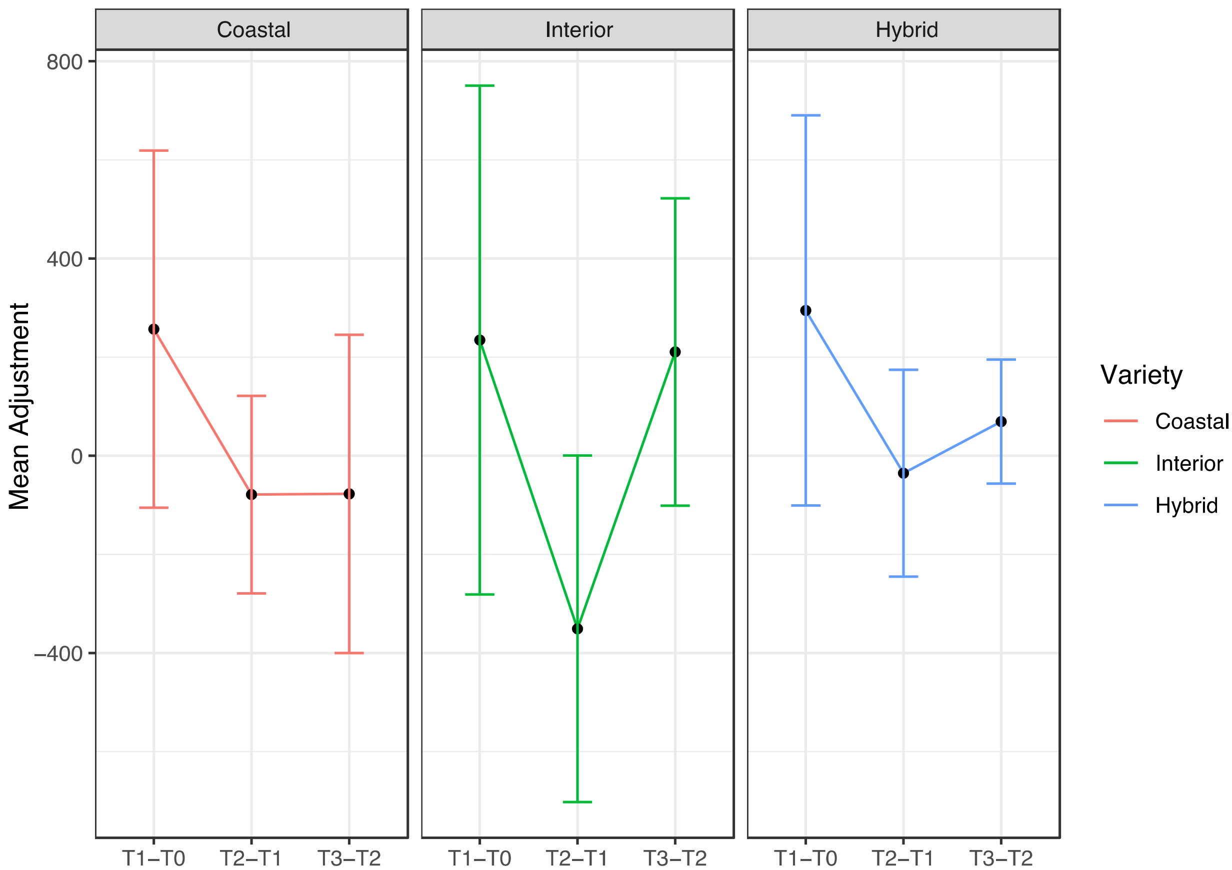

Figure 11 (Supplementary): Lineplot for osmotic adjustment variable calculated from osmotic potential data under well-watered and drought conditions. Osmotic adjustment was calculated by subtracting later times with the previous time period osmotic potential readings. This results in a positive adjustment when osmotic potential increases and a negative adjustment when osmotic potential decreases. Varieties were not significantly different from one another at any time period (p-value > 0.05).

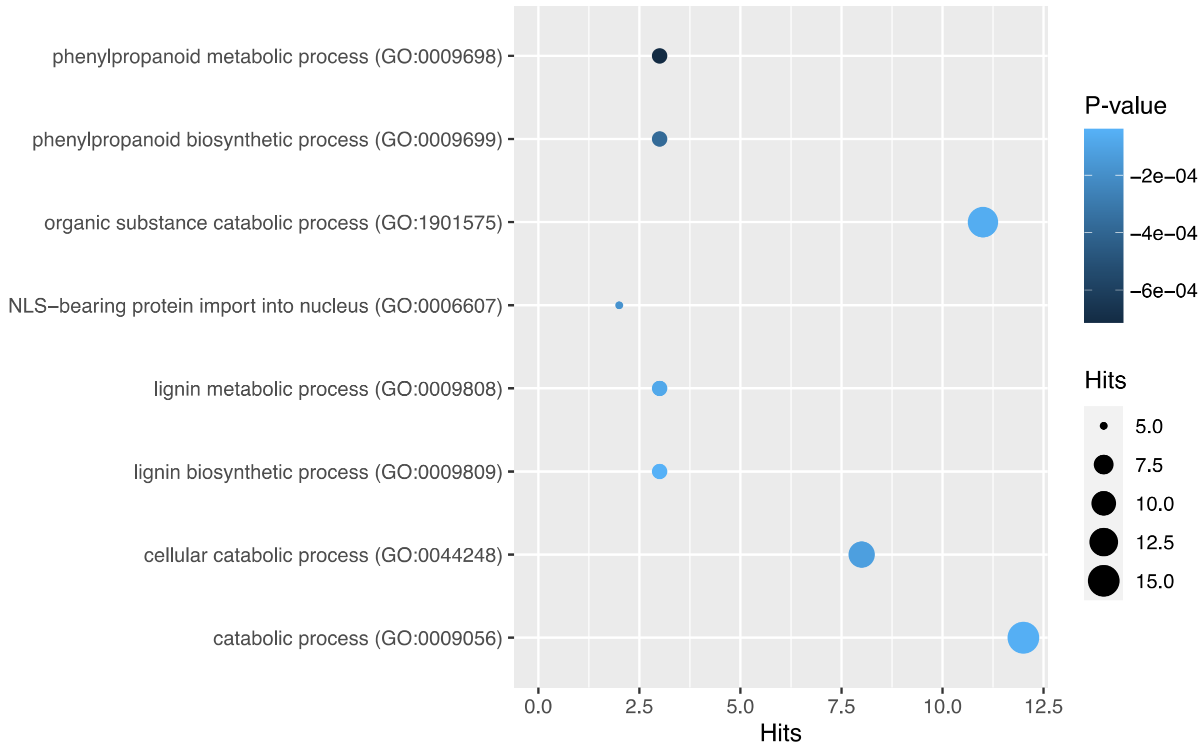

Figure 12 (Supplementary): Results of the enrichment analysis done on protein orthologs from *Arabodopsis thaliana* showing the top 8 results with the lowest 8 p-values (p-value < 6e-04). Proccesses largely include catabolic and metabolic processes of secondary metabolites. The function with the most hits and the lowest p-value is ‘catabolic process.’ These processes are likely involved in similar genetic cascades.

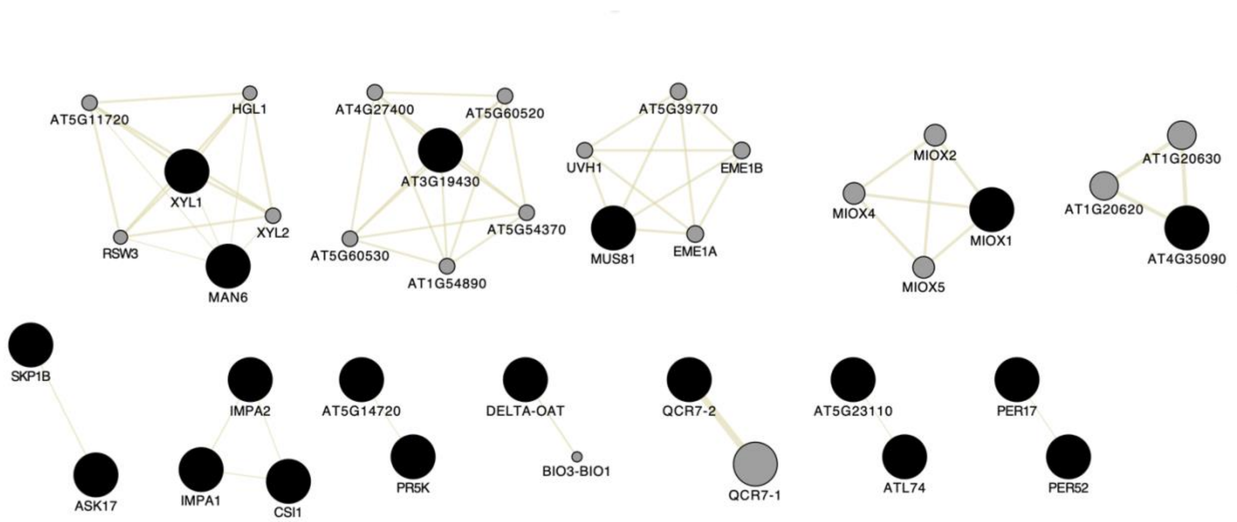

Figure 13 (Supplementary): Protein-protein interaction network based on *Arabodopsis thaliana* ortholog genes produced in Cytoscape. Primary functions include carbohydrate metabolism. (glucosidases), embryogenesis related proteins, DNA replication/repair (endonucleases), oxygenases, and neutralization of reactive oxygen species (ROS) (catalases).

Chapter 3

Management Implications

Commercial value and uses of douglas-fir

Douglas-fir is one of the most important commercial species in North America. The timber industry is an important part of the economy in the Pacific northwest, part of douglas-fir's natural distribution (Reimer, 2021). Douglas-fir is a dominant source of timber in the Pacific northwest. Along with western hemlock forests, douglas-fir accounts for 90% of softwood timberland (Haynes, 2003). Therefore, douglas-fir is an important part of the timber industry in the United States, which produces nearly $300 billion in forest products each year (USFS, 2023). Conserving natural populations of douglas-fir and introducing proper management practices in relation to local adaptation in commercial plantations is of importance to maintain douglas-fir as a key timber species.

Managing for resistance to drought conditions

Previous studies have indicated that douglas-fir may be maladapted to projected future climate conditions , therefore mitigating the effects of climate change on the species should be of great interest as climates continue to shift. Special considerations must be considered for conifer species as they have long life spans with long generation times. Current individuals will likely experience shifts in climate during their lifetime. Long generation times slows the capacity for populations to adapt to new conditions. Overall, genetic studies are a powerful tool that can heavily contribute to active restoration efforts by identifying important genotypes or genes that contribute to local adaptation or commercial success. For instance, previous studies in conifers identified several genes that play a key role in flowering and seed development (Y. Li et al., 2021). Conifers generally have long generation times so understanding the genetic basis of seed production is important to hasten breeding programs or for seed production from favorable individuals. Previous studies of drought tolerance in douglas-fir highlight the importance of genetic association studies using SNP markers, phenotypic traits, and environmental variables (Moran et al., 2017). Linking traits to genomic regions has been proposed as an important contribution to marker-assisted selection in conifers (Isik, 2014). Marker-assisted selection aims to exploit associations between phenotypes and genetic markers to confer adaptive selection. This study identifies genetic markers associated with drought tolerance, water-use efficiency, and growth; all of which could benefit marker-assisted selection to accelerate breeding programs.

Douglas-fir currently faces multiple threats that impact management implications such as increased drought, increased heat and cold stress, and changes in habitat due to climate change. Douglas-fir spans a wide range of climatic conditions due to its large species range with three recognized varieties (coastal, interior, and hybrid) adapted to their respective climate conditions. Hybrids experience the harshest conditions with the least amount of precipitation, most amount of precipitation as snow, and lowest mean annual temperatures. Identifying douglas-fir varieties and populations that are more drought resistant and water use efficient is of great interest to preserve the natural populations of the species and for commercial plantations (Baldi & La Porta, 2022; Moran et al., 2017). Management practices such as assisted migration could greatly benefit from increased understanding of the genetic mechanisms that confer resistant to environmental stressors. Assisted migration is the intentional movement of genotypes by humans during restoration and reforestation with the intent to mitigate the negative effects of climate change.

Studies of commercial plantations in Europe highlight the importance of selecting resistant and climate adapted lines for reforestation efforts (Isaac-Renton et al., 2014). It is important to understand the specific climate conditions that populations of trees are adapted to in order for restoration efforts to be successful. Assisted migration relies on the successful establishment of individuals in potentially novel areas which can heavily rely on local adaptation. Previous studies on whitebark pine (*Pinus albicaulis*), hybrids between white spruce (*Picea glauca*) and engelmann spruce (*Picea engelmanni*), and oyamel fir (*Abies religiosa*) found that assisted migration is possible but has limitations (Sáenz-Romero et al., 2020). The authors highlight that more studies on assisted migration are needed before large operational efforts take place in forest management activities. Furthermore, assisted migration may occur within or outside of the species natural distribution. This can represent restoration or commercial interests respectively; both of which would mitigate the impacts of climate change on douglas-fir.

The intentional introduction of favorable alleles in natural populations can have complex effects on highly polygenic responses such as drought resistance. Identifying genes that have effects on drought responses and water-use efficiency is of great interest to assist in management efforts. Furthermore, this knowledge can be applied to commercial plantations. Identifying trees that may be more suited to the location of the plantation and more resistant to drought or other abiotic stressors can potentially increase yield.

Limitations of management goals

Drought tolerance is a highly polygenic physiological and genetic response in plants. Unintended consequences may arise from assisted migration or genetic breeding programs. Assisted migration should also rely on studies of local adaptation that account for current and future climate conditions for successful establishment (Gray & Hamann, 2011). However, many factors play a role in successful establishment. Individuals may not be adapted to specific soil conditions, such as ectomycorrhizal fungi, limiting the effectiveness of this management strategy (Kranabetter et al., 2015). Plants often have trade-offs between growth and defense (such as defense from abiotic stressors such as drought) or growth and photosynthesis (Evans & Clarke, 2019). Our study identified that hybrid douglas-fir have higher rates of photosynthesis, higher water-use efficiency, and better responses to severe drought than the pure varieties. However, they grow slower on average and may be using more resources on defense rather than growth or reproduction (Obeso, 2002). This is coincident with previous findings that hybrids grow slower and have higher % nitrogen (Compton et al., 2023). Slower growth but greater drought tolerance may not be of great interest to commercial plantations, unless coastal populations become maladaptive due to warming and drier environmental conditions. Larger-scale studies in controlled and field conditions are required to draw definitive conclusions on this complex topic.

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