2019

Association Genetics and Local Adaptation of *Populus trichocarpa* Torr. & Gray

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**ASSOCIATION GENETICS AND LOCAL ADAPTATION OF *POPULUS TRICHOCARPA*
TORR. & GRAY**

Hari Bahadur Chhetri

**Dissertation submitted
to the Eberly College of Arts and Sciences
at West Virginia University
in partial fulfillment of the requirements for the degree of**

**Doctor of Philosophy in
Biology**

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Donna Ford-Werntz, Ph.D.
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Vagner Benedito, Ph.D.**

Department of Biology

**Morgantown, West Virginia
2019**

**Keywords: Quantitative traits, GWAS, heritability, redundancy analysis,
local adaptation, outlier loci, genes, expression networks
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Abstract

Association genetics and local adaptation of *Populus trichocarpa* Torr. & Gray

Hari Bahadur Chhetri

A major goal in plant science is overcoming the recalcitrance of plant biomass to cellulose extraction, to enable efficient production of cellulosic biofuel. We have started to understand the genetic basis of some important traits such as cell wall chemistry, but we do not know anything about the key structural and functional traits such as wood anatomy that greatly affect plant biomass recalcitrance. Furthermore, biofuel feedstocks have to be adapted to varied environmental conditions to ensure high productivity in plantations, but little is known about the molecular mechanisms underlying local adaptation. With the advancement in sequencing and genotyping technologies, association genetics has emerged as a powerful approach for unraveling complex traits in plants, thereby linking the natural variation present in the phenotype with the underlying genotype. Furthermore, the integration of phenotypic, genomic and environmental data has great premise for understanding plant adaptation in the face of climate change.

Because of its rapid growth, hybrid vigor, broad geographic distribution, transformation potential, and the availability of tremendous genetic resources and wide phenotypic variation, *Populus* is a highly desirable genus for biofuel production and other wood products. My dissertation research uses an association genetics approach focused on important anatomical, morphological and physiological traits to address three key questions: (1) What genetic mechanisms underlie variation in morphological and physiological traits in *P. trichocarpa*? (2) What are the factors affecting local adaptation in *P. trichocarpa* and what is the relative contribution of climate and geography variables to population structure? (3) What genes or genomic regions are associated with variation in important functional and structural traits that can be targeted to enhance productivity and reduce recalcitrance of woody bioenergy feedstocks?

My research will enhance understanding of the biology of *Populus trichocarpa* by determining the genetic basis of key agronomic traits such as vessel size and density, leaf area, and stomatal density that affect overall performance under field conditions using genome-wide association study (GWAS). Understanding the genetic basis of these traits is key for developing *Populus* as a biomass feedstock for biofuel production. Furthermore, morphological and structural traits are often tightly correlated with physiological performance. Therefore, another aspect of this study is to unravel the genetic basis of key physiological traits such as leaf chlorophyll content, carbon isotope composition and leaf water potential, and their correlation with morphological traits. This will aid in better understanding of stress tolerance and the overall biology of this species. Furthermore, by performing these studies in plantations that are clonally replicated in three environments, I evaluated the robustness of the associations. Using genotype environment association (GEA) and redundancy analysis (RDA) I identified loci conferring local adaptation in *P. trichocarpa*. Moreover, with RDA analysis I determined the relative contribution of climate and geography in neutral population structure. Similarly, I determined the relative contribution of genomic, climate and geography data in explaining phenotypic variation. A long-term goal of the project is to develop a selection model based on comprehensive genetic and phenotypic information so that the genome enabled breeding value can be estimated. This will enhance the efficiency of *Populus* breeding programs by shortening the breeding cycle and improving the accuracy of selection. This will aid in developing genetically improved trees with high biomass production and reduced recalcitrance to cellulose extraction, thereby furthering the development of the lignocellulosic biofuels industry.

Dedication

To my Parents

Acknowledgements

First of all, I thank my advisor Dr. Stephen P. DiFazio for providing me the opportunity to work in his lab, and for all the guidance and help he provided me through-out this entire research journey. I am who I am today because of him. I thank my committee members Dr. Vagner Benedito, Dr. Donna Ford-Werntz, Dr. Jennifer Hawkins and Dr. Richard Thomas for their valuable comments and suggestions during our meetings.

I thank former postdocs in the lab, Dr. Alejandro Riveros Walker, Dr. Danielle Ellis, Dr. Luke Evans, Dr. Gancho Slavov and the current postdoc Dr. David Macaya-Sanz for their help in fieldwork, statistical analysis and programming. I thank Dr. Jorge Flores, professor at the Department of Biology (now retired) for letting me use his histology facility. I have spent countless hours staring at the microscope in your lab to process my wood anatomy samples.

I thank my lab-mates Dr. Brahma Reddy Induri, Dr. Eli Rodgers-Melnick, Sandy Simon and Ran Zhou for their help in fieldwork and all the great interactions I have had with them over the years.

I thank several undergrads that helped me for collecting data in the field and in the lab for my research - Amanda Emahizer, Luke Stover, Jacob Miller, Patrick Whitehouse, Hoff Lindberg and Savanna Plombon. I thank Sunita Mahat (graduate intern) for helping me with the image analysis using imageJ and matlab.

I thank Dr. Wellington Muchero, Dr. Jay Chen, Dr. Jerry Tuskan, Dr. Jessy Labbe, Lee Gunter, Sarah Jawdy, and Dr. Tony Bryan from Oak-Ridge National Lab for their help in the fieldwork. I thank my teaching mentors Pat Lutsie (now retired), Dr. Katrina Stewart, Dr. Michelle Withers, Dr. Dana Heubert Lima and Dr. Stephanie Young. You have taught me so much to grow as a teacher. I have learned so much from you guys, and you really made the teaching enjoyable.

I cannot thank my wife, Anita enough for her continuous support during the entire period of my graduate studies. She has contributed so much. Thanks for taking care of the family, good food and moral support. Thank you, Elina (daughter) and Elesh (son), you are the joy of my life. At last, but not least, I thank my parents, Krishna and Ganga for all their support and blessings and believing in me.

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Chapter 1. General introduction

1.1 Introduction

Understanding the genetic basis of a trait is of fundamental importance in biology. Various abiotic and biotic factors are known to have an effect on the genetic variation and evolution of a species (Bragg, Supple, Andrew, & Borevitz, 2015; Lind, Menon, Bolte, Faske, & Eckert, 2018; Richardson, Urban, Bolnick, & Skelly, 2014; Sork et al., 2013). Forest trees like *Populus* that occur over a wide geographical range serve as an excellent model system for mechanistic studies of adaptation. The power of advanced sequencing technology coupled with clonally replicated common gardens has helped to accelerate the process of finding links between genotypes and phenotypes. Unraveling the genetic mechanisms underlying key structural and functional traits, thus understanding local adaptation, facilitates the development of *Populus* as a bioenergy crop.

Nevertheless, most morphological, anatomical and physiological traits are complex, and detection of underlying mechanisms is often not straightforward. The traditional way of detecting loci controlling adaptive traits- QTL mapping- is cumbersome and not feasible in many forest trees because of the requirement of a multigenerational pedigree that takes many years to develop. Furthermore, QTL mapping lacks precision because the detected underlying genetic region is not very precise due to lack of recombination, and it lacks generality because the QTL pedigree is not representative of the large genotypic range of populations (Ingvarsson, 2010). In this regard, association genetics has emerged as a powerful tool for assessing the genetic mechanisms of complex traits because it captures the variation present in natural populations (Neale & Savolainen, 2004). One of the biggest advantages of association mapping is that wild natural populations can be used directly, and there is no need to generate a pedigree. Furthermore, loci and alleles underlying the traits can be precisely located and characterized at the nucleotide level, though the realized resolution depends greatly on the background linkage disequilibrium (Balding, 2006). With the vast improvement in sequencing technologies in recent years, whole genome association scans are possible at a low cost. For a tree like *Populus* that has a moderate genome size (about 450 Mb) sequencing hundreds of individuals in a population at a reasonable depth is possible (Evans et al., 2014). However, confounding factors such as population structure can cause spurious correlations, which needs to be taken into account for association studies (Slavov, Leonardi, Adams, Strauss, & DiFazio, 2010).

Forest trees are excellent model systems for studying local adaptation. Forest trees are perennials and have long generation times, span over large geographic areas, and harbor high genetic and phenotypic variation. They often cover large range of environments and show latitudinal clines for various adaptive traits (Grattapaglia, Plomion, Kirst, & Sederoff, 2009; Neale & Kremer, 2011; Savolainen & Pyhäjärvi,

2007). *Populus* is a particularly good model tree genus due to its wide distribution, fast growth, moderate genome size, hybrid vigor, ease of propagation and high ecological and economic value. A wealth of genomic resources is available for the genus. *Populus trichocarpa* is one of the most intensively-studied species in the genus. It is a dominant riparian species that has a distribution from northern California to northern British Columbia. It is a pioneer species that plays an important role in riparian ecosystems (Cronk, 2005; Eckenwalder, Stettler, Bradshaw Jr., Heilman, & Hinckley, 1996), and it is being developed as a biofuel feedstock (Tuskan, 1998). Genomic resources for this species include a whole genome sequence (Tuskan et al., 2006), extensive resequencing data for a large association population (Chhetri et al., 2019; Evans et al., 2014; Weighill et al., 2018), a SNP array targeting 34,000 genome-wide loci (Gerald et al., 2013), exome sequencing (Zhou, Bawa, & Holliday, 2014) and extensive transcriptome libraries (Jansson & Douglas, 2007). Furthermore, high levels of adaptive phenotypic variation is present in *P. trichocarpa* (Chhetri et al., 2019; McKown, Guy, et al., 2014; McKown et al., 2014; McKown, Klápště, et al., 2014). In addition, linkage disequilibrium (LD) in *Populus* is very low on average. Low LD coupled with high-resolution SNP markers in *Populus* can be a big asset for precisely locating QTL alleles in the genome.

Studies on association genetics of forest trees have shown that nucleotide polymorphisms within candidate genes account for significant variation in complex traits in natural populations (Eckert et al., 2009; González-Martínez, Wheeler, Ersoz, Nelson, & Neale, 2007; Ingvarsson, Garcia, Luquez, Hall, & Jansson, 2008; Rellstab, Gugerli, Eckert, Hancock, & Holderegger, 2015; Wegrzyn et al., 2010). However, most of these association studies are either not comprehensive in terms of the traits studied and/or are based on *a priori* selection of targeted genomic regions that ignore a significant part of the genome. In recent years, these efforts have been expanded to the whole genome level and focused on morphological, structural and physiological traits. Numerous genes and genomic regions controlling quantitative traits have been identified in *P. trichocarpa* (Bdeir et al., 2019; Chhetri et al., 2019; Evans et al., 2014; McKown et al., 2014; McKown, Klápště, et al., 2014; Muchero et al., 2015; Wegrzyn et al., 2010). Tremendous progress has been made in finding allelic variants associated with cell wall chemistry (Muchero et al., 2015; Wegrzyn et al., 2010). QTLs for lignin content, syringyl to guaiacyl (S/G) ratio, and 5- and 6-carbon sugars were detected and validated from a QTL and multiple association populations (Muchero et al., 2015). Furthermore, insights have been gained into mechanisms underlying local adaptation, including the role of structural polymorphisms and gene duplication in adaptive trait evolution (Evans et al., 2014).

Forest tree populations typically show local adaptation, in which local genotypes perform better than genotypes from other locations (Jump & Penuelas, 2005; Savolainen, Lascoux, & Merilä, 2013). This poses challenges in the face of rapid climate change because locally advantageous genotypes could

become maladapted. Local populations could respond to climate change by altering genetic composition through gene flow and recombination, which in turn will aid in modifying the structural and functional traits for local adaptation. However, it is unclear if gene flow will be sufficient to respond to the rapid rate of climate change (Aitken, Yeaman, Holliday, Wang, & Curtis-McLane, 2008; Slavov et al., 2004; Xie, Carlson, & Ying, 2012). A combination of comprehensive genomic data and field experiments is required for understanding the genetics of local adaptation (Savolainen, Lascoux & Merila, 2013). There has been some progress in understanding the genetic mechanisms underlying ecologically and economically important traits (Bdeir et al., 2019; Cumbie et al., 2011; Du et al., 2019; Evans et al., 2014; González-Martínez, Huber, Ersoz, Davis, & Neale, 2008; González-Martínez et al., 2007; Ingvarsson, 2010; McKown et al., 2014; Mckown, Klápště, et al., 2014; Muchero et al., 2018, 2015; Neale & Savolainen, 2004; Parchman et al., 2012; Porth et al., 2013; Wang et al., 2018; Wegrzyn et al., 2010; Wei et al., 2014; Zhang et al., 2018), but comprehensive study of morphological, anatomical and physiological traits is still rare in forest trees. We will use an association genetics approach to study key physiological and adaptive traits in *P. trichocarpa*. The complementary aspect of this study is to understand mechanisms underlying local adaptation in *Populus* by employing various differentiation (genome scan), genotype-environment association (GEA) and multivariate methods such as redundancy analysis (RDA) (Capblancq, Luu, Blum, & Bazin, 2018; Duforet-Frebourg, Luu, Laval, Bazin, & Blum, 2016; Forester, Lasky, Wagner, & Urban, 2018; Frichot, Schoville, Bouchard, & François, 2013; Lind et al., 2018; Rellstab et al., 2015).

Better understanding of physical traits (e.g. pulp yield), chemical traits (e.g. cellulose and lignin content), morphological traits [e.g. specific leaf area (SLA) and petiole length], physiological traits (e.g. leaf water potential) as well as anatomical traits (e.g. cell wall thickness and size and distribution of xylem vessels) including their interactions is also of utmost importance to optimize biomass as a major source of bioenergy. Thus far there have been no published studies of the genetic and molecular mechanisms underlying important wood anatomical traits such as xylem vessel size and density. This study aims to provide information for detecting genes responsible for these key anatomical, morphological and physiological traits via an association genetics approach, which may in turn be instrumental in providing a framework for developing *Populus* as a sustainable bioenergy crop. A complementary aspect of this study is to assess the genetic mechanism underlying local adaptation in *Populus*.

1.2 Leaf morphology and function

A wide range of genetic variation has been reported for leaf morphology in *P. trichocarpa* (Gilchrist et al., 2006). Plasticity of leaf traits allows plants to adjust to a wide range of environmental conditions. Trees subjected to drought typically have decreased leaf area, leaf number, and specific leaf area along with decreased photosynthetic rate, transpiration, stomatal conductance (g_s), intercellular CO_2

concentration (c_i), maximum efficiency of PSII (F_v/F_m) and maximum effective quantum yield of PSII (Yield) as reported for some *Populus* species (Xiao, Xu, & Yang, 2008; Xu et al., 2008). *P. trichocarpa* trees in higher latitudes have greater leaf longevity that allows them to compensate for reduced growth and photosynthetic rate (Gornall & Guy, 2007). Trees in high latitudes are also expected to have thicker leaves. Furthermore, the alkene composition of the cuticle has been found to be associated with productivity and water use efficiency in natural *P. trichocarpa* populations (Gonzales-Vigil, Hefer, von Loessl, La Mantia, & Mansfield, 2017). Significant variation in leaf characters among populations has been found in the populations of another important species of the genus, *P. deltoides* in Ontario. Vein numbers in the leaf were shown to have significant correlation with latitude and longitude (Rajora, Zsuffa, & Dancik, 1991). Variation in leaf morphology has great implications for plants in regulating photosynthetic rate, growth and adaptation to a changing climate.

1.3 Stomata

Variation in stomatal size and density in the abaxial and adaxial leaf surface affects stomatal conductance which in turn affects net CO₂ assimilation (A) in plants (Pearce, Millard, Bray, & Rood, 2006). Studies have shown that the stomatal density increases and the size decreases in *P. trichocarpa* and other *Populus* species with an increase in latitude and in xeric conditions (Sparks & Black, 1999). Interestingly, the adaxial leaf stomata in *P. trichocarpa*, although scant in distribution, have shown strong correlation with latitude, thus facilitating higher stomatal conductance and net CO₂ assimilation in the regions with short growing seasons (Gornall & Guy, 2007; McKown et al., 2014). Studies on *P. trichocarpa* and other *Populus* species have shown that more variation in total stomatal densities (abaxial plus adaxial) exists within species than between species. However, abaxial and adaxial stomatal densities and adaxial to abaxial density ratio differed across species suggesting adaptation to different environmental niches (Pearce et al., 2006). Several studies have shown that the regulation of stomatal density and stomatal aperture play a great role in acclimation and adaptation to abiotic stress (Ceulemans, Impens, & Steenackers, 1988; Ceulemans, Praet, & Jiang, 1995). This information can be useful for selecting *Populus* clones suitable for various climatic regions.

1.4 Carbon isotope composition

Carbon isotope composition provides evidence for water use efficiency in plants. There are two ways by which the water use efficiency in plants can be measured. The ratio (A/g_s) of CO₂ assimilation (A) to stomatal conductance (g_s) provides a measure of instantaneous intrinsic water use efficiency (WUE_i) whereas the stable carbon isotope composition ($\delta^{13}\text{C}$) provides a long-term measure of water use efficiency (WUE) in plants and is less prone to short-term environmental fluctuations.

Plants in general have affinity towards the lighter carbon isotope, ¹²C over ¹³C, which is commonly referred to as carbon isotope discrimination. When intercellular CO₂ concentration (c_i) is low,

discrimination decreases and is reflected as a less negative value. The negative value of discrimination is not just due to the intercellular CO₂ concentration inside the leaf. About 27 ppm of the fractionation is due to the affinity of *Rubisco* with ¹²C, and 4 ppm is due to the diffusion properties of the leaf epidermis. The net discrimination, a long-term measure of WUE is calculated as follows (Farquhar, Ehleringer, & Hubick, 1989):

$$\Delta = \delta^{13}C_{air} - \frac{\delta^{13}C_{plant}}{1,000 + \delta^{13}C_{plant}} \times 1,000,$$

where Δ is the net carbon isotope discrimination, and $\delta^{13}C_{air}$ (c_a) and $\delta^{13}C_{plant}$ (c_i) are carbon isotope composition of the source air and the plant, respectively.

More negative values of $\delta^{13}C$ are often associated with lower WUE and higher stomatal conductance (Gornall & Guy, 2007; McCarroll & Loader, 2004). If c_i in the leaf is higher relative to c_a , then it suggests that the stomatal conductance is occurring at a much higher rate than photosynthesis, so that the discrimination against ¹³C is greater, giving low $\delta^{13}C$ values. *P. trichocarpa* clones are known to have strong genetic variation for $\delta^{13}C$ (Gornall & Guy, 2007). Understanding how plants respond to limited water availability measured by net discrimination has important ecological implications.

1.5 Pre-dawn leaf water potential

Predawn leaf water potential (Ψ) is the measure of plant water status which is expected to be in equilibrium with the soil water potential (Fu, Chen, & Li, 2010; Richter, 1997) and represents the energy level of the solution drawn from the soil. The method has been used as a proxy for determining the irrigation timing in crop plants, which is an integrated measure of soil, plant and atmosphere on plant water use. Water potential is a negative number measured in reference to pure water, which has zero water potential. It is measured in bars, where 10 bars is equivalent to 1MPa. A similar measurement, called leaf hydraulic conductance (K_{leaf}), is a measure of the ratio of the flow rate of water to the potential gradient across the leaf and gives an idea of how a plant is responding to the internal and external environment. It is a better measurement than the leaf water potential measurement, but is time consuming (Sack & Holbrook, 2006; Sack & Scoffoni, 2012). Factors such as stomatal conductance and transpiration that affect leaf water potential can have correlations with hydraulic conductance (Sperry, 2000). Drought tolerant species have been shown to have a linear decline in leaf water potential (more negative Ψ value) at 80% loss of K_{leaf} (Scoffoni, McKown, Rawls, & Sack, 2012).

Low water potential in plants can cause stomatal closure and affect photosynthesis. Low water potential in xylem can lead to cavitation affecting the movement of water to the leaves (Sperry, 2000). A study on stem and leaf water potential in *P. euphratica* and *P. russkii* showed variation in moisture retaining capacity between the species (Fu et al., 2010). Another study on *P. tremula* showed a correlation between stomatal conductance and stomatal sensitivity to an increase in leaf water potential (Aasamaa,

Sober, & Rahi, 2001). Under stressed conditions (low water potential) *P. tremula* showed an increased concentration of abscisic acid (ABA) leading to a decrease in stomatal conductance. At water potential lower than -0.7 MPa, *P. balsamifera* showed vulnerability to xylem cavitation (Hacke & Sauter, 1995). *P. trichocarpa* clones have the capability to respond to decreasing leaf water potential by rapidly closing stomata. *P. trichocarpa* populations from high potential evaporation environments have high resistance to drought-induced xylem cavitation (Sparks & Black, 1999).

1.6 Wood anatomy

Wood is primarily comprised of cellulose and lignin, two of the most abundant polymers on earth (Groover, Nieminen, Helariutta, & Mansfield, 2010). The internal anatomy of wood is relatively simple, yet the mechanisms regulating the genetic and molecular basis of wood formation are highly complex. The formation of wood is due to the primary growth that occurs in the early stages of development and the secondary growth that occurs after the primary growth ceases to function (Evert, 2006). Cell walls are a major component of wood and provide rigidity to the cell. The major polymers found in the plant cell wall are cellulose, hemicellulose, pectin and lignin. While lignin is the major component providing mechanical support to the cell and the plant body, it is a major challenge for the enzymatic conversion of polysaccharides to ethanol because of its interference with the enzymatic process (DOE. U.S., 2010).

Anatomically wood is made up of three different xylem tissues in angiosperms – vessels, rays and fibers. Vessels are the main conducting tissues that help in longitudinal transport of water. Rays are physiologically active parenchymatous cells that serve for carbon storage as well as the transport of resources radially between xylem and phloem. Fibers provide the strength. Wood anatomical traits are some of the key functional traits that provide stability and architecture to the tree, provide defense against insects and pathogens, and regulate hydraulic conductivity. Trees with larger vessels generally have low wood density, higher cellulose content and are associated with fast growing ability. In contrast, trees with smaller vessels have high wood density and are associated with slow growth and higher lignin content, but have high survival capability because of hydraulic safety and resistance against damage from herbivores and pathogens. Both size and the number of vessels affect hydraulic conductance in trees (Sperry, Hacke, & Pittermann, 2006). According to the Hagen-Poiseuille law hydraulic conductance of a cylindrical vessel increases with the forth power of the vessel radius (Schuldt, Leuschner, Brock, & Horna, 2013). Trees with larger vessels have higher hydraulic conductance, higher stomatal conductance and more photosynthetic carbon gain (Santiago et al., 2004), but are prone to vessel implosion and cavitation (Hacke, Sperry, Pockman, Davis, & McCulloh, 2001; Hacke, Sperry, Wheeler, & Castro, 2006).

The allocation of resources to different tissue types in the wood depends on abiotic and biotic selection pressures. This would mean a change in the length and density of the vessels, number of ray

cells or the density of fibers that can potentially affect the structural and chemical composition of the wood, ultimately affecting wood recalcitrance and plant performance (Zanne & Falster, 2010).

Manipulating the allocation of resources in poplar wood is important for developing this species as a reliable and sustainable lignocellulosic feedstock for biofuel production.

1.7 Local adaptation in forest trees

Forest trees span over large geographic areas, harbor high genetic and phenotypic variation, and often show latitudinal clines for various adaptive traits (Grattapaglia et al., 2009; Neale & Kremer, 2011; Savolainen et al., 2013; Savolainen & Pyhäjärvi, 2007). Local adaptation is the tendency of plant populations showing better fitness in the native environment compared to the foreign habit. Classical way of testing for local adaptation is by reciprocal experiments where the plants from the two native environments are grown in each other's environment and evaluate the plant fitness. However, experiments like this are time consuming and not feasible, especially for trees. Therefore, recent works in local adaptation in forest trees are focused more on the molecular mechanism underlying local adaptation.

Various methods including differentiation and genotype-environment association (GEA) are widely popular for identifying loci conferring local adaption in forest trees (Rellstab et al., 2015). Differentiation outlier methods (genome scans) allow for detecting loci with strong allele frequency differences among populations without needing environmental or phenotypic data (Duforet-Frebourg et al., 2016; Evans et al., 2014; Günther & Coop, 2013). In contrast GEA methods identify loci based on the associations between genetic and environmental data that are potential drivers of selection (Frichot et al., 2013; Joost et al., 2007; Stucki et al., 2017; Yoder et al., 2014). However, these methods can suffer from high false-positive (De Mita et al., 2013; Meirmans, 2012) and are not powerful enough for detecting recent and weak selection loci (Lind et al., 2018). To overcome these problems, recent studies have used multivariate methods called Redundancy Analysis (RDA) that allow for simultaneously testing the association of loci with the predictor variables (Forester et al., 2018; Legendre & Legendre, 2012). Furthermore, adaptive traits are largely under polygenic control in natural populations (Hollinger, Pennings, & Hermisson, 2019; Pritchard & Di Rienzo, 2010) meaning that selection on adaptive traits might affect hundreds or thousands of loci with a minimum change in allele frequency at a given locus (Le Corre & Kremer, 2012). It is very possible that adaptation is due to covariance among key loci rather than strong differentiation in individual loci, which increase the possibility of small-effect loci remaining undetected from their neutral genetic background (Berg & Coop, 2014; De Villemereuil, Gaggiotti, Mouterde, & Till-Bottraud, 2016; Le Corre & Kremer, 2012; Lind et al., 2018; Rajora, Eckert, & Zinck, 2016). Therefore, local adaptation methods like differentiation (genome scans) and GEA should be complemented with the use of multivariate approaches like RDA to unravel the complexity underlying adaptive genetic polymorphisms (Capblancq et al., 2018; Forester et al., 2018).

With ever growing genomic data and the improvement in various genome scan and other differentiation and GEA methods, determining the molecular signal conferring local adaptation has become increasingly feasible. Several genes controlling cold-hardiness in conifers and genes controlling bud phenology in *Populus* have been identified using association genetics, differentiation, GEA and RDA methods (Capblancq et al., 2018; Eckert et al., 2010; Evans et al., 2014; Fahrenkrog et al., 2017; Vangestel, Eckert, Wegrzyn, St. Clair, & Neale, 2018; Wang et al., 2018). However, whenever possible, an integrated approach that takes into account genomic, environmental and phenotypic data at an appropriate spatial scale should be used for better understanding the mechanism of local adaptation in the face of climate change. A few studies have highlighted the importance of this approach (Eckert et al., 2015; Talbot et al., 2017; Vangestel et al., 2018). Common gardens in general provide a powerful approach for unraveling the genetic basis of quantitative traits in a controlled environment, for testing the interactive effects of environment and genotype on phenotypes, and comparing phenotypic differentiation with neutral population genetic variation. With the increasing availability of genome sequencing data coupled with common garden phenotypic data (Chhetri et al., 2019) and environmental data from the source location of phenotypes, understanding the molecular mechanisms underlying local adaptation in the face of climate change has become more feasible in *Populus*.

1.8 Main objectives

- 1) Understand the genetic mechanisms underlying variation in morphological and physiological traits in *P. trichocarpa*.
- 2) Understand the factors affecting local adaptation in *P. trichocarpa* including the relative contribution of climate and geography variables in population structure.
- 3) Identify candidate genes that can be targeted to enhance productivity and reduce recalcitrance of woody bioenergy feedstocks. Is there an effect of environment in GWAS for leaf morphological traits (compare GWAS for the same traits from two different plantations)?

1.9 Expected significance

Lignocellulosic feedstocks, especially for fast growing trees like *Populus*, are considered to be more reliable and sustainable than starch based feedstocks not only for biofuel production, but also for ecosystem management and carbon balance (DeCicco, 2013; Du et al., 2018; Rubin, 2008; Simmons, Loqué, & Ralph, 2010; Weng, Li, Bonawitz, & Chapple, 2008; Yuan, Tiller, Al-Ahmad, Stewart, & Stewart, 2008). Modification for biomass recalcitrance, increased biomass production, and efficient conversion of biomass into ethanol are the main challenges that need to be overcome before *Populus* trees can be commercialized for biofuel production. In recent years much progress has been made in unraveling the underlying genetic mechanisms of complex traits in *Populus* and other forest trees. However, most prior studies are based on candidate gene or other approaches that ignore a significant part of the genome

that might potentially be affecting the trait. Furthermore, these studies are limited either by the number of traits being studied or by the sample size (limited power). We have started to learn the underlying genetic mechanism that controls the chemical composition in the cell wall in *Populus*, but we do not know anything about the underlying genetic mechanisms regulating the most important anatomical and architectural traits such as vessel and fiber density, ray cell numbers, and cell wall thickness that affect the hydraulic conductivity and carbon storage in trees. Moreover, understanding local adaptation and how these functional and structural traits interact with the environment is very important for selecting the high performing trees for various environmental conditions.

This study is an attempt to provide a comprehensive survey of genetic mechanisms underlying morphological, anatomical and physiological traits that affect overall plant productivity. Unraveling the genetic architecture of complex traits relevant to whole plant productivity including anatomical, morphological and physiological traits would enhance the understanding of the biology of this species. We arguably have the largest forest tree population for genome-wide association studies. Whole genome resequencing data are available for more than 1000 genotypes. A complementary aspect of this study is to assess the local adaptation in *P. trichocarpa*. Understanding the genetic mechanisms underlying local adaptation using genotype-environment association (GEA) and multivariate approaches such as Redundancy Analysis (RDA) would provide a better understanding of polygenic adaptation in *Populus*. Furthermore, understanding the genetic and environmental components driving local adaptation is key for accelerated domestication and ultimately developing *P. trichocarpa* as a bioenergy crop.

The research is expected to identify genes and gene families related to vessel size and density, and other important plant productivity and biofuel related traits. Functional analysis of the detected polymorphisms and genes will allow better understanding of the phenotype. The knowledge thus gained can be transformed to optimize *P. trichocarpa* as a bioenergy feedstock for biomass production and efficient sugar release from lignocellulosic biomass. This knowledge can be transferred to other commercial trees such as *Eucalyptus* and bioenergy crops such as switchgrass and sorghum.

The present study will complement other association studies of complex traits such as bud phenology, bud flush, tree diameter and height in the same population. Extensive data collected for some of the traits such as SLA and stomatal density in more than one common garden will validate the association of a particular trait with the genomic region. This may also provide an avenue for research ultimately leading to developing *Populus* as a sustainable bioenergy crop. Transgenic plants with reduced lignin content, increased cellulose content, increased water use efficiency and increased biomass are possible by overexpressing or knocking out genes responsible for quantitative variation in these traits. Furthermore, understanding the molecular mechanism underlying local adaption would be important for better understanding the biology and sustainable use and management of this species. Finally, the data

generated from this research can be incorporated into a marker-aided breeding program to accelerate the domestication of these crops for biofuels and other end uses.

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Chapter 2. Multitrait genome-wide association analysis of *Populus trichocarpa* identifies key polymorphisms controlling morphological and physiological traits

Note: This chapter is taken directly from my publication:

*“Multitrait genome-wide association analysis of *Populus trichocarpa* identifies key polymorphisms controlling morphological and physiological traits”, New **Phytologist** 2019, 223: 293-309. Published 7 March 2019. DOI: 10.1111/nph.15777.*

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2.1 Summary

- Genome-wide association studies (GWAS) have great promise for identifying the loci that contribute to adaptive variation, but the complex genetic architecture of many quantitative traits presents a substantial challenge.
- We measured 14 morphological and physiological traits and identified SNP-phenotype associations in a *Populus trichocarpa* population distributed from California to British Columbia. We used whole genome re-sequencing data of 882 trees with more than 6.78 million SNPs, coupled with multitrait association to detect polymorphisms with potentially pleiotropic effects. Candidate genes were validated with functional data.
- Broad-sense heritability (H^2) ranged from 0.30 to 0.56 for morphological traits and 0.08 to 0.36 for physiological traits. A total of 4 and 20 gene models were detected using the single trait and multitrait association methods, respectively. Several of these associations were corroborated by additional lines of evidence, including co-expression networks, metabolite analyses, and direct confirmation of gene function through RNAi.
- Multitrait association identified many more significant associations than single trait association, potentially revealing pleiotropic effects of individual genes. This can be particularly useful for challenging physiological traits like water-use efficiency or complex traits like leaf morphology, for which we were able to identify credible candidate genes by combining multitrait association with gene co-expression and co-methylation data.

2.2 Non-technical summary

We used markers from across the entire genome together with combinations of related traits to identify candidate genes that potentially control important adaptive traits in the black cottonwood tree. Several of these genes were corroborated by functional analysis, including a possible transcriptional regulator of drought responses, as well as a gene involved in cell wall composition that affects leaf size. This study provides a framework for direct functional annotation of genes, which remains a major challenge for recalcitrant model organisms like forest trees.

2.3 Introduction

A long-standing question in evolutionary biology is the role of selection in shaping the spatial and temporal patterns of phenotypic variation (Weigel & Nordborg, 2015). In the era of genomics, it is now possible to identify the molecular mechanisms underlying phenotypic variation on the landscape. Owing to their wide geographical distribution and climatic gradients, forest trees are an excellent model system for testing how genetic drift, and selection affect genetic variation within a species (Neale & Savolainen, 2004; Ingvarsson & Street, 2011; Neale & Kremer, 2011; Ingvarsson *et al.*, 2016). Forest trees generally have large effective population sizes, extensive gene flow, high genetic variation, and local adaptation (Neale & Savolainen, 2004; González-Martínez *et al.*, 2006; Ingvarsson & Street, 2011). About 31% of the total land area on earth is occupied by forests, which are of great ecological and economic importance (MacDicken *et al.*, 2016), so understanding the factors affecting variation in traits that are important for environmental adaptation is of utmost importance, particularly in the context of rapidly changing climates (Aitken *et al.*, 2008).

Association genetics has emerged as a major tool for identifying the genomic regions underlying traits of interest (Ingvarsson & Street, 2011). Using natural populations that have undergone many generations of recombination between ancestral haplotypes allows identification of the genomic region affecting a trait at fine scale. Nevertheless, one of the major downsides of association mapping is the requirement of large numbers of loci and individuals (Visscher *et al.*, 2017). With recent advances in sequencing technologies, acquiring genomic data at a whole-genome scale has become much more feasible. Nevertheless, despite the high heritability of many morphological traits, only a small proportion of heritability is explained by single nucleotide polymorphisms (SNPs) in most GWAS analyses, suggesting insufficient statistical power (Solovieff *et al.*, 2013). In fact, power analyses indicate that most association studies in forest trees are orders of magnitude too small to detect the effects of alleles of small effect and low frequency (Visscher *et al.*, 2017), which collectively account for a large fraction of the heritability of complex traits (Boyle *et al.*, 2017).

While increasing sample size of GWAS populations is clearly desirable, it is costly and, in some cases, may not be feasible. Alternatively, approaches that can improve the power of GWAS from the same inputs can be used. One approach is to use gene and pathway-based analysis, where GWAS is performed on a set of SNPs or genes (Kim *et al.*, 2016). Another option is to take a multitrait approach, where GWAS is performed with multiple related traits combined in a multivariate framework. Recently, the latter approach has gained some popularity because it offers substantial increase in power compared to the standard univariate approach (Porter & O'Reilly, 2017). One of the big advantages of multitrait GWAS is that missing information in one of the phenotypes in the multitrait set can be complemented by the other phenotypes (Ritchie *et al.*, 2015). The increased power of multitrait GWAS depends in part on

correlation among traits (Porter & O'Reilly, 2017) and the combination of weak genetic effects across the traits (Casale *et al.*, 2015). Multitrait GWAS also takes advantage of pleiotropic effects of polymorphisms, thereby increasing statistical power even when the traits have low correlation (Broadaway *et al.*, 2016; Hackinger & Zeggini, 2017). Finally, unlike analyses based on principal components, multitrait GWAS effectively captures indirect genetic effects whereby a SNP affects one phenotype through its effects on a functionally-related phenotype (Stephens, 2013; Porter & O'Reilly, 2017).

Here we used the model species *P. trichocarpa* to explore the utility of multitrait GWAS to detect genetic variants controlling adaptive traits. The genus *Populus* has a wide distribution in the northern hemisphere and are dioecious, wind pollinated, and highly heterozygous. *Populus* are also fast growing, easy to propagate, and demonstrate interspecific hybrid vigor, all of which makes the genus a model system with high economic potential for production of forest products and biofuels (Jansson & Douglas, 2007; Rubin, 2008). *P. trichocarpa* is from section Tacamahaca (Eckenwalder, 1996) and is distributed from central California to northern British Columbia (BC). It is the first tree genome to be sequenced (Tuskan *et al.*, 2006) and a tremendous amount of genetic resources are available, including abundant transcriptomes (Sjödin *et al.*, 2009; Geraldine *et al.*, 2011; Zhang *et al.*, 2018b) and re-sequencing data (Slavov *et al.*, 2012; Evans *et al.*, 2014), as well as multiple association populations in replicated plantations (Evans *et al.*, 2014; McKown *et al.*, 2014c; Holliday *et al.*, 2016). From these studies, and others, it has been shown that climate plays a major role in shaping genetic variation and driving selection within this species. We show here that multitrait GWAS is a substantially more powerful approach than single trait GWAS in identifying molecular determinants of quantitative traits, though much remaining heritable variation remains to be identified.

2.4 Materials and methods

2.4.1 Plantation establishment and phenotyping

The *P. trichocarpa* association population consists of 1084 trees collected from natural populations in western Washington, Oregon, California, and BC (Figure 2.1). The trees were clonally propagated from stem cuttings and planted in a common garden in Corvallis, OR in July of 2009 (Evans *et al.*, 2014). The plantation consists of 3 blocks in a completely randomized design and the trees were planted at 2 m x 3 m spacing. The plantation was coppiced in December of 2010 and again in December of 2013. Coppiced plants were allowed to re-sprout and grow for one season, after which they were pruned to a single leader in January of the following year (2012 and 2015, respectively).

In December 2013, 759 trees were sampled for carbon isotope analysis. Wood cores (12 mm) were taken from breast height of the tree and the 2012 growth ring was selected for analysis. Cross section of the wood tissue representing the entire growth ring (about 1.2 to 1.8 mg) representing early, intermediate and

late wood were sampled. The wood samples were oven-dried at 65°C for at least 72 h, weighed and wrapped in a tin capsule before sending to the Appalachian Ecology Lab in Frostburg, Maryland for analysis. Carbon isotope composition ($\delta^{13}\text{C}$) was estimated as follows:

$$\delta^{13}\text{C} = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000,$$

where, R_{sample} and R_{standard} are the $^{13}\text{C}/^{12}\text{C}$ ratios in a sample and standard, respectively.

In July of 2014 leaf characteristics were measured for 1056 trees (one complete block plus a subset of replicates, Table 2.1). The first and second fully expanded leaves (counting from the apex) were collected from a branch receiving full sunlight. One of the leaves was used for measuring petiole length and diameter with a digital caliper and then scanned using a hand-held scanner. Images were analyzed to estimate leaf area, leaf length, leaf width and leaf perimeter using *imageJ* software (Shindelin *et al.*, 2015). Dry weights were determined for the same leaves for estimates of specific leaf area (SLA). Leaf chlorophyll (SPAD) was assessed using a SPAD 502 Plus meter (Spectrum Technologies) with an average of 3 replicate measures on leaf section. Abaxial stomatal density was measured by applying clear nail polish to the broadest part of the leaf close to the midrib. A clear piece of tape was then used to capture an imprint of the epidermal leaf surface. These were mounted on slides and the number of stomata in 1 mm² area in 4 random fields was counted at 400x magnification. Pre-dawn leaf water potential was measured for 964 trees using the cut petiole method (Scholander *et al.*, 2016). Measurements were made on a fully-expanded leaf from the middle of the canopy. Leaves were collected between the hours of 2 AM and 5 AM and a pressure bomb was used in the field to measure the pressure of N₂ gas required to force sap from the cut petiole. Height was measured following the 2015 growing season.

2.4.2 Statistical analyses

All measurements were checked for recording errors and outliers were removed. Data were checked for normality. The phenotypic values were adjusted for any within garden microsite variation using Thin Plate Spline (*tps*) regression using the *Tps* function of the *fields* package in R. Using *tps*-adjusted phenotypic values, broad-sense heritabilities were estimated for all traits using the genotypes with replicate clonal measurements. Variance components were estimated by fitting the model with the *lmer* and *ranef* functions of the *lme4* package in R, with genotype as a random effect, and error estimated from the residuals of the model:

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + \sigma_E^2}$$

Genetic correlation between traits was estimated using Best Linear Unbiased Predictors (BLUPs) from the same model. Using the clonal *tps*-adjusted values, the Pearson correlation was performed using the *cor* function of the *stats* package in R. The *prcomp* function of the *ggbiplot* package in R was used to

estimate the relationships of the phenotypes using linear combinations (Principal Components) of the original phenotypic values. Leaf Water Potential was not included in the PCA due to low heritability.

2.4.3 Genotypic data

Preparation of the genotypic data was as described in Evans *et al.* (2014) and Weighill *et al.* (2018). Briefly, whole-genome re-sequencing was performed for 1053 trees using Illumina genetic analyzers at the DOE Joint Genome Institute. Pairwise relatedness was calculated using GCTA (Yang *et al.*, 2011), taking population structure into account. Trees related more closely than first cousins were removed from the analyses. The remaining 882 individuals were used for all subsequent analyses. A genetic relationship matrix was estimated for the remaining trees using GEMMA, and used as a covariate in GWAS analyses. Principal Components (PCs) of all resequencing data were estimated using *smartpca* from EIGENSOFT v6.1.4 and the first 60 PCs were selected as potential covariates for the association tests. Stepwise regression using the *step* function with default selection criteria (i.e. both backward and forward selection) of the MASS package in R was used for selecting PCs that were significantly associated with each phenotype or group of phenotypes. All significant PCs were used as covariates for GWAS (Supplementary Table S2.1). Finally, SNPs with minor allele frequency ≤ 0.05 and markers with severe departures from Hardy-Weinberg expectations were removed.

2.4.4 Test for association

Association tests were performed using GEMMA (Zhou & Stephens, 2012, 2014). Phenotypic BLUPs, genetic relationship matrix, significant PC axes of the genotypic data and 6,781,211 SNPs (remaining SNPs after filtering with MAF less than 0.05) were used for the association test. Single trait GWAS was run for 14 phenotypes (Table 2.1). The tested model was:

$$y = W\alpha + x\beta + u + \epsilon,$$

where y is an n -vector of phenotypic BLUP values, where n is the number of individuals tested; W is an $n \times c$ matrix of covariates; α is a c -vector of corresponding coefficients, where c is the number of principal coordinate axes used; x is an n -vector of marker genotypes, β is the effect size of the marker, u is an n -vector of random effects that includes a relatedness matrix and ϵ is an n -vector of errors.

2.4.5 Trait selection for multitrait GWAS

Multitrait combinations were created based on genetic correlations among phenotypes as well as hypothesized structural and functional relationships of the traits. The latter can be important even in the absence of genetic correlations (Stephens, 2013). Pairwise genetic correlations were performed (Figure 2.2; Supplementary Table S2.2) and the functional relationships were assessed through relevant literature for the phenotypes before forming 12 multitrait sets (Table 2.2). For example, leaf area, leaf dry weight, leaf length and leaf width were combined to form a multitrait set because these traits are highly intercorrelated and represent the leaf as a structural unit. Likewise, tree height, leaf area and petiole length

were combined because the traits are inter-correlated and all affect interception of photosynthetically active radiation. We also combined traits that did not have high genetic correlations, but presumably had functional relationships. For example, we combined carbon isotope composition, leaf water potential and stomatal density because these traits together provide a broader picture of water-use efficiency (WUE), abiotic stress and gas exchange in plants. Pre-dawn leaf water potential is a measure of water retaining capacity of the plants. Measurement of $\delta^{13}\text{C}$ composition in wood provides a measure of integrated WUE. Higher composition of $\delta^{13}\text{C}$ is related to lower carbon isotope discrimination, which in turn is related to high water use efficiency. The gas exchange process in plant leaves is regulated by stomata in the leaves and the number and density and number of stomata is key for this mechanism. Multitrait association was conducted with GEMMA using the same model as for single trait associations, except y is an $n \times d$ matrix of d phenotypes for n individuals.

2.4.6 Analyses of association results

Determining a significance cutoff is one of the biggest challenges for high-dimension analyses like GWAS (Sham & Purcell, 2014). Here we have chosen a uniformly conservative approach to facilitate comparisons among GWAS methods. We used a P -value cutoff ($\alpha \leq 0.05$) based on the Bonferroni correction criterion of 7.37×10^{-9} and a more liberal P -value cutoff of 1×10^{-7} to identify suggestive associations. These were later cross-referenced to other sources of evidence to highlight robust associations (see below). For the purpose of summarizing the results, significant SNPs within 10 kb of one another were merged and counted as a single significant locus. Gene models that were closest to significant SNPs were identified based on v3 of the *P. trichocarpa* genome. Annotation information was obtained from Phytozome, including expression level in different plant tissues and annotations of putative gene functions (Goodstein *et al.*, 2012). Percentage of variance explained (PVE) by SNPs was estimated using the formula in Shim *et al.* (2015).

2.4.7 Network analysis

To gain further insight into possible biological functions of candidate genes identified by the GWAS analysis, we examined the position of the genes in networks constructed for the same population that was used in this paper. The networks were based on the following: gene co-expression using the JGI Plant Gene Atlas for *P. trichocarpa* (<https://phytozome.jgi.doe.gov>); GWAS of metabolite profiles determined by GC-MS (Tschaplinski *et al.*, 2012), and methylation data for multiple tissues in *P. trichocarpa* (Vining *et al.*, 2012). Details of the underlying data and network construction can be found in Weighill *et al.* (2018). Briefly, candidate genes identified from single and multitrait GWAS were used as a seed to identify subnetworks that were potentially related to the gene. A merged network was created by combining metabolites at False Discovery Rate (FDR) of 0.1, significant GWAS SNPs linked to the

corresponding phenotypes and the co-expression and co-methylation subnetworks. The networks were visualized in Cytoscape version 3.6.1 (Shannon *et al.*, 2003).

2.4.8 Functional analysis of the GAUT9 candidate gene

Detailed functional characterization was conducted for one of the genes highlighted by the multitrait GWAS and network analyses presented here in order to provide experimental validation of the functional roles inferred by our analyses. A 123 bp fragment comprising portions of the coding region and 3'-untranslated region of *Potri.004G111000* (*PtGAUT9.1*) was amplified via PCR from a *P. trichocarpa* cDNA library using the following gene specific primers: *PtGAUT9.1*-F (CACCCCCGGGTTTGGCCTTTAGACGAATTCC) and *PtGAUT9.1*-R (TCTAGAGTGACAATAATGATCGGATCCA). The fragment was cloned into an RNAi cassette and transferred to a binary vector for Agrobacterium-mediated transformation of the *P. deltoides* clone WV94, as previously described (Biswal *et al.*, 2015, 2018a). Measurement of leaf traits was carried out on 10 plants each of 3-month-old WT, empty vector control, and *PdGAUT9.1*-KD lines. RNA isolation and quantitative RT-PCR were performed as previously described (Biswal *et al.*, 2015, 2018a). Briefly, total RNA was isolated using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA). The primers *PdGAUT9.1*-qRT-F (GTGCTTGGCCTCGGATATAA) and *PdGAUT9.1*-qRT-R (GAAACATGAAACCTTGGCTTGA) were used to amplify the target gene, *PdGAUT9.1*. The closely-related *PdGAUT9.2* gene was also evaluated to demonstrate the specificity of down-regulation using the primers qRT-F (GCGGCATCAATGGTGGATTA) and *PdGAUT9.2*-qRT-R (TTCTATTCCTCGCCACTCTCTC).

We also evaluated the impacts of the associated polymorphism on gene expression in the association population using RNAseq data from developing xylem, as described by Zhang *et al.* (2018b). Briefly, normalized gene expression was determined using TopHat2, Cufflinks, and Featurecounts, and normalized via DESeq2, followed by Pearson's correlation with the genotype.

2.5 Results

2.5.1 Physiological and morphological trait variation

Broad-sense heritabilities ranged from 0.297 to 0.561 for morphological traits, and from 0.080 to 0.363 for physiological traits (Table 2.1). Pre-dawn leaf water potential had low broad-sense heritability that was not significantly different from 0 (Table 2.1). Although TPS regression was used to correct for microsite variation, physiological traits like pre-dawn leaf water potential appear to be very sensitive to environmental conditions and the micro-climatic conditions at the time of sampling.

Most morphological traits were highly inter-correlated whereas physiological traits were generally not inter-correlated, which is consistent with expectations due to the high measurement error for the latter (Figure 2.2; Supplementary Table S2.2). We performed Principal Component Analysis (PCA) to

further explore the relationships among traits within the population. The first principal component (PC1) explained more than 47% of the total variation. PC1 and PC2 together explained 61% of the total variation. Morphological traits were positively weighted toward the PC1 axis (Figure 2.3), which also shows a slight negative correlation with the latitude of the provenance ($r=-0.17$, $P\leq 0.001$). Specific leaf area and stomatal density were negatively weighted for PC2, while SPAD was positively weighted along this axis (Figure 2.3; Supplementary Table S2.3). PC2 generally separated the Columbia population from BC and the core subpopulations (Figure 2.3). Most morphological traits were correlated with the latitude of origin (Supplementary Table S2.4).

2.5.2 SNP-trait associations

We conducted single trait GWAS with 6.78 million SNPs for the 14 morphological and physiological traits. We did not identify any SNP that passed Bonferroni correction ($P<7.37\times 10^{-9}$). However, we identified a total of 4 SNPs (Table 2.1; Figure 2.4a) that passed a suggestive association P -value cutoff of 1×10^{-7} . These associated SNPs were within or close to four *P. trichocarpa* gene models (Table 2.3). PVE of significant SNPs ranged from 3.45% to 4.35% (Supplementary Table S2.5), though this is likely to be inflated since it is estimated in the discovery population.

Multitrait GWAS for a total of 12 sets of traits identified 5 SNPs that passed the Bonferroni correction P -value cutoff and a total of 32 SNPs that passed the suggestive association P -value cutoff of 1×10^{-7} (Figure 2.4b; Table 2.4). These SNPs were within or close to 22 *P. trichocarpa* gene models (Table 2.4). PVE of these SNPs ranged from 0.0003% to 4.35% for the individual traits comprising the multitrait set (Supplementary Table S2.5).

To facilitate the presentation, we divided the multitrait association results into the following three categories based on the correspondence of the multitrait and the single trait results: 1) multitrait GWAS with increased power for the same (or nearby) SNP positions as in the single trait GWAS (Figures 2.5a,b; Supplementary Figure S2.1); 2) multitrait GWAS with increased power, but with different genomic positions than the single trait GWAS (Figures 2.5c,d; Supplementary Figures S2.2 & S2.3) multitrait GWAS with reduced power for some loci, but with the same (or nearby) SNP positions as in the single trait GWAS (Figures 2.5e,f; Supplementary Figure S2.3). Each category contained 4 multitrait combinations. QQ plots showed a clear improvement for multitrait association compared to the corresponding single trait association (Supplementary Figures S2.4-S2.6).

To provide further evidence for the involvement of the associated SNPs in trait variation, we integrated our GWAS results with other independent datasets, including leaf metabolite levels, gene expression and tissue-specific methylation. Three genes that were significantly associated with carbon-isotope, leaf area and stomatal density (CI-LA-SD) showed interesting linkages to co-expressed genes and/or metabolites, including a potential regulatory network mediated by YABBY transcription factors,

and a possible regulatory network that is associated with phenolic composition (Figure 2.6; Supplementary Tables S2.6 & S2.7). Similarly, the multitrait associations for leaf shape (leaf area-leaf dry weight-leaf length-leaf width, and specific leaf area-leaf aspect ratio) were linked by association with common candidate genes and revealed two large co-expression networks as well as a group of co-expressed enzymes that affect cell wall characteristics (Figure 2.7).

2.5.3 Direct evidence of the role of *GAUT9* in determining leaf area in *Populus*

One of the genes associated with leaf morphology (LA-LD-LL-LW) was Potri.004G111000, annotated as *galacturonosyltransferase 9 (GAUT9)*. The polymorphism was 1.9 kb downstream of the end of the predicted stop codon (Supplementary Table S2.6). The next closest gene is nearly 33 kb away from the SNP, so Potri.004G111000 is the most likely gene to be impacted by this polymorphism. Consistent with this, the associated polymorphism was significantly correlated with the expression of Potri.004G111000 in developing xylem samples from the association population (Supplementary Figure S2.7a, $r=0.169$, $P<0.001$), but not in fully expanded leaves (Supplementary Figure S2.7b, $r=0.035$, ns). Lack of correlation in leaves is likely due to sampling of the wrong developmental stage, but this requires further investigation.

In the process of studying the role of Potri.004G111000 in the recalcitrance of *P. deltoides* xylem, multiple *P. deltoides* RNAi lines were generated and leaf characteristics of wild type, vector control and three RNAi *PdGAUT9.1*-KD lines were compared (Figure 2.8). Reducing the *GAUT9.1* transcript level by 51-60% (Figure 2.8c) resulted in a 43-66% increased leaf length and leaf width at all developmental time points analyzed in 3-month old greenhouse grown plants (Figures 2.8d-f).

2.6 Discussion

Identification of the genetic underpinnings of adaptive trait variation has been an elusive goal of forest tree research for more than a century (Wheeler *et al.*, 2015). Such efforts have been greatly enhanced in the age of genomics, which potentially enables identification of sequence variants controlling heritable variation. The genus *Populus* has been a focus of much of this effort due to the tremendous investment in genetic and genomic resources in recent years (Jansson & Douglas, 2007). Previous studies have demonstrated that *P. trichocarpa* contains substantial heritable variation that has been shaped by the combined effects of demographic history and selection (Slavov *et al.*, 2012; Evans *et al.*, 2014; Gerald *et al.*, 2014; Holliday *et al.*, 2016). However, studies focused on GWAS of individual complex traits have mostly failed to uncover variants that control a majority of the genetic variation in *P. trichocarpa* (Evans *et al.*, 2014; Gerald *et al.*, 2014; McKown *et al.*, 2014b, 2018), most likely due to a lack of power to detect variants of small effect and/or low allele frequency (Visscher *et al.*, 2017). Here we attempt to compensate for these problems by using a larger GWAS population and by performing multitrait GWAS

coupled with multiple lines-of-evidence to support the roles of marginally-associated loci in the target phenotypes.

2.6.1 Morphological and physiological trait correlations and influence of geography

Largely consistent with previous studies (Evans *et al.*, 2014; McKown *et al.*, 2014a; Holliday *et al.*, 2016), correlations of morphological and physiological traits with latitude in our study suggests that the variation in adaptive traits in *P. trichocarpa* is partly driven by geography. There was a negative correlation of tree height with latitude, indicating that northern provenances grew poorly in our test site. Many leaf traits were also correlated with height as well as latitude, so the functional relationships among these traits cannot be readily discerned. Based on the correlation coefficients, leaves became smaller and thicker with low abaxial stomatal density and high chlorophyll content for trees from higher latitudes. Several other *Populus* studies in common gardens have reported higher nitrogen content, stomatal conductance and photosynthetic assimilation in northern trees (Gornall & Guy, 2007; McKown *et al.*, 2014a; Soolanayakanahally *et al.*, 2015; Elmore *et al.*, 2017; Momayyezi & Guy, 2017). Furthermore, Gornall & Guy (2007) and McKown *et al.* (2014b) found a negative correlation between abaxial stomata density and latitude, but they further indicated that the northern *P. trichocarpa* trees were amphistomatous, with adaxial stomata density increasing with the latitude. Most trees used in our study lacked adaxial stomata, likely reflecting the more southerly distribution of our collection (data not shown).

For the most part we found no clear relationships between wood $\delta^{13}\text{C}$ and leaf traits or latitude of origin. This was unlikely to be due to excessive experimental error because these traits all showed significant broad sense heritability. This is consistent with other published reports for *Populus*. For example, a previous field study of *P. trichocarpa* revealed no correlation between $\delta^{13}\text{C}$ of wood and location of origin for a wide variety of morphological and physiological traits (McKown *et al.*, 2014a). However, in a greenhouse study of *P. trichocarpa*, intrinsic WUE was correlated with photosynthetic assimilation and leaf mass area (Momayyezi & Guy, 2017). Similarly, *P. balsamifera* showed a positive correlation of wood and leaf $\delta^{13}\text{C}$ with latitude in a greenhouse study (Soolanayakanahally *et al.*, 2009) and no correlation with latitude in a field study (Soolanayakanahally *et al.*, 2015). Monclus *et al.* (2009) found a correlation between $\delta^{13}\text{C}$ and productivity traits (fresh biomass, height and circumference) for *P. deltoides* $\times$ *P. trichocarpa* hybrids but no correlation of leaf $\delta^{13}\text{C}$ and productivity for *P. deltoides* $\times$ *P. nigra* hybrids (Monclus *et al.*, 2005). The variability in these results may be due to the effect of environments of the common gardens or variation in the genotypic responses to drought (Soolanayakanahally *et al.*, 2015).

2.6.2 Enhanced power with multitrait GWAS

Despite a relatively large sample size and the use of whole genome SNP data, single trait GWAS for 14 traits revealed only 4 loci with suggestive associations, and collectively these explain only a very small percentage of variance in the phenotypes. In contrast, multitrait GWAS for 12 combinations of a subset of these traits identified 32 SNPs in or near 20 genes. It appears that the overall power of the analysis was moderately improved for most trait combinations compared to the corresponding single trait GWAS. The power of multitrait GWAS depends on multiple factors, including SNP effect size, direction of effect (positive vs. negative), percentage of variance explained (PVE) by the SNP, and trait correlations (Zhou & Stephens, 2014; Porter & O'Reilly, 2017). Additionally, because many of our traits were measured without replication, the multitrait analyses may have provided more accurate estimates of the underlying phenotypes due to covariance of some of the traits. Although we used the presumed functional relationship and the correlations of the traits to form the multitrait sets for this study, we cannot determine the relative impacts of these factors in these specific analyses, though this has been explored elsewhere through simulation studies (Zhou & Stephens, 2014; Porter & O'Reilly, 2017). Nevertheless, we can gain some insights by examining the PVE of the significant SNPs from multitrait GWAS in the corresponding single trait analyses to indirectly infer the contribution of each of the component traits. In the following section we explore this using case studies from each of the three categories defined above.

2.6.3 Category 1

This category includes multitrait GWAS with increased power for the same (or nearby) SNP positions as in the single trait GWAS. The multitrait GWAS that includes carbon isotope, leaf area and stomatal density (CI-LA-SD) well represents this category (Figure 2.5a). The multitrait GWAS detected 4 suggestive SNPs, each of which was in approximately the same position as a non-significant peak from at least one of the corresponding single trait GWASs. Examination of the effect plot for the SNP with the highest significance (Figure 2.5b) suggests that the multitrait GWAS may be capturing a pleiotropic effect in this case, since two of the traits, $\delta^{13}\text{C}$ and stomatal density, both have higher means for homozygotes for the major allele compared to the other genotype classes. A similar pattern is evident for the peaks on Chr01 and Chr02, though in these cases the allelic effects are in opposite configurations for leaf area and stomatal density (Supplementary Figure S2.8), possibly reflecting a weak negative correlation between these traits.

Examination of provisional annotations and direct functional linkages for the genes closest to the associated SNPs provides further insight into the possible mechanisms by which these loci affect these three traits. For example, Potri.002G145100, a putative YABBY-1 related axial regulator, was co-expressed with 12 other genes, including another YABBY-5 transcription factor and a WUSCHEL-related homeobox gene family member, possibly representing a large regulatory network (Figure 2.6).

Another possible regulatory network contained Potri.001G411800, an EF-Hand Calcium Binding Domain protein. In *P. trichocarpa*, Potri.001G411800 has moderate expression in early and late dormant bud, early male and female development, root tip, young, immature and first fully expanded leaves (Goodstein *et al.*, 2012). This gene was co-expressed with 6 other genes, including another EF-hand family protein, Potri.011G129100. Calcium is an important second messenger in eukaryotes and has important roles in cell signaling and response to biotic and abiotic stresses and developmental cues (Sanders *et al.*, 2002; Chen *et al.*, 2015; Ranty *et al.*, 2016; Zhu, 2016; Edel *et al.*, 2017). The EF-hand motif is the most common and highly conserved calcium-binding motif (Lewit-Bentley & Réty, 2000; Zeng *et al.*, 2017).

This co-expression network provides further evidence that Potri.001G411800 is involved in responses to abiotic stress. It is co-expressed with a late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein (Potri.009G158900), a group that has a major role in responses to drought, salinity and, osmotic and temperature related stresses (Gao & Lan, 2016; Magwanga *et al.*, 2018). Potri.001G411800 is also associated with 10 different metabolites in the same population, including several that are related to plant development and stress responses (Figure 2.6). For example, at least five of the metabolites are identified as flavonoids or flavonoid glycosides, including caffeoyl-quercetin glycoside, coumaroyl caffeoyl glycoside, and catechol glycoside. Flavonoids are known to have antioxidant properties that are induced under abiotic and biotic environmental stresses (Hernández *et al.*, 2009). Quercetin glycosides also play an important role in plant growth and development (Parvez *et al.*, 2004). More importantly, they are known to have a role in osmotic adjustment wherein the deleterious effect of water deficit is minimized by the active accumulation of solutes such as glycosides and phenolics as a response to drought (Tschaplinski *et al.*, 2019).

2.6.4 Category 2

This category includes cases in which the multitrait GWAS had increased power, but the associated loci did not overlap with peaks in the single trait GWAS. The multitrait set that includes leaf area, leaf dry weight, leaf length and leaf width (LA-LD-LL-LW) well represents this category (Figure 2.5c). We detected 10 significant SNPs in the multitrait GWAS compared to none in the corresponding single trait GWAS, and the peaks were largely non-overlapping. The locus with the highest association in the multitrait analysis showed similar patterns of genotypic means for all four traits, consistent with an additive effect (Figure 2.5d). Most of the other cases had low minor allele frequency and high variation among phenotypes for homozygotes for the minor allele (Supplementary Figure S2.9; Supplementary Table S2.5). This is likely a case where joint estimation of the variances provided more power to detect differences among the genotypic classes, resulting in significant multitrait associations for loci that showed no association with the individual component traits.

This case study also provides an example of the use of multiple lines of evidence to provide further support for relatively weak associations (Weighill *et al.*, 2018). For example, the SNP Chr04_9996091 had a P -value of 4.72×10^{-8} , which does not pass a strict FDR with $\alpha=0.05$, so we classified this as a “suggestive” association. However, we have multiple lines of evidence that the closest gene model, Potri.004G111000, is involved with leaf development. This gene encodes a putative galacturonosyltransferase that is moderately expressed in the first fully expanded leaf, young and immature leaves, and pre-dormant and fully open vegetative buds (Goodstein *et al.*, 2012). The closest *Arabidopsis thaliana* homolog of this gene, AT3G02350, encodes a gene annotated as galacturonosyltransferase 9 (*GAUT9*), for which enzyme activity has not yet been established. *GAUT9* belongs to the *GAUT* gene family of proven and putative pectin homogalacturonan (HG) galacturonosyltransferases (Sterling *et al.*, 2006; Atmodjo *et al.*, 2013; Biswal *et al.*, 2018b; Voiniciuc *et al.*, 2018). Down-regulation of the *PdGAUT9.1* gene caused increased leaf length and width in both developing and mature leaves of greenhouse-grown *P. deltoides*, confirming the role of the gene in leaf development in *Populus*.

Other *GAUT* genes have also been shown to affect cell wall properties and leaf size in *Populus*. Downregulation of a *GAUT12* homolog in *P. deltoides* showed decreased xylan and pectin content in the cell wall and increased biomass yield (Biswal *et al.*, 2015), while over-expression showed a reduction in overall plant productivity and resulted in smaller leaves, reduced xylem cell numbers and size, and an increase in the amount of xylose and galacturonic acid in the cell wall (Biswal *et al.*, 2018b). Downregulation of *GAUT4* in *P. deltoides* resulted in decreased pectic homogalacturonan and rhamnogalacturonan II and increased plant height, diameter, leaf area, and biomass (Biswal *et al.*, 2018a). Additional evidence supporting increased leaf growth with decreases in pectin is provided by reports of increased expansion of *Arabidopsis* rosette leaves resulting from overexpression of polygalacturonase, an enzyme that degrades pectic homogalacturonan (Rui *et al.*, 2017).

Co-expression analysis lends further support for the involvement of this gene in cell wall biosynthesis. Potri.004G111000 (*GAUT9*) was co-expressed with 17 other gene models and one metabolite that are cell-wall related (Figure 2.7; Supplementary Tables S2.6 & S2.7). For example, Potri.004G123500, is annotated as a Uridine diphosphate (UDP) glycosyltransferase (UGT) superfamily. In *P. trichocarpa*, this gene had moderate expression in pre-, early and late dormant buds, young and immature and first fully expanded leaves, and stem nodes and internodes (Goodstein *et al.*, 2012). Another gene, Potri.010G102300, encodes a xyloglucan endotransglucosylase that is a member of the Glycoside Hydrolase Family 16 and which is also expected to affect cell wall properties (Nishikubo *et al.*, 2011; Yang *et al.*, 2014).

Two other genes with suggestive associations to leaf morphology characteristics were co-expressed with a large number of other genes with potential regulatory functions. Potri.008G144100 is similar to the AAA-ATPase subunit of the 26S proteasome complex, and it was co-expressed with eight other genes with putative roles in protein degradation or synthesis, plus an ATP-dependent caseinolytic protease (Potri.018G018800) potentially involved in lipid processing (Figure 2.7; Supplementary Table S2.7). Another gene associated with leaf morphology, Potri.001G173900, encodes a Tudor-like RNA-binding protein with conserved ENT and Agenet domains. There is emerging evidence that the latter domain may be involved in transcriptional regulation in *Arabidopsis* (Zhang *et al.*, 2018a). This gene was co-expressed with 49 other genes with putative roles in protein degradation or RNA regulation, as well as nine genes with annotations related to carbohydrate metabolism (Supplementary Tables S2.6, S2.7). These two genes are excellent candidates as master regulators of leaf morphology, possibly mediated by cell wall modification.

Drost *et al.* (2015) identified a major QTL peak on Chr10 for leaf width in an interspecific *P. trichocarpa* x *P. deltoides* pseudobackcross family. This peak is in close proximity to one of our GWAS peaks for LA-LD-LL-LW. An ADP-ribosylation factor GTPase (*PtARF1*) was the prime candidate gene in the hybrid family based on eQTL analysis and functional assays (Drost *et al.*, 2015). However, this gene was over 200kb from the closest associated SNP in our study (Potri.010G254700, a leucine-rich repeat transmembrane protein kinase). Furthermore, *PtARF1* did not appear in our networks, indicating that it was not even weakly associated with leaf morphology in our population, and was not co-expressed or co-methylated with any weakly associated genes. This may indicate that different mechanisms control leaf morphology within *P. trichocarpa* compared to interspecific hybrids. However, the hypothesized mechanism for *PtARF1* focuses on its role in vesicle-mediated trafficking of the PIN protein to regulate auxin gradients (Drost *et al.*, 2015), which is broadly consistent with the genes in our network that affect cell wall extensibility and carbohydrate metabolism. Intriguingly, the co-expression network of Potri.001G173900 includes a gene (Potri.017G101100) for which the best homolog in *Arabidopsis* (AT3G02260) is a putative calossin-like protein required for polar auxin transport.

2.6.5 Category 3

This case study includes multitrait GWAS sets that had lower top SNP-trait association signals compared to the corresponding single trait GWAS for some loci. The multigrain GWAS set that included specific leaf area and leaf aspect ratio (SL-AR) well represents this category (Figure 2.5e). We detected four loci with suggestive associations for multitrait GWAS compared to 2 for single trait GWAS for specific leaf area (Figure 2.5e). In the case of locus Chr01_38557469, the association for SL was stronger than that for the AR-SL combination, and that locus explained 3.78% of the variation in SL (Supplementary Table S2.5). In contrast, there was no hint of an association for AR at that locus, possibly

due to high variation in the minor allele homozygous individuals (Figure 2.5f). The loci for which the multitrait association showed the lowest P -value follow a similar pattern to those from Categories 1 and 2, with both traits showing differences among the genotypes (Supplementary Figure S2.10). For the AR-SL multitrait we detected a total of 4 gene models, out of which one gene model overlapped with the single trait GWAS for specific leaf area (Figure 2.7). One of these, Potri.008G014000, belongs to the HSP-20 like chaperones superfamily. This gene is co-expressed with 49 other genes in *P. trichocarpa*, including 22 with putative roles in protein degradation or RNA processing, suggesting that this is another important regulatory network for leaf morphology (Supplementary Table S2.7).

2.6.6 Comparison with previous GWAS studies in *P. trichocarpa*

We compared all genes identified from single trait as well as the multitrait GWAS with the previous GWAS studies in *P. trichocarpa* using 34K *Populus* SNP array data (McKown *et al.*, 2014b,c), but despite the moderate (0.2 to 0.4) heritabilities of most of the comparable traits such as leaf traits, height, chlorophyll content and stomatal density in these studies, none of the 22 genes we identified in our study overlapped with the previous studies. This might be due to the difference in the genotypes and the common garden used for our study, or to higher phenotyping error in the present study. It also likely reflects the more targeted genome sampling in the previous studies, which only assayed 3543 genes that were pre-selected based on annotations and other functional information (Geraldes *et al.*, 2011). Nevertheless, we believe that the whole genome resequencing of 882 trees used in our study allowed the detection of robust genetic variants underlying some phenotypic traits. Some corroboration for these associations was provided by patterns of expression and co-expression, intersection with genetic control of metabolites, and direct confirmation of mutant phenotypes.

2.7 Conclusion

We have presented one of the most comprehensive GWAS studies to date for *P. trichocarpa* in terms of the size of the SNP dataset and the number of genotypes. Taking advantage of the natural variation present in the population and the power of multitrait association, we detected candidate genes that were associated with adaptive morphological and physiological traits. Some of these may represent genes with potentially pleiotropic effects on adaptive traits including leaf morphology, and water use efficiency. These have great potential for further functional characterization and can be a suitable target for breeding programs as they capture functional and structural relationships among the traits that are not apparent with single trait GWAS. Furthermore, the network analysis added an extra layer of information that provided further independent lines of evidence supporting the involvement of these genes in their associated phenotypes and provides clues about possible mechanisms of action. This is an important step in functional annotation, which remains a major challenge for recalcitrant model organisms like forest trees.

2.8 Tables and figures

Table 2.1 Broad-sense heritability and the number of SNP-trait associations for morphological and physiological traits in *P. trichocarpa*.

Trait	H^2 (TPS) ^a	H^{2b}	N ^c	Total trees ^d	SNPs <1×10 ^{-7e}	PCs ^f	Chip_ H^2 (+/- CI) ^g
Morphology							
Height (HT)	0.363	0.320	876	2378 (851)	0	27	1 (+/- 0.002)
Leaf area (LA)	0.344	0.336	794	1056 (262)	0	23	0.793 (+/- 0.265)
Leaf aspect ratio (AR)	0.462	0.477	794	1056 (262)	0	20	0.61 (+/- 0.312)
Leaf dry weight (LD)	0.371	0.360	844	1094 (250)	0	26	0.751 (+/- 0.251)
Leaf length (LL)	0.370	0.360	794	1056 (262)	0	22	0.766 (+/- 0.262)
Leaf perimeter (LP)	0.362	0.351	794	1056 (262)	0	22	0.79 (+/- 0.262)
Leaf width (LW)	0.344	0.346	794	1056 (262)	0	25	0.76 (+/- 0.266)
Petiole diameter (PD)	0.297	0.184	839	1124 (285)	0	20	0.62 (+/- 0.263)
Petiole length (PL)	0.561	0.562	839	1124 (285)	0	23	0.881 (+/- 0.28)
Specific leaf area (SL)	0.371	0.376	784	1010 (226)	2	19	0.746 (+/- 0.257)
Stomatal density (SD)	0.500	0.493	813	1064 (251)	1	16	0.834 (+/- 0.267)
Physiology							
Carbon isotope (CI)	0.363	0.375	681	759 (78)	0	15	0.292 (+/- 0.337)
Leaf water potential (WP)	0.080	0.000	823	964 (141)	0	15	0.322 (+/- 0.319)
SPAD ₂₀₁₄ (SP)	0.310	0.297	839	1124 (285)	1	17	0.566 (+/- 0.331)

^aBroad Sense Heritability with Thin Plate Spline correction (TPS) correction applied to the phenotypic data

^bBroad Sense Heritability without TPS correction.

^cNumber of genotypes.

^dNumber of ramets sampled, with replicates in parentheses

^eSNPs with P -values<1×10⁻⁷ (suggestive significant SNPs)

^fNumber of SNP PC covariates used in multitrait GWAS

^gMean Chip Heritability values for phenotypes with confidence interval (CI)

Note: All broad sense heritability estimates were significantly different from 0 except for WP.

Table 2.2 List of traits for multitrait associations in *P. trichocarpa*.

Trait Combination	Abbreviation	popN ^a	PCs ^b
Carbon isotope, height, leaf area, petiole length	CI_HT_LA_PL	632	14
Carbon isotope, leaf area, stomatal density	CI_LA_SD	603	12
Carbon isotope, leaf area, SPAD, stomatal density	CI_LA_SD_SP	600	12
Carbon isotope, leaf water potential	CI_WP	673	6
Carbon isotope, leaf water potential, stomatal density	CI_SD_WP	638	8
Height, leaf area, petiole length	HT_LA_PL	791	8
Height, petiole diameter, petiole length	HT_PD_PL	839	13
Leaf area, leaf dry weight, leaf length, leaf width	LA_LD_LL_LW	788	14
Leaf area, SPAD, stomatal density	LA_SD_SP	755	14
Leaf aspect ratio, specific leaf area	AR_SL	780	9
Leaf dry weight, petiole diameter, SPAD	LD_PD_SP	831	7
Petiole diameter, petiole length, specific leaf area	PD_PL_SL	781	17

^aNumber of unique genotypes^bNumber of SNP PC covariates used in multitrait GWAS**Table 2.3** Genes identified from *P. trichocarpa* single trait GWAS.

Gene model ^a	Trait	<i>P</i> -value ^b	Functional annotation ^a
Potri.001G371800	Specific leaf area	3.95E-08	NA ^c
Potri.004G111000	Specific leaf area	9.76E-08	galacturonosyltransferase 9
Potri.008G111800	Stomatal density	8.93E-08	18S pre-ribosomal assembly protein gar2-related
Potri.010G098400	SPAD	4.84E-08	Tetratricopeptide repeat (TPR)-like superfamily protein

^aGene models are annotated using v3.1 of the *P. trichocarpa* genome^bSNP *P*-values < 1 × 10⁻⁷^cNA, Not available

Table 2.4 Genes identified from *P. trichocarpa* multitrait GWAS.

Gene model ^a	Trait	<i>P</i> -value ^b	Functional annotation ^a
Potri.001G173900	Leaf area, leaf dry weight, leaf length, leaf width	1.61E-08	Plant tudor-like RNA-binding protein
Potri.001G174300	Leaf area, leaf dry weight, leaf length, leaf width	9.15E-08	NA ^c
Potri.001G189300	Leaf area, leaf dry weight, leaf length, leaf width	3.30E-08	NA
Potri.001G371800	Leaf aspect ratio, specific leaf area	7.96E-08	NA
Potri.001G411800	Carbon isotope, leaf area, stomatal density	2.41E-08	EF-hand calcium-binding domain containing protein
Potri.002G055400	Leaf area, leaf dry weight, leaf length, leaf width	3.28E-08	Phytochrome interacting factor 4
Potri.002G145100	Carbon isotope, leaf area, stomatal density	2.62E-08	Plant-specific transcription factor YABBY family protein
Potri.003G165400	Leaf aspect ratio, specific leaf area	6.62E-08	Gem-like protein 5
Potri.004G111000	Leaf area, leaf dry weight, leaf length, leaf width	4.72E-08	Galacturonosyltransferase 9
Potri.004G153400	Leaf aspect ratio, specific leaf area	6.59E-08	Similar to RAS-related GTP- binding protein
Potri.005G097900	Leaf area, SPAD, stomatal density	4.29E-08	Similar to oxidoreductase; 2OG- Fe(2) oxygenase family protein
Potri.006G132500	Leaf area, leaf dry weight, leaf length, leaf width	1.57E-12	Ribosomal protein L4/L1 family
Potri.006G134200	Carbon isotope, leaf area, SPAD, stomatal density	5.29E-08	Lysine-ketoglutarate reductase/saccharopine dehydrogenase bifunctional enzyme
Potri.008G121700	Carbon isotope, leaf water potential, stomatal density	5.43E-09	NA

Potri.008G144100	Leaf area, leaf dry weight, leaf length, leaf width	5.37E-08	Regulatory particle triple-A ATPase 6A
Potri.009G015500	Carbon isotope, leaf area, stomatal density	8.76E-09	Mitochondrial transcription termination factor family protein
Potri.012G065600	Leaf area, petiole length, height	3.24E-08	Leo1-like family protein
Potri.014G136400	Petiole diameter, petiole length, specific leaf area	2.66E-08	LRR receptor-like serine/threonine-protein kinase RKF3-related
Potri.016G071700	Carbon isotope, leaf area, SPAD, stomatal density	1.36E-08	NA
Potri.019G021600	Leaf area, leaf dry weight, leaf length, leaf width Carbon isotope, height, leaf area, petiole length	8.87E-08 1.56E-08	FtsH extracellular protease family

^a Gene models are annotated using v3.1 of the *P. trichocarpa* genome.

^b SNP *P*-values < 1×10^{-7}

^c NA, Not available

Figure 2.1 Source locations of 882 *P. trichocarpa* genotypes sampled in this study (colored dots). The trees were grown in a common garden in Corvallis, Oregon (black star).

Figure 2.2 Pairwise Pearson genetic correlation of selected morphological and physiological traits (traits with at least 681 genotypes) measured in the *P. trichocarpa* common garden in Corvallis, Oregon. The color spectrum, bright red to bright blue represents highly positive to highly negative correlations and the number represents the correlation values. Best Linear Unbiased Predictor (BLUP) adjusted values were used. *P*-values are provided in Supplementary Table S2.2. SPAD represents leaf greenness. AR, aspect ratio; SLA, specific leaf area.

Figure 2.3 PCA biplot showing the first and second principal components with individual *P. trichocarpa* genotypes (the points) colored by provenance as in Figure 2.1, and relative weightings of the explanatory variables indicated by vectors. BC, British Columbia; OR, Oregon.

Figure 2.4 *P. trichocarpa* SNP-trait association peak counts at 10kb intervals. (a) Single trait GWAS (b) Multitrait GWAS.

Figure 2.5 Manhattan plots comparing GEMMA univariate and multivariate GWAS in *P. trichocarpa*.

The colors of the dots correspond to single trait or multitrait associations. P -values are converted to $-\log_{10}(P\text{-value})$. SNPs above red lines passed Bonferroni correction test ($P < 7.37 \times 10^{-9}$), SNPs above blue lines are considered suggestive associations ($P < 1 \times 10^{-7}$). Only SNPs with $P < 1 \times 10^{-3}$ are plotted. (a) Carbon isotope, stomatal density, and leaf area. (b) Allelic effects of SNP near Potri.009G015500. (c) Leaf area, leaf dry weight, leaf length, leaf width, and leaf area. (d) Allelic effects of SNP near Potri.009G015500. (e) Leaf aspect ratio and specific leaf area. (f) Allelic effects of SNP near Potri.009G015500.

leaf dry weight, leaf length, and leaf width. (d) Allelic effects of SNP near gene Potri.006G132500. (e) Leaf aspect ratio and specific leaf area. (f) Allelic effects of SNP near gene Potri.001G371800. SNPs depicted in Figures b, d, and f are circled in the corresponding Manhattan plots and the error bars in b, d, and f represent $\pm$ standard error for re-scaled phenotypic values.

Figure 2.6 Merged network for carbon isotope, leaf area and stomatal density (CI_LA_SD) in *P. trichocarpa*. Networks of co-expressed genes were based on RNAseq data for 14 tissue types from the Phytozome *Populus* gene atlas. Networks of associated metabolites were based on GWAS for the same population that was used here (Weighill *et al.*, 2018).

Figure 2.8 Effects of *PdGAUT9.1* down-regulation on leaf size in *P. deltoides*. (a) Gene model for *PtGAUT9.1* (*Potri.004G111000*) from *Populus trichocarpa* v3.0 genome. Black boxes indicate the 5' and 3' untranslated regions (UTRs); purple boxes indicate exons and lines indicate introns. The indicated RNAi targeted sequence was 123bp. The sequences used for quantitative RT-PCR are indicated by arrows. (b) Schematic representation of *PtGAUT9.1* RNAi silencing construct used to generate *P. deltoides* *PdGAUT9.1*-KD transgenic lines. (c) Relative transcript abundance of *PdGAUT9.1* (*Potri.004G111000*) and *PdGAUT9.2* (*Potri.017G106800*) as determined by quantitative RT-PCR

analysis of leaf RNA from greenhouse-grown 3-month-old poplar WT, vector control (V. control.1) and *PdGAUT9.1-KD* lines (KH28.1, KH28.3 and KH28.12). Expression of *PdGAUT9.1* in poplar WT was set to 1 and *18S rRNA* was used as a reference gene. Error bars are SE, n = 6. Differences were tested by one-way ANOVA ($P < 0.05$; $P < 0.001$). (d) Leaf phenotype (the sixth leaf from the apex) of *P. deltoides* control (WT and VC) and *PdGAUT9.1-KD* line (KH28.12) from 3-month-old plants. (e) Length and (f) width of leaves from different developmental stages of three different 3-month-old *GAUT9-KD* transgenic lines (KH28.1, KH28.3 and KH28.12). Every other leaf of ten plants was measured starting with the 2nd leaf from the apex. The error bars represent $\pm$ standard error for leaf length and leaf width.

2.9 Supplementary tables and figures

Supplementary Table S 2.1 SNP PC covariates used in *P. trichocarpa* single and multitrait GWAS analyses.

	Trait	SNP PC covariates*	SNP PC count
Single-trait	Height (HT)	1,3-7,10,12-13,16-18,20-22,26-28,31,35-36,38,42,44,49,51,57	27
	Leaf area (LA)	1,3-7,10-13,16,20,22,28,32,38,44-45,47,49,50,57,60	23
	Leaf aspect ratio (AR)	2-7,9,13,21-22,34-35,41-43,47,49-50,57-58	20
	Leaf dry weight (LD)	1,3,4,6-7,9,10-13,16,19-22,32,36,38,42-45,47,49,51,57	26
	Leaf length (LL)	1,3-7,10,12-13,15-17,20,22,28,32,38,44,46-47,51,60	22
	Leaf perimeter (LP)	1,3-7,10,12,13,17,20,22,25,28,32,38,44,46-47,49,57,60	22
	Leaf width (LW)	1-4,6-7,9-13,16,20-22,28,32,38,41,43,47,49,50,57,60	25
	Petiole diameter (PD)	1-4,6-7,11-13,18,22,28,32,36,43,46-47,51,57,60	20
	Petiole length (PL)	1-7,11-13,15-17,22,27,32,40,46-47,50-51,54,57	23
	Specific leaf area (SL)	1-3,5,7,10-11,13,16,19,24,36,40-43,50,55,59	19
	Stomatal density (SD)	1-2,5-6,13,18,22,24,34,39,46,48,50,52,57-58	16
	Carbon isotope (CI)	2-3,6-8,15-16,21-22,28,31,47,48,53,59	15
	Leaf water potential (WP)	1,3-4,7,9,12,17,20,22,28,33-34,47,49,52	15
	SPAD ₂₀₁₄ (SP)	1,4,6-7,15,19,21,24,29-30,32,40,42-43,52,54,60	17
Multi-trait	Carbon isotope, height, leaf area, petiole length (CI_HT_LA_PL)	1-7,12-13,22,25,38,47,57	14
	Carbon isotope, leaf area, stomatal density (CI_LA_SD)	3-5,7,12-13,18,25,38,39,47,57	12
	Carbon isotope, leaf area, SPAD, stomatal density (CI_LA_SD_SP)	3-5,7,12-13,18,25,38,39,47,57	12
	Carbon isotope, leaf water potential (CI_WP)	1-2,7,8,15,28	6
	Carbon isotope, leaf water potential, stomatal density (CI_SD_WP)	1-3,4,6,18,22,57	8
	Height, leaf area, petiole length (HT_LA_PL)	1,3-6,18,22,57	8
	Height, petiole diameter, petiole length (HT_PD_PL)	1,3-6,7,11-13,16,22,32,47	13
	Leaf area, leaf dry weight, leaf length, leaf width (LA_LD_LL_LW)	1,3-4,6-7,12-13,22,32,38,44,47,49,57	14
	Leaf area, SPAD, stomatal density (LA_SD_SP)	3-5,7,12-13,18,32,38,47,49-50,57,60	14
	Leaf aspect ratio, specific leaf area (AR_SL)	1,3,5,7,10-11,36,40,59	9
	Leaf dry weight, petiole diameter, SPAD (LD_PD_SP)	1,6-7,29,32,43,60	7
	Petiole diameter, petiole length, specific leaf area (PD_PL_SL)	1-7,10,13,17,22,27,32,36,42,47,50	17
* PC covariates were selected based on the stepwise regression using both forward and backward selection methods			

Supplementary Table S 2.2 Pearson pairwise correlation of morphological and physiological traits collected in *P. trichocarpa* association population; the numbers below diagonal represent correlation values; the numbers above diagonal represent *P*-values. Red and blue colors indicate positive and negative correlations or *P*-values, respectively.

Trait type	Traits	Morphology										Physiology					Traits	Trait type
		Height (HT)	Leaf area (LA)	Leaf aspect ratio (AR)	Leaf dry weight (LD)	Leaf length (LL)	Leaf perimeter (LP)	Leaf width (LW)	Petiole diameter (PD)	Petiole length (PL)	Specific leaf area (SL)	Stomatal density (SD)	Carbon isotope (CI)	Leaf water potential (WP)	SPAD (SP)			
Morphology	Height (HT)	NA	0	0	0	0	0	0	0	0	0	0.782	0	0.079	Height (HT)	Morphology		
	Leaf area (LA)	0.489	NA	0	0	0	0	0	0	0.681	0	0.285	0	0	Leaf area (LA)			
	Leaf aspect ratio (AR)	-0.356	-0.443	NA	0	0.001	0	0	0	0.004	0.252	0.368	0.002	0.274	Leaf aspect ratio (AR)			
	Leaf dry weight (LD)	0.407	0.91	-0.399	NA	0	0	0	0	0	0	0.087	0	0	Leaf dry weight (LD)			
	Leaf length (LL)	0.437	0.904	-0.114	0.821	NA	0	0	0	0.399	0	0.606	0	0	Leaf length (LL)			
	Leaf perimeter (LP)	0.493	0.963	-0.342	0.871	0.952	NA	0	0	0.942	0	0.41	0	0	Leaf perimeter (LP)			
	Leaf width (LW)	0.513	0.944	-0.695	0.857	0.754	0.883	NA	0	0.455	0	0.215	0	0	Leaf width (LW)			
	Petiole diameter (PD)	0.417	0.778	-0.402	0.769	0.719	0.764	0.764	NA	0	0.001	0.963	0	0	Petiole diameter (PD)			
	Petiole length (PL)	0.417	0.691	-0.401	0.642	0.623	0.684	0.689	0.589	NA	0.623	0	0.219	0	0		Petiole length (PL)	
	Specific leaf area (SL)	0.14	-0.014	-0.098	-0.318	-0.029	0.002	0.025	-0.116	0.017	NA	0	0.695	0.564	0		Specific leaf area (SL)	
Physiology	Stomatal density (SD)	-0.124	-0.22	-0.039	-0.293	-0.271	-0.231	-0.159	-0.219	-0.128	0.241	NA	0	0.176	0	Stomatal density (SD)	Physiology	
	Carbon isotope (CI)	-0.009	-0.036	0.03	-0.058	-0.017	-0.028	-0.042	0.002	-0.042	0.013	-0.12	NA	0.377	0.116	Carbon isotope (CI)		
	Leaf water potential (WP)	0.175	0.164	-0.103	0.153	0.128	0.155	0.161	0.141	0.142	0.02	-0.05	-0.03	NA	0.931	Leaf water potential (WP)		
	SPAD (SP)	0.059	0.252	-0.037	0.378	0.291	0.262	0.217	0.322	0.163	-0.436	-0.23	0.053	0.003	NA	SPAD ₂₀₁₄ (SP)		
Note: NA = Not applicable																		

Note: NA = Not applicable

Supplementary Table S 2.3 PCA loadings of the traits of 13 morphological and physiological traits used in PCAbiplot (Figure 2.3) collected in *P. trichocarpa* association plantation in Corvallis, OR. Red and blue colors indicate positive and negative loadings, respectively. The first five PCs explain more than 83% of the variation in the traits. PC1, PC2, PC3, PC4 and PC5 explain 47%, 14%, 9%, 8% and 6% of the total variation, respectively.

Traits	PC1	PC2	PC3	PC4	PC5
Carbon isotope	-0.011	0.103	0.512	-0.737	0.390
Height	0.207	-0.213	0.211	-0.123	-0.536
Leaf AR	-0.171	0.358	0.471	0.516	0.184
Leaf area	0.392	-0.058	0.036	0.064	0.093
Leaf dry weight	0.372	0.148	-0.126	0.010	0.031
Leaf length	0.353	0.088	0.275	0.293	0.167
Leaf Perimeter	0.383	-0.021	0.139	0.153	0.123
Leaf width	0.375	-0.174	-0.138	-0.133	0.005
Petiole diameter	0.335	0.041	-0.027	-0.035	0.088
Petiole length	0.287	-0.108	-0.033	0.033	0.085
SLA	-0.053	-0.571	0.392	0.111	0.114
SPAD	0.113	0.513	-0.233	-0.138	0.175
Stomatal density	-0.118	-0.387	-0.362	0.101	0.645

Supplementary Table S 2.4 Pearson correlation of morphological and physiological traits with latitude of origin in *P. trichocarpa*.

Traits	Correlation with latitude (r)	P-value
Height (HT)	-0.292	<0.001*
Petiole length (PL)	-0.082	0.017*
Petiole diameter (PD)	-0.001	0.976
Leaf area (LA)	-0.092	0.009*
Leaf perimeter (LP)	-0.086	0.016*
Leaf length (LL)	-0.026	0.458
Leaf width (LW)	-0.125	<0.001*
Leaf aspect ratio (AR)	0.147	<0.001*
Leaf dry weight (LD)	0.047	0.175
Specific leaf area (SL)	-0.256	<0.001*
SPAD (SP)	0.176	<0.001*
Stomatal density (SD)	-0.175	<0.001*
Leaf water potential (WP)	-0.058	0.096
Carbon isotope (CI)	0.026	0.504
PC1 of all traits except leaf feret and leaf water potential	-0.171	<0.001*
PC2 of all traits except leaf feret and leaf water potential	0.409	<0.001*
*Significant P-values		

Note: Supplementary Tables S2.5, S2.6 and S2.7 are too large to fit here, but are available online at this link: <https://nph.onlinelibrary.wiley.com/doi/10.1111/nph.15777>.

Supplementary Figure S 2.1 Manhattan plots comparing GEMMA univariate and multivariate GWAS in *P. trichocarpa*. The colors of the dots correspond to single trait or multitrait (purple) associations. P -values are converted to $-\log_{10}(P\text{-value})$. SNPs above red lines passed Bonferroni correction test ($P < 7.37 \times 10^{-9}$), SNPs above blue lines are considered suggestive associations ($P < 1 \times 10^{-7}$). Only SNPs with $P < 1 \times 10^{-3}$ are plotted. (a) Stomatal density (green), carbon isotope (red), and pre-dawn leaf water potential (blue). (b) Height (green), leaf area (blue), and petiole length (red). (c) Height (green), petiole diameter (blue), and petiole length (red).

Supplementary Figure S 2.2 Manhattan plots comparing GEMMA univariate and multivariate GWAS in *P. trichocarpa*. The colors of the dots correspond to single trait or multitrait (purple) associations. P -values are converted to $-\log_{10}(P\text{-value})$. SNPs above blue lines are considered suggestive associations ($P < 1 \times 10^{-7}$). Only SNPs with $P < 1 \times 10^{-3}$ are plotted. (a) Leaf area (blue), stomatal density (green), and SPAD (red). (b) Leaf area (blue), petiole length (green), height (yellow), and carbon isotope (red). (c) Leaf area (blue), stomatal density (green), SPAD (yellow), and carbon isotope (red).

Supplementary Figure S 2.3 Manhattan plots comparing GEMMA univariate and multivariate GWAS in *P. trichocarpa*. The colors of the dots correspond to single trait or multitrait (purple) associations. P -values are converted to $-\log_{10}(P\text{-value})$. SNPs above blue lines are considered suggestive associations ($P < 1 \times 10^{-7}$). Only SNPs with $P < 1 \times 10^{-3}$ are plotted. (a) Carbon isotope (red), pre-dawn leaf water potential (blue). (b) Leaf dry weight (red), petiole diameter (green), SPAD (blue). (c) Petiole diameter (green), petiole length (red), SLA (blue).

Supplementary Figure S 2.4 QQ-plot for single trait GWAS in *P. trichocarpa* for carbon isotope composition (a), leaf area (b), and stomatal density (c) and the corresponding multitrait GWAS with all 3 traits (d). For corresponding Manhattan Plots, see Figure 2.5a.

Supplementary Figure S 2.5 QQ-plot for single trait GWAS in *P. trichocarpa* for leaf area (a), leaf dry weight (b), leaf length (c) and leaf width (d) and the corresponding multitrait GWAS with all 4 leaf traits (e). See Figure 2.5c for the corresponding Manhattan plots.

Supplementary Figure S 2.6 QQ-plot for single trait GWAS in *P. trichocarpa* for leaf aspect ratio (a) and specific leaf area (b), and the corresponding multitrait GWAS with both traits (c). See Figure 2.5e for the corresponding Manhattan plots.

Supplementary Figure S 2.7 Pearson correlation of *GAUT9* (Potri.004G111000) gene expression in (a) leaf and (b) developing xylem of *P. trichocarpa* with genotype at locus Chr04_9996091. Big boxes represent interquartile range with black horizontal bar within the box representing median expression

value. The lower and upper whiskers represent 1.5 times the interquartile range, and the circles represent outliers.

Supplementary Figure S 2.8 Allelic effects plots for single traits underlying the carbon isotope-leaf area-stomatal density multitrait association analysis in *P. trichocarapa*. Locus position is indicated above the plot. The error bars represent standard errors for re-scaled phenotypic values. (a) Locus Chr01_43644555; (b) Locus Chr01_43644561; (c) Locus Chr02_10821727; (d) Locus Chr02_10821794.

Supplementary Figure S 2.9 Allelic effects plots for single traits underlying the LA_LD_LL_LW association analysis in *P. trichocarpa*. Locus position is indicated above the plot. Numbers in parentheses below the plot are the number of observations of each genotype. Error bars represent standard errors for re-scaled phenotypic values. LA, leaf area; LD, leaf dry weight; LL, leaf length; LW, leaf width. (a) Locus Chr01_14742176; (b) Locus Chr01_14769998; (c) Locus Chr02_3703017; (d) Locus Chr04_9996091; (e) Locus Chr06_10894444; (f) Locus Chr06_10894482; (g) Locus Chr08_9674908; (h) Locus Chr10_16268015; (i) Locus Chr10_16268028; (j) Locus Chr19_2437823.

Supplementary Figure S 2.10 Allelic effects plots for single traits underlying the AR_SLA association analysis in *P. trichocarpa*. Locus position is indicated above the plot. Numbers in parentheses below the plot are the number of observations of each genotype. Error bars represent standard errors for re-scaled phenotypic values. AR, aspect ratio; SLA, specific leaf area. (a) Locus Chr01_38557469; (b) Locus Chr03_17588967; (c) Locus Chr03_17588972; (d) Locus Chr08_752568.

2.10 References

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Chapter 3. Local adaptation in *Populus trichocarpa*

3.1 Abstract

Unraveling the complex patterns of adaptation in forest ecosystems by exploiting the association between genetic and environmental heterogeneity across populations is of increasing interest. Clear understanding of local adaptation can help in effective optimization of productivity and stress tolerance traits, sustainable use and management of forest resources, conservation strategies and domestication of commercial species. Here we integrated genomic, geoclimatic and phenotypic data and used three conceptually different methods for testing the evidence for local adaptation in *Populus trichocarpa*: (1) correlation of phenotypic traits with geoclimatic variables; (2) genotype-environment association (GEA) analysis; and (3) a multivariate ordination method called redundancy analysis (RDA). We identified genomic outlier loci and underscored the relative contribution of climate and geography on neutral population structure and the relative contribution of climate, geography and genomic data (SNPs) on adaptive traits. Out of 67 genes identified based on outlier loci in GEA, 32 of them were shared with the genes identified from climate and phenotypic RDA models. Climate and geography matrices individually explained 2.57% and 5.66% of the variation, respectively in the SNP eigenvector matrix that defines neutral population structure in the climate RDA model. Similarly, individual SNP eigenvector, climate and geography predictor variable matrices explained 21.66%, 12.31% and 16.63% of the variance in the adaptive trait matrix in the phenotypic RDA model. Based on these approaches we identified multiple loci conferring local adaptation including anthocyanin and fatty acid pathways related genes that are involved in biotic and abiotic stress tolerance, defense and growth and development mechanisms in plants. This study provides invaluable insights on the power of using multivariate methods in integrating genomic, geoclimatic and phenotypic data to unravel the genetic mechanisms underlying local adaptation in *P. trichocarpa*. The findings from this study can be useful for developing conservation strategies and management of forest resources and complex trait optimization for commercial use.

3.2 Introduction

Understanding the complex patterns of adaptation in forest ecosystems is of increasing interest (Bragg, Supple, Andrew, & Borevitz, 2015; Rellstab, Gugerli, Eckert, Hancock, & Holderegger, 2015; Sork et al., 2013). Natural selection has a great role in shaping the patterns of genetic diversity and adaptation. Genetic differentiation leading to local adaptation depends on the strength of selection, biotic and abiotic factors, the relative roles of selection and gene flow, demographic history, and spatial and environmental variation. Knowledge of spatial distribution of adaptive genetic variation and its influence on morphological, phenological and physiological traits is crucial in the face of climate change (Neale & Kremer, 2011). Clear understanding of local adaptation can help in effective optimization of productivity

and stress tolerance traits, sustainable use and management of forest resources, conservation strategies and domestication of commercial species. Forest ecosystems are at risk and long-lived immobile organisms like trees respond much slower to the changing climate regime and may suffer adaptational lag (Aitken, Yeaman, Holliday, Wang, & Curtis-McLane, 2008). However, the mechanisms of local adaptation are not very well understood.

Forest trees are excellent model systems for studying local adaptation. Forest trees have long generation times, harbor high genetic and phenotypic variation, and often cover a large range of environments and show geographic clines for adaptive traits (Neale & Kremer, 2011; Savolainen, Lascoux, & Merilä, 2013). Spatial patterns of genetic variation are affected by the interaction among habitat heterogeneity (geographic, edaphic and environmental), migration and selection (Richardson, Urban, Bolnick, & Skelly, 2014). Fitness related traits are affected by the shift in allele frequencies due to local selective forces, which can disrupt local adaptation (Vangestel, Eckert, Wegrzyn, St. Clair, & Neale, 2018). Despite the belief that the gene flow homogenizes the populations by swamping the adaptive alleles, there is evidence that forest trees exhibit local adaptation at the fine spatial scales (Eckert et al., 2015; Slavov et al., 2009; Slavov & Zhelev, 2010). Local adaptation with gene flow is particularly effective when selection is strong, and the environment is heterogeneous (Lind et al., 2017).

Local adaptation is the tendency of plant populations to show higher fitness in their native environment compared to a foreign habitat. Various methods are in use for understanding the mechanisms of local adaptation: 1) common garden experiments, especially reciprocal transplant experiments that test for the performance of all accessions in all of the sampled home environments; 2) Phenotype-environment correlations; 3) Genetic differentiation (outlier detection) methods that solely require genomic data at the population level; 4) GEA methods that consider associations between genomic and environmental data; and 5) Multivariate ordination methods that simultaneously test for the association of loci with predictor variables. Reciprocal transplant experiments are the classical way for testing whether the genotypes do better in their native environments compared to foreign environments. Furthermore, common gardens in general are useful for unraveling the genetic basis of quantitative traits, for testing the effects of the environment on genotypes and comparing phenotypic differentiation with neutral population genetic variation. However, recent work on local adaptation is more focused on genetic differentiation and GEA methods for identifying the loci conferring local adaptation (Lind, Menon, Bolte, Faske, & Eckert, 2018).

Outlier detection methods mainly differ in their computational efficiency and the type of data they can use (reviewed in Capblancq, Luu, Blum, & Bazin, 2018; Forester, Lasky, Wagner, & Urban, 2018; Lind et al., 2018). The accuracy of these methods depends on the sample size, the nature of the data and the model used to correct for spatial variation (Lotterhos & Whitlock, 2015). The main difficulty is to distinguish true selection signatures from false positives due to population structure and demographic

history such as population expansion, population bottleneck and isolation by distance (Jensen, Kim, DuMont, Aquadro, & Bustamante, 2005; Meirmans, 2012). Moreover, it should be noted that there is no universal method that works for all sample sizes and data types. Differentiation outlier methods (genome scans) allow for detecting loci with strong allele frequency differences among populations without needing environmental or phenotypic data (Evans et al., 2014), although there is a discordance among results of different gene scan methods. In contrast, GEA methods identify loci based on the associations between genetic and environmental data that are potential drivers of selection. This allows for individual level sampling in contrast to the population level sampling in the differentiation methods, thereby providing an explicit link between the genomic loci and predictor variable (for e.g. environmental variable). However, these methods can suffer from high false-positive rates if not correctly controlled for multiple testing (De Mita et al., 2013; Meirmans, 2012). Univariate mixed-effect models that test for the association of one locus at a time with the predictor variables are the most popular methods in this class (Frichot, Schoville, Bouchard, & François, 2013; Joost et al., 2007; Stucki et al., 2017).

Recently, GEA and related methods have gained popularity in the field of landscape genomics. The main goal of landscape genomics is to use genomic data to determine the relative effects of demographic history, environmental heterogeneity, selection and migration on adaptive evolutionary processes (Sork et al., 2013; Storfer, Patton, & Fraik, 2018). However, most of the current landscape genomic studies have limited power, largely due to limited sample size. Because of this, there is not much congruence across studies in terms of the adaptive loci identified in forest trees (Ćalić, Bussotti, Martínez-García, & Neale, 2016). Such discrepancies across studies could reflect parallel evolution (Geraldes et al., 2014), but robust conclusions are limited due to the low power for detecting adaptive loci in the studies conducted thus far (Ćalić et al., 2016). In addition to large sample sizes, robust landscape genomics inferences require homogeneous sampling from across the range of the species distribution and refined computationally efficient methods. Furthermore, combining common garden studies with landscape genomics is a better approach for validation of candidate genes and increased power of detection (Bragg et al., 2015; De Villemereuil, Gaggiotti, Mouterde, & Till-Bottraud, 2016; Flanagan, Forester, Latch, Aitken, & Hoban, 2018; Prunier, Verta, & Mackay, 2016). Power and inference can also be improved by integrating genotype, phenotype and environment data. Furthermore, multivariate approaches that analyze many loci simultaneously may provide better estimation of the correlations with predictor variables. These methods are effective in identifying multilocus selection outliers as they test for groups of markers covarying in response to environmental predictors. Furthermore, whenever possible, integrated approaches that take into account of genomic, environmental and phenotypic data from the same population sample are most desirable (Eckert et al., 2015; Talbot et al., 2017; Vangestel et al., 2018).

Recently, the ordination method known as Redundancy Analysis (RDA) has gained popularity for analyzing multivariate genomic, geoclimatic and phenotypic data (Brauer, Hammer, & Beheregaray, 2016; Capblancq et al., 2018; Forester, Jones, Joost, Landguth, & Lasky, 2016; Forester et al., 2018; Gugger, Liang, Sork, Hodgskiss, & Wright, 2018; Hofmeister, Werner, & Lovette, 2019; Lasky et al., 2012; Meirmans, 2015; Meirmans, Godbout, Lamothe, Thompson, & Isabel, 2017; Rellstab et al., 2015; Sork et al., 2016; Talbot et al., 2017). RDA is widely used in community ecological studies, but its application in the field of landscape genomics is relatively recent, though it is probably the best method for integrating all of this information (Eckert et al., 2015; Vangestel et al., 2018). RDA is a family of ordination methods that allow for the representation of complex biological information in a reduced number of dimensions. Indirect ordination methods like PCA are used for reducing the dimensionality of a set of data (Cavalli-Sforza, 1996), but they do not explicitly take predictors into account. Direct ordination methods like RDA account for predictor variables and are effective at detecting multilocus selection by testing how a group of SNPs covary in response to environmental predictors (Rellstab et al., 2015). RDA uses constrained ordination to model a set of predictor variables such that it restricts the decomposed orthogonal response matrix to the combinations of predictors (Jombart, Pontier, & Dufour, 2009; Legendre & Legendre, 2012) and uses unconstrained ordination axes to model the response data (e.g. genetic variation). RDA allows for the detection of weak multilocus selection signals while controlling for false positives (Le Corre & Kremer, 2012; Savolainen et al., 2013; Tiffin & Ross-Ibarra, 2014). It not only allows detection of loci under selection but also allows for partitioning the variance in the response matrix into predictor variables (redundant variance). RDA provides greater statistical power than PCA based genome scan methods (Capblancq et al., 2018), outlier differentiation methods, GEA methods and other multivariate methods such as random forests, and works well for a range of sample sizes, selection levels, demographic histories and sampling designs (Forester et al., 2018). An advantage of outlier analysis in RDA is it allows identification of loci correlated with the multivariate environmental gradients experienced by plants that may be important to local adaptation, as opposed to testing climate variables individually (Hancock et al., 2011).

With ever growing genomic data and the improvement in genome scan and other differentiation and GEA methods, it is increasingly feasible to determine the loci conferring local adaptation. Several genes controlling cold-hardiness in conifers have been identified using association genetics, differentiation and GEA methods (Eckert et al., 2010; Evans et al., 2014; Vangestel et al., 2018). Some studies have also taken advantage of common garden studies where outlier loci identified from genome scan methods have been shown to control physiological and phenological traits (Capblancq et al., 2018; Evans et al., 2014; Gonzales-Vigil, Hefer, von Loessl, La Mantia, & Mansfield, 2017). Hundreds of genomic regions showing evidence of recent positive and/or divergent selection and enrichment for

associations with adaptive traits that displayed patterns consistent with natural selection have been identified in *Populus trichocarpa* (Evans et al., 2014; Geraldles et al., 2014; Holliday, Zhou, Bawa, Zhang, & Oubida, 2016; Zhou, Bawa, & Holliday, 2014). The generally low LD in forest trees warrants the use of high density marker data to detect genetic variants underlying local adaptation (Neale & Ingvarsson, 2008).

Here we used resequencing data from 869 unrelated trees to understand the genetic mechanisms underlying local adaptation in *P. trichocarpa*. We take advantage of a common garden in Corvallis, OR to better understand the relative effects of environment and genotype on adaptive traits. Both climate and ecologically important traits vary extensively across the range of *P. trichocarpa* and some variation likely represents local adaptation (adaptation to climate). We built upon the previous population genomics study of *P. trichocarpa* (Evans et al., 2014) with a larger data set and sophisticated statistical methods. We used three conceptually different methods for testing for evidence of local adaptation. First, we tested for the correlation of phenotypic traits with climate variables of the source locations. Second, we used univariate, multivariate and PC-based GEA to identify loci potentially conferring local adaptation. Third, we used RDA to answer three major questions: 1) What is the relative contribution of climate and geography in explaining neutral population genetic structure?; 2) What is the relative contribution of genomic, climate and geographic predictors in explaining the variation in adaptive traits?; and 3) Which predictor variables significantly explain the variation in the genomic matrix and the adaptive trait matrix in the RDA models? Finally, we used this analysis to highlight outlier loci that potentially confer local adaptation in *P. trichocarpa*.

3.3 Methods

3.3.1 Plantation establishment and sampling

The *P. trichocarpa* association population consists of 1100 trees collected from natural populations in western Washington, Oregon, British Columbia, and California. The trees were clonally propagated from stem cuttings and planted in a common garden in Corvallis, Oregon in July of 2009 (Evans et al., 2014). The plantation consists of three blocks in a completely randomized design, and the trees were planted at 2 m x 3 m spacing. The plantation was coppiced in December of 2010 and again in December of 2013. Coppiced plants were allowed to re-sprout and grown for one season, after which they were pruned to a single leader in January of the following year (2012 and 2015, respectively). All 10 phenotypic traits [bud set, bud flush, carbon isotope, height, leaf area, leaf aspect ratio, petiole length and diameter, specific leaf area (SLA), chlorophyll content (SPAD) and stomatal density] reported here were measured 6 to 18 months after the second coppice except for bud set which was measured before December 2010.

In June of 2014 leaf characteristics were measured for 1056 trees. The first and second fully expanded leaves (counting from the apex) were collected from a branch receiving full sunlight. One of the leaves was used for measuring petiole length and diameter with a digital caliper, and then scanned using a hand-held scanner. Images were analyzed to estimate leaf area, leaf length, leaf width, leaf perimeter, and leaf feret diameter using *imageJ* software (Schindelin, Rueden, Hiner, & Eliceiri, 2015). Dry weights were determined for the same leaves for estimation of specific leaf area (SLA). Leaf chlorophyll was estimated using a SPAD 502 Plus meter (Spectrum Technologies) using the average of 3 replicate measures on different parts of the leaf. The other leaf was used for measuring abaxial stomatal density. The details for estimating stomatal density are described in Chhetri et al. (2019). In 2015, 2378 trees were measured for height. We measured bud set for 2169 trees (838 genotypes) in 2010 and bud flush for 2428 trees (869 genotypes) in 2013. Details of the phenotypic data collected from 2014 is described in Chhetri et al. (2019).

3.3.2 Stable carbon isotope analysis

In 2013, 759 trees were sampled for carbon isotope analysis. Wood cores were taken from breast height of the tree and the previous year's growth ring were selected for analysis. A cross section of the wood tissue representing the entire growth ring (about 1.2 to 1.8 mg) representing early, intermediate and late wood was sampled. Detailed methods for processing wood samples for carbon isotope analysis is described in Chhetri et al. (2019).

3.3.3 Phenotypic data imputation

We imputed missing phenotypic data using the *phenix* function of the *phenix* package in R. *Phenix* imputes missing phenotypic data using a Bayesian matrix factorization model by accounting for the relatedness among the individuals (Dahl et al., 2016).

3.3.4 Environmental data collection

Climate and geography vary considerably across the range of distribution of *P. trichocarpa* (Supplementary Figure S3.1) The climate and geography data (26 variables, Tables 3.1 & 3.2) from the source location of 869 *P. trichocarpa* accessions from 1990 to 2010 were obtained from ClimateWNA (T. Wang, Hamann, Spittlehouse, & Murdock, 2012). The average values across the years were used for all analyses.

3.3.5 Correlation of adaptive traits with climate

We first obtained genotypic best linear unbiased predictors of 11 phenotypic traits by running the mixed model analysis using the *lmer* function of the *lme4* package in R and ran Pearson correlation of phenotypic traits with 26 climate variables and their first 4 PCs that explained more than 90% of the total variation cumulatively (Table 3.2). We also performed correlation of all phenotypic traits with geoclimate variables and their first four PCs (Table 3.3).

3.3.6 Test for the association of climate variables with the SNPs in the genome

In order to identify the genetic variants that potentially drive local adaptation in *P. trichocarpa* we performed a genome-environment association (GEA) analysis using single and multirait and PC-based GEMMA (X. Zhou & Stephens, 2012, 2014). The statistical test for association was performed for six climate variables – mean annual precipitation (MAP), mean annual temperature (MAT), mean warmest month temperature (MWMT), relative humidity (RH), summer heat:moisture index (SHM) and continentality [TD, the difference between MWMT and mean coldest month temperature (MCMT)]. Pairwise correlations were run for all 26 climatic variables and only one variable from the group of highly correlated variables ($r > 0.8$) was selected for GWAS. The association test was also performed with the first two PCs of the 22 climate and 3 geography (latitude, longitude and elevation) variables that cumulatively explained more than 80% of the total variation. Furthermore, a multitrait association was performed for a set of all six selected climate variables. Because the mean annual radiation (MAR) dataset was incomplete, we removed it from the PC based GWAS.

The statistical test of association was performed with 869 re-sequenced unstructured individuals (Figure 3.1) using Genome-wide Efficient Mixed Model Association package (GEMMA, X. Zhou & Stephens, 2012). The model tested was:

$$y = W\alpha + x\beta + u + \epsilon,$$

where y is an n -vector of phenotypic BLUP values, where n is the number of individuals tested; W is an $n \times c$ matrix of covariates; α is a c -vector of corresponding coefficients, where c is the number of principal coordinate axes used; x is an n -vector of marker genotypes, β is the effect size of the marker, u is an n -vector of random effects that includes a relatedness matrix and ϵ is an n -vector of errors.

A genetic relationship matrix estimated using GEMMA was used to control for the effects of relatedness in the association test. Phenotypic association was tested with 6.41 million SNPs that had $MAF \geq 0.05$.

To correct for multiple testing, we used the Bonferroni correction method, but as this method is very stringent for a large number of tests, we relaxed our P -value cutoff from 7.417×10^{-9} (Bonferroni correction threshold) to $P < 1 \times 10^{-7}$, which we designated as a suggestive association threshold. Adjacent significant SNPs were aggregated into peaks using a 20kb moving window.

3.3.7 Multivariate association: redundancy analysis

Redundancy analysis (RDA) is a constrained ordination method that combines multiple linear regression and principal component analysis. It is an extension of multiple regression where the variation in a multivariate Y response matrix is explained by a multivariate X matrix of explanatory variables. RDA constructs a matrix Y' of fitted genetic or phenotypic values estimated from the regression of each locus or phenotype and performs PCA on the matrix Y' to produce canonical/constrained axes that are linear combinations of the predictors (Legendre & Legendre, 2012; van den Wollenberg, 1977). Variables

in the original response matrix are modeled as a function of predictor variables producing as many constrained axes as predictors. We performed redundancy analysis with two-fold objectives – first, in order to examine the effect of spatial and climate variation on the SNPs in the *P. trichocarpa* genome, and second, to test for association of multivariate phenotypic data with multivariate SNP, climate and geographical variables. SNP eigenvectors were generated using the program *smartpca* for the same 869 *P. trichocarpa* accessions used for GEMMA association with 11.1 million SNPs after removing loci with $MAF < 0.01$ (Patterson, Price, & Reich, 2006). The 113 significant eigenvectors from *smartpca* were used for all RDA analyses.

For our first objective, we ran three RDA models – one with the SNP eigenvectors (EVs) as the response matrix constrained by the climate variables (model 1), second with SNP EVs as the response matrix constrained by the matrix of climate and spatial (geography or space) variables (model 2) and the other with the SNP EVs as the response matrix constrained by the climate variables with spatial variables used as a covariate (model 3). We used the 113 SNP eigenvectors as a response matrix and the five climate variables (MAP, MAT, MWMT, SHM and RH) out of the six variables selected for GEA above as the explanatory variables for model 1. We removed TD from the RDA model because the variance inflation factor (VIF) for a few of the climate variables was greater than 10 when TD was included as one of the predictor variables, indicating multicollinearity. The same 5 climate variables selected for the climate RDA model 1 were used in the other two climate RDA models and a phenotypic RDA model (model 2, 3 and 4 below). We ran third degree polynomials on geographical variables (latitude and longitude) and selected the spatial variables (named as Space in the model) based on the stepwise regression (forward selection criteria) using *ordistep* function of the *vegan* package in R. We performed RDA analysis with the *rda* function of the *vegan* package (vegan 2.5-2) in R 3.5.1 (Oksanen et al., 2018; R Core Team, 2017).

SNP EVs ~ Clim (RDA model 1)

SNP EVs ~ Clim + Space (RDA model 2)

SNP EVs ~ Clim + Condition (Space) (RDA model 3)

For our second objective, we ran the RDA model with a response matrix of 11 phenotypic traits constrained on the matrices of 113 significant SNP eigenvectors and 5 climate variables with the matrix of spatial variables as a covariate.

Pheno ~ SNP EVs + Clim + Condition (Space) (RDA model 4)

For each of the significant (phenotypic RDA model) or outlier SNP EVs (climate RDA model) identified from each RDA model, we selected the top 0.1% SNPs from the tails of distribution of the SNP loadings (weightings) and considered those as the candidate for outlier loci.

3.4 Results

3.4.1 Trait heritabilities and genetic correlation of phenotypes with geography and climate variables

Broad-sense heritabilities of the phenotypic traits showed the presence of substantial genetic variation. The heritability estimates ranged from 0.297 to 0.825 for all traits. Overall, the heritability estimates were higher for phenology traits (H^2 for bud set and bud flush were 0.628 and 0.825 respectively) compared to morphological and physiological traits. Based on the pairwise correlation of geoclimate variables of the source locations of the *P. trichocarpa* accessions, in general, temperature, AHM, SHM and CMD decreased with latitude whereas TD and RH had positive correlation with latitude (Table 3.1). EMT, Eref, CMD and eFFP increased with temperature, whereas PAS decreased with temperature (Table 3.1). Bud set and bud flush had significant correlations with tree height and most of the other morphological traits (Table 3.3). While bud set and bud flush had significant negative correlation to each other, most traits including bud set, bud flush and tree height were strongly positively correlated with many climate and geographical variables, and PC1 of the 26 geo-climatic variables (Table 3.2 & 3.3). Bud flush and tree height had significant negative correlation with latitude whereas bud set had a significant positive correlation with latitude. Furthermore, tree height had a strong and significant correlation with temperature and MCMT and negative correlation with DD_0 and DD_18. Leaf area and SLA showed a similar trend as tree height – they had significant negative correlation with latitude and positive correlation with temperature. SLA and stomata had positive correlation with CMD, EXT and Eref whereas SPAD had negative correlation with MCMT. Leaf traits had moderate but significant correlations with selected climate and geographical variables, and PC1 of the 26 geo-climatic variables. Furthermore, stomatal density had significant negative correlation with relative humidity and positive correlation with EXT, Eref and CMD (Table 3.3).

3.4.2 Detection of outlier loci using genotype-environment association (GEA)

Using single and multitrait association of six climate variables and single trait association of PC1 and PC2 of 26 geoclimate variables with the 6.78 million SNPs, we identified a total of 422 significant SNPs (48 SNP peaks) in the vicinity of 66 genes based on a Bonferroni correction threshold of 5% significance level ($P < 7.417 \times 10^{-9}$). Fourteen of these genes were shared across the three genotype-environment association (GEA) detection methods – single and multitrait and PC based associations (Figure 3.2a). Multitrait association detected the highest number of genes (48 genes, 99 significant SNPs) followed by single trait association (44 genes, 121 significant SNPs) and PC based single trait association (23 genes, 52 significant SNPs) (Figure 3.2a). With a relaxed P -value cutoff ($P < 1 \times 10^{-7}$), 833 SNPs (137 SNP peaks) were detected, and these were near 172 genes (Figure 3.2b).

3.4.3 Genes identified by genotype-environment association (GEA)

We identified some interesting genes across the genome for GEA with all (single, multitrait and PC based) GWAS methods. We found several genes in chromosome 10 for single trait (MAP, RH, SHM and TD), multitrait and PC2 GWAS (Figure 3.3; Supplemental Figure 3.2) including Potri.010G079500 and Potri.010G079600 code for a very long chain beta-ketoacyl-CoA synthase. The best match of this gene is KCS11 in *Arabidopsis*, which is potentially involved in cuticular wax biosynthesis. As expected, multitrait GWAS provided more power and detected genes with significant SNP peaks in several chromosomes (Figure 3.3), but single trait and PC based GWAS also detected genes with significant SNP peaks in various chromosomes (Table 3.4). For example, both PC1 and MAT GWAS revealed a strong peak near two genes: Potri.015G063400 (ABC transporter family protein) and Potri.015G063300 (bHLH family protein) on chromosome 15. Furthermore, PC2 and RH GWAS detected a gene, Potri.016G017400 that is related to the anthocyanin biosynthesis pathway.

3.4.4 Redundancy analysis (RDA)

3.4.4.1 Association of genome-wide SNPs with geography and climate variables

RDA analysis with the response matrix of 113 SNP EVs constrained on five climate variables (MAP, MAT, MWMT, SHM and RH) (model 1) showed that all climate variables significantly explained variation in the SNP EV response matrix. RDA model 2 with 113 SNP EVs constrained on five climate and 9 space variables showed that all predictor variables were significant. From RDA model 1, thirteen EVs (EVs 1 to 8, 10, 11, 14, 15 and 37) were identified as outliers from the tails (>3 standard deviations) of the distribution of EVs in each significant RDA axis. From RDA model 2, 17 EVs (all EVs identified in RDA model 1 plus EVs 18, 30, 32 and 33) were identified as outliers using the same criterion. Furthermore, there was a moderate population (river) level grouping of genotypes due to RDA axis 1 (Figure 3.4a & b) and RDA axis 3 (Supplemental Figure S3.3a, b, c & d) for both RDA model 1 and RDA model 2. Similarly, the partial RDA analysis with the 113 SNP EVs constrained on five climate variables conditioned on spatial variables (RDA model 3) showed that the explanatory matrix of climate variables significantly explained the variation in the response matrix of SNP eigenvectors. We identified five outlier SNP eigenvectors (EVs 2, 3, 5, 8 and 32) from the tails of the distribution of each significant RDA axis (Figure 3.5a; Supplemental Figure S3.5a-d). Compared to RDA model 1 and 2, there was no population level separation of genotypes due to RDA axis 1 and 3 in RDA model 3 (Figure 3.4c; Supplemental Figure S3.3e & f). Comparing the three RDA models, among population genetic variation due to climate variables is confounded by geography (Figure 3.4a-c)

Partitioning the variation explained by constrained variables, climate explained only 2.6% whereas spatial variables (latitude and longitude) explained 5.6% of the variation in the constrained SNP

eigenvectors, while both climate and geography shared less than 1% of the variation explained by the constrained variables (Figure 3.7a).

3.4.4.2 Association of phenotypic traits with genome-wide SNPs, geography and climate variables

Partial RDA analysis with the response matrix of 11 adaptive traits constrained on 113 SNP eigenvectors and five climatic variables conditioned on the matrix of spatial variables (RDA model 4) showed that the explanatory (predictor) variables significantly explained the variation in the phenotypic trait matrix. Out of all explanatory variables (113 SNP eigenvectors and 5 climate variables), 17 SNP eigenvectors significantly explained the variation in the phenotypes (Supplemental Figure S3.4). For the full phenotypic RDA model, (phenotypic matrix constrained on 113 SNP EVs, 5 climate variables and 7 space variables) we identified 16 SNP eigenvectors as significant. EVs 59 and 62 that were significant in the partial model were not significant in the full model, but an additional eigenvector EV61 was significant in full RDA model compared to the partial phenotypic RDA model.

Partitioning the variance explained by the constrained axis into the matrices of three explanatory variables, about 22% of the total variation in the RDA model was explained by the explanatory (constrained) variables that included 113 SNP eigenvectors, 5 climate variables and the matrix of spatial variables. SNP eigenvectors explained most of the variation with 21.6% while the other two predictor matrices, climate (5 variables) and the spatial matrix explained 12.3% and 16.6% of the total variation, respectively (Figure 3.7b).

3.4.5 Comparison between Clim and Pheno RDA, and GEA

A total of 7807 genes (48822 SNPs) were identified based on the selection of top 0.1% SNP loadings (weightings) from 5 significant SNP eigenvectors detected by the Clim RDA model (for e.g. EV32, Figure 3.5a). Comparing these genes with the genes from GEA analysis, 35 (based on Bonferroni cutoff of 7.417×10^{-9} for GEA SNPs) and 79 genes (based on a suggestive association cutoff of $P < 1 \times 10^{-7}$) were shared across the methods.

A total of 16782 genes (139988 SNPs) were identified based on selection of the top 0.1% SNP loadings (weightings) from 17 significant SNP eigenvectors detected by the Pheno RDA model (for e.g. EV1, Figure 3.5b). Comparing these genes with the genes from GEMMA GEA analysis, 49 (based on Bonferroni cutoff of $P < 7.417 \times 10^{-9}$ for GEA SNPs) and 118 genes (based on a suggestive association cutoff of $P < 1 \times 10^{-7}$) were shared across the methods. All methods (Clim and Pheno RDA, and GEA) shared a total of 32 genes at $P < 7.417 \times 10^{-9}$ for GEA SNPs (Table 3.4; Figure 3.6).

3.4.6 Comparing Pheno RDA model with simple multiple regression models

With the phenotypic RDA model, we detected 17 significant SNP eigenvectors that significantly explained the variation in the matrix of 11 phenotypic traits (Figure 3.5b; Supplemental Figure S3.4, S3.6a-o). Comparing the simple multiple regression of each of the individual phenotypes in the RDA model with the phenotypic model, we found that 70 predictor variables including SNP eigenvectors, climate and space variables were significant in all simple multiple regression models combined. However, only 17 of them (all were SNP eigenvectors) were significant in the phenotypic RDA model. This difference is mainly driven by significant predictors from multiple regression for stomata, bud set and bud flush where most of the predictor variables that significantly explained the variation in the simple multiple regression were not significant in RDA model (Figure 3.8).

3.5 Discussion

Local adaptation is prevalent in forest trees such as *Populus*, despite large populations with weak neutral population structure (Aitken et al., 2008; Savolainen et al., 2013; Savolainen & Pyhäjärvi, 2007). Local adaptation is particularly intriguing for wind pollinated trees like *Populus* where pollen can travel long distances (Slavov et al., 2009). Nevertheless, multiple studies have attempted to underscore the mechanisms of local adaptation in *Populus* (Capblancq et al., 2018; Evans et al., 2014; J. Wang et al., 2018). Here we attempted to provide the evidence for local adaption in *P. trichocarpa* by taking advantage of the phenotypic data collected in a common garden in Corvallis, OR and using combination of multiple data combinations (genotypic, phenotypic and geoclimatic data) and methods (RDA and GEA). We built upon the previous evidence of local adaption based on differentiation and GWAS methods (Evans et al., 2014; Chhetri et al., 2019) by incorporating larger genomic, phenotypic and geoclimatic data. We assessed the evidence of selection using the correlation of phenotypic traits with geoclimatic variables, identified outlier loci conferring local adaption using Genotype Environment Association (GEA) and used multivariate canonical redundancy analysis (RDA) to identify loci conferring local adaptation. We also used the latter method to partition the variation in neutral population structure into geography and climate predictor variables and the variation in phenotypic data into genomic, geographic and climatic predictor variables and highlighted their relative effects on local adaptation.

3.5.1 Genetic variation (phenotypic trait) and phenotype environment correlation

The presence of substantial heritable genetic variation in the phenotypic traits we studied here adds credibility to the genetic correlations of the phenotypic traits with the environmental variables and the multivariate regression of phenotypic traits with SNP PCs, climate and geography as predictor variables. Multiple studies have shown that climate is a driver of local adaption in *Populus* (Evans et al., 2014; Slavov & Zhelev, 2010). Our results from this study suggest the same with significant genetic

correlations of many of our phenotypic traits with climate of the source locations (Table 3.3). In congruence with other studies (Mckown et al., 2014), most of these traits and first and second PCs of the whole genome re-sequencing data have statistically significant correlations with latitude. Genetic correlations between the phenotypic traits and climate variables are likely confounded by geography and should be interpreted with caution (Chhetri et al., 2019).

However, for two reasons the confounding effects of geography on the relationships of phenotypic traits and the neutral genetic variation is less severe than previously thought in *Populus*. First, some phenotypes such as tree height, bud flush and bud set had much stronger correlations with environmental variables in our study compared to other studies (Mckown et al., 2014; Oubida et al., 2015). Second, SNP PC1 and SNP PC2 explain only 1.64 and 0.76% of the total SNP variation in our study (Supplementary Table S3.1) which is lower than previously reported (Mckown et al., 2014; Slavov et al., 2012), suggesting that our data reveal underlying patterns that were not previously detected. In support of this hypothesis, the correlations of SNP PC1 and SNP PC2 with latitude are not as strong as previously reported with smaller sample sizes (Mckown et al., 2014; Slavov et al., 2012).

Phenotypic traits significantly correlated with climate variables and climate PC axes are largely consistent with previous studies (Chhetri et al., 2019; Evans et al., 2014; Mckown et al., 2014). Bud flush was positively correlated, and bud set was negatively correlated with PC1 of 26 geo-climatic variables. PC1 of geoclimate variable is driven by the positive loadings of temperature related variables such as MAT, MCMT, DD5, DD18, MWMT, and moisture index related variables such as SHM and AHM and negative loadings of precipitation (MSP and MAP) related variables, latitude and elevation, and DD_18 and PAS (Table 3.2). These traits are quite important for growth, as early bud flush and late bud set were correlated with increased tree height.

Pairwise correlation of phenotypic traits and climate variables in our study suggest that taller trees generally have larger leaves, longer petioles, larger petiole diameter, but lower stomatal density in abaxial leaf surfaces. However, the contribution of stomatal patterning to tree growth is not clear as the wood carbon isotope composition that is indicative of abiotic stress tolerance showed no correlation with geoclimate as well as other phenotypic traits. Furthermore, there was no consistency across studies in the relationship of most physiological traits. Low trait heritabilities reported for most of these physiological traits makes it harder to make predictions on tree growth and stress tolerance in *P. trichocarpa* (Chhetri et al., 2019; Mckown et al., 2014).

3.5.2 GEA outlier detection

We found multiple genes with annotations consistent with their involvement in important plant functions on chromosome 10 and chromosome 16. For example, Potri.016G017400, is 255 bp upstream of a SNP that was associated with PC2 of 26 geoclimate variables ($P=3.75 \times 10^{-9}$), as well as in the

multitrait GWAS of 6 climate variables ($P=8.78 \times 10^{-8}$). Potri.016G017400 encodes a putative glucosyl/glucuronosyl transferase, which is from the anthocyanin biosynthesis pathway. Anthocyanins are a group of secondary metabolites that have antioxidant properties. They play important roles in pigmentation and in defense against biotic and abiotic stresses. Genes involved in the anthocyanin biosynthesis pathway have been well characterized in the Brassicaceae family including *Arabidopsis* spp. and *Brassica* spp. (Guo et al., 2014; Misyura, Colasanti, & Rothstein, 2013; Shi & Xie, 2014) and the Solanaceae family including *Solanum lycopersicum*, *Capsicum* spp. and *Solanum tuberosum* (Liu et al., 2018). In *Populus* Potri.016G017400 is highly expressed in pre- and early dormant bud, spring and winter apical bud, and early male developmental processes (Figure 3.5).

On chromosome 10, Potri.010G079500 and Potri.010G080200 were close to major peaks in all GWAS analyses (single and multitrait, and PC-based methods). Two of these GWAS hits were within the coding regions of the genes - one was a non-synonymous SNP within the coding region of the Potri.010G079500 gene and the other was a synonymous SNP within the coding region of Potri.010G080200. Both of these genes are putative very long chain beta-ketoacyl-CoA synthase genes, which play a major role in the fatty acid biosynthesis pathway. Potri.010G079500 is highly expressed in young and first fully expanded leaves, late female development organs, stem treated with ammonia, nitrate and urea, female receptive organ, mid male developmental stage and apical and fully open bud. Potri.010G080200 is highly expressed in male and female early developmental stages, early and mid-spring apical buds, stem node and internode, young and immature leaves and root tips (Figure 3.6). The Potri.010G079500 gene also appears to be important for the alkene composition of cuticular wax in *P. trichocarpa* (Gonzales-Vigil et al., 2017). The gene is downregulated in trees with low alkene composition. Non-alkene producing trees had reduced growth and higher disease susceptibility, emphasizing the adaptive importance of this trait (Gonzales-Vigil et al., 2017).

3.5.3 Outlier detection using RDA

Because our SNP data set was large, we used an indirect approach for detecting the loci conferring local adaption. We used significant SNP PCs (eigenvectors) from the PCA analysis as a matrix of genetic data in the RDA analysis. Therefore, instead of identifying outlier loci directly from the RDA models, we identified outlier PCs that were potentially reflecting outlier SNP loadings (weightings) from the tails of the distribution. We extracted the top 0.1% of the SNPs from the tails of the distribution of the loadings and considered these as outlier loci. We identified 32 genes close to these outlier loci that were in common with the sets identified by the two RDA models and the GEA method. However, only one SNP PC was in common between the 5 and 17 outlier SNP PCs from the climate and phenotypic RDA models. The large number of genes we identified here (both for neutral population genetic structure and phenotypic traits) is not surprising considering that adaption in forest trees is highly polygenic, and the

adaptive mechanism is different depending on the type of selective forces and the adaptive trait itself (Boyle, Li, & Pritchard, 2017; Eckert et al., 2009; Evans et al., 2014; Holliday, Wang, & Aitken, 2013). Under this scenario, selection can affect hundreds or thousands of loci such that the allele frequency of a given locus may not be altered much (Le Corre & Kremer, 2012), thus requiring a powerful method like RDA to identify the presumed weak selection signal.

The RDA analysis for SNP, climate and geography matrices suggests that a very small portion of among-population variation (7.6%) is explained by climate and geography in *P. trichocarpa*, out of which a large proportion of the variation is shared by both climate and geography variables. As climate alone explains only about 2.6% of the total variation, the large portion of variation in the SNP eigenvector matrix is due to population structure. A large proportion of the among population variance (92.4%) remained unexplained. Part of the missing variance may be attributed to historical demographic processes or other environmental variables such as pH and soil characteristics not included in the RDA model. Nevertheless, all five of the climate variables in all Clim RDA models (RDA model 1, 2 and 3) significantly explained the variation in the SNP eigenvector response matrix.

Both climate and geography explained 12.3% and 16.6% of the variation respectively in the phenotypic matrix justifying the effect of climate in adaptive traits. This also matches with the significant correlations of some phenotypic traits with climate and geography (especially latitude) variables showing spatial clinal variation. Variance partitioning in the phenotypic RDA model showed that SNP data accounted for 21.664% of the phenotypic variation, although SNP data alone explained only 3.891% of the total phenotypic variation.

Simulation studies have shown that RDA has greater statistical power than GEA methods such as LFMM (Frichot et al., 2013), differentiation methods such as PCAdapt (a non-constrained ordination method, Luu, Bazin, and B. 2017), and other constrained ordination methods (Capblancq et al., 2018; Forester et al., 2018). Moreover, RDA-based methods have low false positive rates coupled with high true-positive rates compared to other general linear models (GLM) and LFMM methods and RDA is better than other methods in picking up weak selection signals (Forester et al., 2016, 2018). Given that adaptation is not always driven by strong differentiation at the individual loci and it is very likely that the covariance among key loci has a major role, small-effect single locus signatures can remain undetected from their neutral genomic background (Berg & Coop, 2014; De Villemereuil et al., 2016; Le Corre & Kremer, 2012; Lind et al., 2017; Rajora, Eckert, & Zinck, 2016). Thus identification of adaptive loci is challenging and the use of multivariate statistics like RDA to unravel the complexity underlying adaptive genetic polymorphisms is warranted (Capblancq et al., 2018; De Kort et al., 2014; Forester et al., 2018; Lasky et al., 2012; Meirmans, 2012; Meirmans et al., 2017; Talbot et al., 2017; Vangestel et al., 2018). RDA methods identified outlier loci that were undetected in other genome scans (PCAdapt) and GEA

(LFMM) methods in *P. trichocarpa* (Capblancq et al., 2018). Using a combination of trait-climate correlation, GEA and RDA methods we provided here a comprehensive assessment of the relative roles of geoclimatic, genomic and phenotype variation methods in understanding the genetic mechanisms underlying local adaption.

Local adaptation to varied environmental conditions allows trees to respond to the changing climate regime and can help in overcoming the potential adaptational lag. This study is an important step towards unraveling the evolutionary mechanisms in forest trees. Genomic signals identified here can have important implications in breeding programs and effective optimization of productivity traits for developing *Populus* as a biofuel feedstock. Furthermore, these findings can be invaluable for developing management and conservation strategies and sustainability of forest resources in the face of climate change.

3.6 Conclusion

Integrating genomic, environmental and phenotypic data we provided here a comprehensive assessment of the genetic basis of local adaption in *P. trichocarpa*. With the correlation of phenotypic traits with geography and climate variables we showed that the phenotypic traits have an environmental gradient that coincides in part with the latitudinal gradient. Using single trait, multitrait and PC-based GEA methods we detected adaptive signals that are common across all methods and identified key genes that potentially affect physiological functioning and adaptation in *P. trichocarpa*. We complemented our GEA analysis with a conceptually different multivariate ordination method, RDA, where we identified genomic outliers (genes) from both phenotypic and climate RDA models that overlapped with the genes identified from the GEA methods. Variance partitioning of the response matrices in both climate and phenotypic models underscores the importance of geographical variation in neutral population genetic structure (climate RDA model) and adaptive genetic variation (phenotypic RDA model). These findings have important implications for effective optimization of productivity traits, and developing sustainable management and conservation strategies of forest trees. Given that local adaption is believed to be under polygenic control, outlier loci detection methods should incorporate multivariate methods that allow for simultaneous detection of adaptive genomic signals covarying with the adaptive forces, or at least the univariate methods should be complemented with multivariate methods. Furthermore, to gain better insights into the adaption of tree populations it is important to integrate genotypic, environmental and phenotypic data whenever possible. Nevertheless, given the polygenic nature of the adaption we need larger sample size from across the range of distribution range of tree species at the whole genome level to accurately estimate the genome-wide signals contributing to local adaption.

3.7 Tables and figures

Table 3.1 Pearson correlation (r) between 26 geo-climate variables and PCs 1 – 4 of 26 geo-climate variables of the source location of *P. trichocarpa* genotypes. Values greater than 0.13 or less than -0.13 are significant based on the Bonferroni correction criteria at 5% significance level.

Geo-clim																												
Longitude	GEOGRAPHY																											
Latitude	-0.515																											
Elevation	-0.197	0.348	Elevation																									
MAT	0.530	-0.780	-0.806	MAT																								
MWMT	0.464	-0.527	-0.665	0.771	MWMT																							
MCMT	0.476	-0.798	-0.711	0.952	0.563	MCMT																						
TD	-0.352	0.704	0.538	-0.774	-0.210	-0.926	TD																					
MAP	-0.216	0.369	0.378	-0.423	-0.367	-0.327	0.221	MAP																				
MSP	-0.133	0.515	0.229	-0.369	-0.373	-0.283	0.165	0.846	MSP																			
AHM	0.293	-0.554	-0.457	0.591	0.539	0.486	-0.330	-0.929	-0.843	AHM																		
SHM	0.106	-0.529	-0.252	0.398	0.551	0.263	-0.061	-0.724	-0.905	0.817	SHM																	
DD_0	-0.513	0.722	0.750	-0.938	-0.598	-0.968	0.872	0.221	0.172	-0.392	-0.178	DD_0																
DD5	0.514	-0.747	-0.797	0.973	0.868	0.865	-0.627	-0.497	-0.445	0.659	0.507	-0.839	DD5															
DD_18	-0.527	0.780	0.807	-0.999	-0.749	-0.960	0.795	0.415	0.356	-0.580	-0.377	0.948	-0.964	DD_18														
DD18	0.458	-0.595	-0.617	0.793	0.944	0.610	-0.291	-0.455	-0.454	0.607	0.605	-0.586	0.897	-0.768	DD18													
NFFD	0.491	-0.713	-0.814	0.977	0.666	0.976	-0.850	-0.350	-0.265	0.503	0.266	-0.970	0.913	-0.982	0.686	NFFD												
bFFP	-0.438	0.537	0.863	-0.922	-0.736	-0.864	0.686	0.393	0.248	-0.524	-0.277	0.863	-0.905	0.921	-0.744	-0.938	bFFP											
eFFP	0.485	-0.694	-0.763	0.957	0.662	0.959	-0.832	-0.309	-0.226	0.472	0.238	-0.944	0.899	-0.960	0.699	0.986	-0.933	eFFP										
FFP	0.468	-0.624	-0.829	0.955	0.714	0.926	-0.769	-0.359	-0.243	0.509	0.265	-0.917	0.918	-0.956	0.736	0.978	-0.984	0.982	FFP									
PAS	-0.522	0.668	0.820	-0.928	-0.665	-0.894	0.755	0.519	0.379	-0.616	-0.353	0.902	-0.879	0.933	-0.655	-0.928	0.872	-0.877	-0.890	PAS								
EMT	0.467	-0.751	-0.729	0.945	0.587	0.988	-0.901	-0.279	-0.230	0.445	0.226	-0.974	0.858	-0.953	0.618	0.983	-0.880	0.975	0.942	-0.889	EMT							
EXT	0.475	-0.718	-0.551	0.755	0.899	0.567	-0.263	-0.452	-0.538	0.616	0.670	-0.555	0.851	-0.735	0.888	0.607	-0.591	0.572	0.593	-0.635	0.546	EXT						
Eref	0.504	-0.906	-0.573	0.865	0.745	0.777	-0.582	-0.518	-0.576	0.682	0.623	-0.720	0.894	-0.858	0.786	0.756	-0.653	0.717	0.696	-0.768	0.733	0.909	Eref					
CMD	0.165	-0.631	-0.277	0.458	0.538	0.329	-0.145	-0.770	-0.940	0.844	0.955	-0.225	0.562	-0.441	0.601	0.313	-0.286	0.276	0.287	-0.410	0.275	0.729	0.734	CMD				
MAR	0.292	-0.285	0.123	0.117	0.033	0.142	-0.156	0.032	-0.073	0.025	0.050	-0.156	0.087	-0.118	0.062	0.104	-0.002	0.091	0.046	-0.071	0.153	0.160	0.205	0.096	MAR			
RH	-0.122	0.453	-0.040	-0.145	-0.185	-0.045	-0.027	0.493	0.582	-0.499	-0.560	-0.082	-0.250	0.134	-0.275	0.028	-0.086	0.084	0.087	0.091	0.069	-0.537	-0.569	-0.705	-0.076	RH		
PC1	0.53107	-0.809	-0.7791	0.987	0.7929	0.9215	-0.729	-0.542	-0.504	0.7003	0.5289	-0.89	0.9802	-0.983	0.8255	0.9446	-0.898	0.921	0.925	-0.925	0.909	0.801	0.902	0.585	0.120	-0.235		
PC2	-0.0874	-0.148	0.2186	-0.149	0.1443	-0.297	0.4154	-0.608	-0.759	0.5657	0.7704	0.3991	0.0086	0.1706	0.2024	-0.315	0.281	-0.346	-0.32	0.153	-0.36	0.367	0.261	0.788	0.010	-0.758		
PC3	0.13245	0.2437	-0.1619	0.023	0.5752	-0.219	0.5224	0.257	0.2594	-0.185	-0.003	0.1042	0.1693	0.005	0.4612	-0.066	-0.118	-0.037	0.045	0.088	-0.16	0.376	0.005	-0.085	-0.090	0.111		
PC4	-0.4649	0.4586	-0.4076	-0.027	0.0248	-0.075	0.1015	-0.369	-0.173	0.262	0.1319	0.0652	0.0027	0.0282	0.0031	0.0465	-0.243	0.045	0.151	-0.105	-0.03	-0.23	-0.29	0.015	-0.363	0.385		

Table 3.2 PCA loadings of 26 geo-climate variables for the first 4 PCs. PC1, PC2, PC3 and PC4 explained 62.44%, 17.13%, 6.02% and 5.20% of the total variation, respectively. Dark blue and dark red colors indicate high positive and high negative values, respectively.

Geo-climate variable	Abbreviation	PC1	PC2	PC3	PC4
Mean Annual Temperature (°C)	MAT	0.245	-0.071	0.001	-0.023
Degree-days Above 5°C	DD5	0.243	0.003	-0.070	-0.125
Number of Frost-Free Days	NFFD	0.234	-0.150	-0.011	0.067
Frost Free Period	FFP	0.229	-0.151	-0.115	0.016
Mean Coldest Month Temperature (°C)	MCMT	0.229	-0.141	0.109	0.148
Date of End of FFP	eFFP	0.228	-0.165	-0.020	0.044
Extreme Minimum Temperature of 30 Years	EMT	0.226	-0.171	0.074	0.109
Hargreaves Reference Evaporation	Eref	0.225	0.124	0.166	-0.092
Degree-days Above 18°C	DD18	0.206	0.095	-0.151	-0.356
Extreme Maximum Temperature of 30 Years	EXT	0.200	0.174	0.018	-0.367
Mean Warmest Month Temperature (°C)	MWMT	0.197	0.067	-0.202	-0.441
Annual Heat:Moisture Index	AHM	0.175	0.269	-0.102	0.234
Hargreaves Climatic Moisture Deficit	CMD	0.146	0.374	0.019	0.064
Longitude	Long	0.133	-0.043	0.306	-0.263
Summer Heat:Moisture Index	SHM	0.132	0.367	-0.069	0.027
Mean annual radiation	MAR	0.032	0.006	0.586	-0.147
Mean annual relative humidity	RH	-0.059	-0.363	-0.215	0.009
Mean Annual Summer Precipitation (mm)	MSP	-0.126	-0.362	0.006	-0.244
Mean Annual Precipitation (mm)	MAP	-0.135	-0.287	0.149	-0.331
Continentalty (difference between MWMT and MCMT)	TD	-0.181	0.197	-0.223	-0.378
Elevation (m)	Elev	-0.193	0.104	0.349	-0.017
Latitude	Lat	-0.202	-0.071	-0.365	-0.037
Degree-days Below 0°C	DD_0	-0.221	0.189	-0.081	-0.056
Date of Beginning of FFP	bFFP	-0.222	0.135	0.198	0.010
Precipitation as Snow (mm)	PAS	-0.230	0.073	0.045	-0.108
Degree-days below 18°C	DD_18	-0.244	0.081	-0.009	0.001

Table 3.3 Pearson correlation between phenotypic traits and geo-climate variables and PCs of 26 geo-climate variables. Values greater than 0.12 or less than -0.12 are significant at the 5% Bonferroni correction level of significance. Dark blue and dark red colors indicate high positive and high negative *P*-values, respectively.

Geo-climate*/Trait	Bud flush	Bud set	Height	Leaf area	Petiole length	Petiole diameter	Leaf aspect ratio	Specific leaf area	Stomatal density	Carbon isotope	SPAD
Long	0.374	-0.435	0.427	0.343	0.309	0.233	-0.165	0.012	-0.088	0.031	-0.017
Lat	-0.508	0.61	-0.418	-0.149	-0.179	-0.096	0.212	-0.329	-0.17	0.026	0.175
Elev	-0.256	0.293	-0.229	-0.072	-0.149	-0.033	0.123	-0.131	-0.035	0.12	0.091
MAT	0.456	-0.555	0.431	0.194	0.238	0.115	-0.224	0.223	0.07	-0.085	-0.155
MWMT	0.358	-0.301	0.21	0.078	0.093	0.069	-0.151	0.082	0.093	-0.023	-0.086
MCMT	0.418	-0.587	0.471	0.221	0.26	0.126	-0.236	0.25	0.039	-0.082	-0.17
TD	-0.332	0.556	-0.462	-0.225	-0.264	-0.117	0.209	-0.256	-0.004	0.089	0.161
MAP	-0.339	0.252	-0.192	-0.014	-0.058	-0.023	0.025	-0.137	-0.031	-0.035	-0.008
MSP	-0.314	0.285	-0.174	0.067	0.01	0.036	0.02	-0.222	-0.117	-0.057	0.036
AHM	0.413	-0.373	0.248	0.04	0.081	0.04	-0.074	0.19	0.075	-0.006	-0.05
SHM	0.317	-0.247	0.101	-0.086	-0.052	-0.046	-0.034	0.197	0.137	0.039	-0.053
DD_0	-0.351	0.556	-0.457	-0.239	-0.273	-0.138	0.232	-0.219	-0.018	0.072	0.154
DD5	0.478	-0.5	0.372	0.151	0.196	0.094	-0.203	0.197	0.098	-0.084	-0.143
DD_18	-0.452	0.56	-0.436	-0.2	-0.243	-0.117	0.224	-0.226	-0.065	0.087	0.156
DD18	0.41	-0.342	0.234	0.073	0.107	0.061	-0.16	0.106	0.113	-0.044	-0.109
NFFD	0.397	-0.548	0.444	0.218	0.256	0.126	-0.23	0.211	0.022	-0.086	-0.152
bFFP	-0.37	0.431	-0.367	-0.184	-0.218	-0.117	0.208	-0.128	0.017	0.095	0.116
eFFP	0.386	-0.541	0.429	0.221	0.253	0.134	-0.234	0.187	0.011	-0.082	-0.149
FFP	0.385	-0.492	0.403	0.205	0.238	0.127	-0.225	0.159	-0.003	-0.09	-0.134
PAS	-0.388	0.506	-0.424	-0.208	-0.243	-0.121	0.176	-0.188	0.002	0.042	0.1
EMT	0.374	-0.562	0.453	0.22	0.254	0.126	-0.242	0.233	0.029	-0.066	-0.166
EXT	0.458	-0.373	0.239	0.051	0.094	0.037	-0.143	0.201	0.211	-0.038	-0.127
Eref	0.537	-0.535	0.366	0.12	0.164	0.073	-0.178	0.283	0.185	-0.059	-0.157
CMD	0.393	-0.311	0.154	-0.069	-0.02	-0.035	-0.04	0.238	0.179	0.023	-0.068
MAR	0.112	-0.201	0.163	0.101	0.131	0.038	-0.066	0.114	0.056	0.032	-0.055
RH	-0.37	0.15	-0.054	0.073	0.033	0.052	-0.024	-0.174	-0.226	0.045	0.049
PC1	0.483	-0.559	0.424	0.174	0.219	0.107	-0.212	0.238	0.081	-0.066	-0.147
PC2	0.163	0.048	-0.121	-0.184	-0.165	-0.102	0.101	0.085	0.158	0.071	0.038
PC3	-0.049	0.232	-0.188	-0.05	-0.073	-0.008	0.011	-0.2	0.028	-0.01	0.035
PC4	-0.206	0.213	-0.163	-0.138	-0.102	-0.085	0.083	-0.084	-0.117	-0.006	0.076
Bud flush	NA	-0.340	0.312	0.190	0.190	0.145	-0.142	0.187	0.067	0.036	-0.070
Bud set	NA	NA	-0.514	-0.287	-0.304	-0.201	0.217	-0.266	-0.061	0.097	0.134

Table 3.4 Genes identified from *P. trichocarpa* single and multitrait GWAS shared by climate and phenotype RDA models.

Gene model ^a	Trait ^b	P-value ^c	Functional annotation
Potri.004G163400	RH	2.25E-10	similar to Vacuolar ATP synthase 16 kDa proteolipid subunit 1/3/5
Potri.005G095500	TD	7.38E-13	similar to unnamed protein product (<i>Fragaria x ananassa</i> , co-ortholog of several CAD genes
Potri.006G082100	multitrait*	3.62E-10	similar to expressed protein in <i>Arabidopsis thaliana</i> ; similar to expressed protein in <i>Arabidopsis thaliana</i> ; [ortholog of At3g53670,At2g37480,]
Potri.006G082200	multitrait	6.39E-09	NA
Potri.006G170700	multitrait	1.09E-10	PTHR23257:SF331 - OCTICOSAPEPTIDE/PHOX/BEM1P DOMAIN-CONTAINING PROTEIN KINASE
Potri.006G171200	multitrait	7.98E-10	PTHR11926//PTHR11926:SF221 - GLUCOSYL/GLUCURONOSYL TRANSFERASES
Potri.008G131700	multitrait	1.58E-09	ORG: <i>Malus x domestica</i> , co-ortholog of AAQ96165, AAQ96164, At1g14920, At2g01570, AAQ54509
Potri.008G210400	TD	4.87E-09	NA
Potri.008G210500	TD	3.41E-10	NA
Potri.008G210600	TD	1.42E-09	NA
Potri.010G079300	PC2**	2.34E-10	NA
Potri.010G079400	PC2	1.47E-11	NA
Potri.010G079500	multitrait	4.30E-30	NA
Potri.010G079600	multitrait	6.80E-29	PTHR13288 - DNA-DAMAGE REPAIR PROTEIN DRT111
Potri.010G079700	PC2	6.48E-20	NA
Potri.010G079800	TD	1.56E-09	NA
Potri.010G080000	multitrait	4.88E-38	NA
Potri.010G080100	TD	8.84E-11	NA

Gene model ^a	Trait ^b	P-value ^c	Functional annotation
Potri.010G080200	multitrait	4.09E-42	NA
Potri.010G080300	multitrait	6.24E-19	PTHR22773:SF88 - NADH-UBIQUINONE OXIDOREDUCTASE CHAIN 4
Potri.010G080400	multitrait	5.78E-24	NA
Potri.010G080500	multitrait	1.79E-16	NA
Potri.010G080600	multitrait	5.12E-12	PF04720 - PDDEXK-like family of unknown function (PDDEXK_6)
Potri.014G104800	MWMT	8.71E-11	no apical meristem (NAM) family protein; similar to no apical meristem (NAM) - <i>Petunia hybrida</i> ; similar to EMBL:PHDNANAM (co-ortholog of At3g61910, At2g46770)
Potri.014G106400	MWMT	1.36E-09	similar to Thylakoid membrane phosphoprotein 14 kDa; similar to chloroplast precursor
Potri.015G016700	RH	1.93E-09	PF02362 - B3 DNA binding domain (B3)
Potri.016G011500	multitrait	7.81E-14	Transporting two-sector ATPase / Mitochondrial ATPase
Potri.016G017400	PC2	3.75E-09	PTHR11926//PTHR11926:SF267 - GLUCOSYL/GLUCURONOSYL TRANSFERASES
Potri.016G090400	multitrait	1.46E-11	PF00651//PF03000 - BTB/POZ domain (BTB) // NPH3 family (NPH3)
Potri.016G095100	PC2	6.55E-11	PTHR23180:SF293 - ADP-RIBOSYLATION FACTOR GTPASE-ACTIVATING PROTEIN AGD7
Potri.017G143000	multitrait	2.14E-09	protein kinase family protein; similar to protein kinase domain containing protein (co-ortholog of At5g24010)
Potri.018G055100	multitrait	1.69E-09	PTHR32263:SF8 - INACTIVE POLY [ADP-RIBOSE] POLYMERASE SRO2-RELATED

^aGene models are annotated using v3 of the *P. trichocarpa* genome

^bFull trait names provide in Table 2

^cSNP p values < 1×10⁻⁹

*Multitrait set that includes MAP, MAT, MWMT, RH, SHM and TD climate variables

**PC2 of all 26 geoclimate variables listed in Table 2

Figure 3.1 Source locations of 869 *P. trichocarpa* genotypes sampled in this study (purple dots). The trees were grown in a common garden in Corvallis, Oregon (black star).

Figure 3.2 Genes detected by genotype environment association (GEA) across the methods – single trait, multitrait and PC-based. (a) $P < 7.417 \times 10^{-9}$ (Bonferroni correction threshold). (b) $P < 1 \times 10^{-7}$.

Figure 3.3 Single trait, multitrait and PC based association showing the association of geo-climate variables with the SNPs in chromosome 10 – Manhattan (left) and QQ plots (right). Numbers 1 to 19 represent chromosomes; scaffolds are the reads that did not align to any of the 19 chromosomes. (a) and (b) Summer heat:moisture index (SHM) (c) and (d) Mean annual precipitation (MAP) (e) and (f) Multitrait GWAS of six climate variables – MAP, MAT (mean annual temperature), MWMT (mean warmest month temperature), RH (relative humidity), SHM and TD [temperature difference between MWMT and MCMT (mean coldest month temperature)]. (g) and (h) PC2 of 25 geo-climate variables (includes latitude, longitude and elevation, and 22 climate variables). Red horizontal line indicates Bonferroni correction threshold ($P=7.417 \times 10^{-9}$) and blue horizontal line indicates suggestive association threshold ($P=1 \times 10^{-7}$). Nearest gene to the highlighted SNP/s (red circle) is Potri.010G080200, which is a very long chain beta-ketoacyl-CoA synthase with potential involvement in cuticular wax biosynthesis.

Figure 3.4 Triplots showing RDA on SNP eigenvectors constrained by the matrix of climate variables (a), climate and space variables (b) and climate variables with space as covariates (c). Both RDA1 and RDA2 axes were significant in all RDA models. It appears that the separation of *P. trichocarpa* populations in the RDA axis 1 in the climate only RDA model is due to the confounding effect of spatial variation. Circles indicate 869 *P. trichocarpa* genotypes (color coded by populations, the rivers). Triangles indicate SNP eigenvectors with outlier eigenvectors (based on a 3 standard deviation cutoff selected from the tails of the distribution of RDA axes) in black. Blue arrows indicate the influence of climate variables on RDA axis.

Figure 3.5 Square loadings of top 0.1% SNPs from one of the 5 significant eigenvectors (EV32) detected from the climate RDA analysis (a) and one of the 17 outlier eigenvectors (EV1) detected from the phenotypic RDA analysis (b). Numbers 1 to 19 represent chromosomes; scaffolds are the reads that did not align to any of the 19 chromosomes. Gray and yellow colors represent positive and negative square loadings, respectively. Red and blue circles indicate top 100 square loadings corresponding to positive and negative loadings, respectively. Climate RDA (EV32) identified the same gene (circled red) that was identified in GEA. Phenotypic RDA (EV1) also identified a similar gene, Potri.010G80300 (circled blue) just 100 bp upstream of the Potri.01G080200.

Figure 3.7 Partitioning of variance components in RDA analyses. (a) SNP ~ Clim + Geo model. 7.6% of the total variation is explained by climate and geography (matrix of spatial variable). (b) Pheno ~ SNP + Clim + Geo model. 22% of the total variation is explained by SNP EVs, climate and geography.

Figure 3.8 Comparison of significant explanatory variables (brown) from RDA model with all 11 traits and the corresponding simple multiple linear regression models. EVs represent SNP eigenvectors, Y represents a space variable, MAT, MWMT, and RH are climate variables.

3.8 Supplementary tables and figures

Supplementary Table S 3.1 Pearson correlation (r) of significant SNP eigenvectors from Clim and Pheno RDA models (Model 3 and 4) with selected geo-climate and first 4 PCs of 26 geo-climate variables. Values with variable color coding are r values with less than or greater than 0.12 being significant based on Bonferroni correction criteria at 5% level of significance.

SNP EV	% Var*	Longitude	Latitude	Elevation	MAT	MWMT	MAP	SHM	RH	Clim_PC1	Clim_PC2	Clim_PC3	Clim_PC4
EV1	1.639	0.258	0.468	0.143	-0.231	-0.187	0.179	-0.440	0.430	-0.271	-0.349	0.111	0.124
EV2	0.755	-0.286	0.568	0.124	-0.409	-0.259	0.456	-0.490	0.224	-0.467	-0.238	0.343	0.063
EV3	0.564	-0.569	0.311	0.360	-0.478	-0.288	0.195	0.128	0.028	-0.443	0.243	-0.029	0.144
EV5	0.407	-0.228	0.015	0.092	-0.025	0.118	0.252	-0.088	0.105	-0.047	-0.129	0.231	-0.024
EV6	0.284	0.276	-0.229	-0.140	0.262	0.166	0.146	-0.052	0.032	0.226	-0.245	0.051	-0.240
EV8	0.238	-0.167	0.089	-0.230	0.071	-0.061	-0.201	0.012	-0.033	0.073	-0.020	-0.151	0.294
EV18	0.199	-0.007	-0.074	0.025	0.048	-0.117	0.127	-0.176	-0.036	0.015	-0.163	-0.141	-0.140
EV22	0.189	-0.016	-0.093	-0.065	0.053	-0.038	0.012	-0.043	-0.052	0.043	-0.045	-0.111	-0.054
EV23	0.187	-0.048	0.047	0.026	-0.052	-0.027	-0.056	0.017	-0.030	-0.040	0.065	-0.001	0.055
EV27	0.181	0.004	0.006	-0.010	-0.024	-0.014	0.056	0.018	-0.024	-0.025	0.017	0.036	-0.037
EV32	0.177	0.024	-0.051	-0.067	0.077	0.130	-0.149	0.213	-0.156	0.100	0.180	0.036	0.039
EV44	0.166	0.001	-0.020	0.056	-0.014	0.010	0.040	-0.008	0.014	-0.013	0.004	0.027	-0.058
EV46	0.165	0.036	0.004	-0.034	0.032	-0.005	0.099	-0.066	0.160	0.013	-0.152	0.012	0.020
EV59	0.156	-0.014	-0.016	-0.011	0.012	-0.021	0.000	0.016	0.043	0.012	-0.021	-0.048	0.035
EV60	0.156	0.008	0.008	-0.024	-0.002	-0.001	0.007	-0.021	0.007	-0.004	-0.010	0.009	0.003
EV62	0.155	0.014	-0.041	-0.007	0.040	-0.025	0.080	-0.036	0.070	0.026	-0.108	-0.045	-0.034
EV72	0.152	0.012	-0.035	0.016	0.025	-0.022	0.068	-0.026	0.050	0.013	-0.074	-0.044	-0.042
EV93	0.144	-0.004	0.000	-0.036	0.027	0.043	-0.029	0.034	-0.009	0.030	0.017	0.024	0.038
EV106	0.141	-0.024	0.007	-0.014	0.000	0.002	-0.015	0.006	-0.008	0.000	0.011	0.003	0.019
EV107	0.141	-0.021	0.018	-0.029	0.012	0.022	-0.023	-0.001	0.010	0.011	-0.002	0.019	0.047

*Percentage of total variance explained by SNP eigenvectors

(a)

(b)

(c)

(d)

(e)

(f)

(g)

Supplementary Figure S 3.1 Geography and climate maps for the range of *P. trichocarpa* distribution. (a) Elevation; (b) Mean annual temperature (MAT); (c) Mean coldest month temperature (MCMT); (d) Mean warmest month temperature (MWMT); (e) Summer heat:moisture index (SHM); (f) Annual heat:moisture index (AHM); (g) Mean annual relative humidity (RH).

Supplementary Figure S 3.2 Single trait GWAS showing the association of climate variables with the SNPs in the genome – Manhattan (left) and QQ (right) plots. Numbers 1 to 19 represent chromosomes; scaffolds are the reads that did not align to any of the 19 chromosomes. (a) and (b) MAT (Mean annual temperature); (c) and (d) MWMT (Mean warmest month temperature); (e) and (f) TD (Temperature difference between MWMT and MCMT (Mean coldest month temperature)); (g) and (h) RH (Mean annual relative humidity); (i) and (j) PC1 of 25 geo-climate variables (includes latitude, longitude and elevation, and 22 climate variables). Red horizontal line indicates Bonferroni correction threshold ($P=7.417 \times 10^{-9}$) and blue horizontal line indicates suggestive association threshold ($P=1 \times 10^{-7}$).

Supplementary Figure S 3.3 RDA triplot with the response matrix of 113 SNP eigenvectors constrained by the matrix of climate variables [(a) and (b)], climate and space variables [(c) and (d)] and climate variables conditioned on space variables [(e) and (f)]. Circles indicate 869 *P. trichocarpa* genotypes (color coded by population). Triangles indicate SNP eigenvectors with outlier eigenvectors (based on a 3 standard deviation cutoff selected from the tails of the distribution of RDA axes) in black. Blue arrows indicate the influence of climate variables on RDA axis.

Supplementary Figure S 3.4 RDA triplot with the response matrix of 11 phenotypic traits (large colored circles) constrained by the matrices of 113 SNP eigenvectors and 5 climate variables with the spatial matrix used as covariate. Arrows indicate variable containing SNP eigenvectors and climate with blue indicating climate variables and red indicating significant variables in the RDA model. Small gray circles indicate 869 *P. trichocarpa* genotypes.

Supplementary Figure S 3.5 Square loadings of top 0.1% SNPs of outlier eigenvectors (EVs) detected from the climate RDA analysis (a) EV2 (b) EV3 (c) EV5 (d) EV8. Numbers 1 to 19 represent chromosomes; scaffolds are the reads that did not align to any of the 19 chromosomes. Gray and yellow colors represent positive and negative square loadings, respectively. Red and blue indicate top 100 square loadings corresponding to positive and negative loadings, respectively.

(c)

(d)

(e)

(f)

(g)

(h)

(i)

(j)

Supplementary Figure S 3.6 Square loadings of top 0.1% SNPs of outlier eigenvectors (EVs) detected from the phenotypic RDA analysis (a) EV6 (b) EV18 (c) EV22 (d) EV23 (e) EV27 (f) EV31 (g) EV44 (h) EV46 (i) EV59 (j) EV60 (k) EV62 (l) EV72 (m) EV93 (n) EV106 (o) EV107. Numbers 1 to 19 represent chromosomes; scaffolds are the reads that did not align to any of the 19 chromosomes. Gray and yellow colors represent positive and negative square loadings, respectively. Red and blue indicate top 100 square loadings corresponding to positive and negative loadings, respectively.

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Chapter 4. Genome-wide association study of wood anatomical, wood chemistry and morphological traits in *Populus trichocarpa*

4.1 Abstract

To understand the genetic mechanisms underlying wood anatomy, wood chemistry and morphological traits in *Populus trichocarpa*, we used 869 unrelated genotypes from a common garden in Clatskanie, OR that were previously collected from across the distribution range in western North America. Using GEMMA mixed model analysis, we tested for the association of 27 phenotypic traits and 19 multitrait combinations with 6.741 million SNPs covering the entire genome. Broad-sense trait heritabilities ranged from 0.117 to 0.477. As expected, traits were correlated within the trait types - anatomical traits were correlated to each other and morphological traits were correlated to each other. Most traits were significantly correlated with geoclimatic variables suggesting the role of climate and geography in shaping the variation of this species. We identified a total of 20 and 33 gene models from single and multitrait GWAS, respectively. Two SNPs from single trait GWAS and 10 SNPs from multitrait GWAS passed a Bonferroni threshold of 7.417×10^{-9} , leading to the identification of two and eight nearby candidate genes, respectively. We have presented here one of the most comprehensive GWAS analyses for *P. trichocarpa* to date including the first GWAS for wood anatomical traits for this species. We identified important genes related to defense mechanisms and abiotic stress tolerance with wood anatomical and wood chemistry GWAS and genes involved in light and hormone signaling pathways with morphological trait GWAS. The identified genes have great potential for optimizing traits for lignocellulosic biofuel production.

4.2 Introduction

It is of increasing interest to identify the molecular variants underlying adaptive and morphological trait variation in plant populations. Loci highlighted by such analyses have great potential for optimizing the trait of interest through genetic engineering or breeding, thereby producing trees with increased productivity, enhanced abiotic stress tolerance, and/or improved quality of end products. Because of their wide geographical distribution and climatic gradients, large effective population sizes, and high genetic variation, forest trees are excellent model systems for understanding local adaptation and the genetic architecture of the complex traits (González-Martínez, Ersoz, Brown, Wheeler, & Neale, 2006; Ingvarsson, Hvidsten, & Street, 2016; Neale & Kremer, 2011; Neale & Savolainen, 2004; Street & Ingvarsson, 2011). In this regard, efforts have been made to optimize the ecologically and economically important tree *Populus* for lignocellulosic biofuel production. Vast amounts of genomic and phenotypic resources are available for the genus. Several large-scale genome-wide association studies have identified the underlying genetic architecture related to morphological, physiological, wood chemistry and disease

resistance traits (Bdeir et al., 2019; McKown, Klápště, et al., 2014; Muchero et al., 2015; Zhang et al., 2018). Furthermore, the biology of wood formation, cell wall ultrastructure and composition, and cell wall recalcitrance are fairly well studied (Allwright et al., 2016; Du et al., 2016, 2018; Du, Pan, Xu, Li, & Zhang, 2013; Du et al., 2019; Escamez et al., 2017; Fahrenkrog et al., 2017; Gandla, Martín, & Jönsson, 2018; Groover, Nieminen, Helariutta, & Mansfield, 2010; Johnson, Kim, Ralph, & Mansfield, 2017; Muchero et al., 2015; Porth et al., 2015; Porth, Klápště, et al., 2013; Porth, Ranjan, et al., 2013; Studer et al., 2011; Wegrzyn et al., 2010; Xi, Song, Sun, Shen, & Li, 2017). However, the genetic architecture underlying wood anatomical traits such as vessel size and density is relatively unknown, despite the importance of these traits for cell wall composition and the overall performance of the tree.

Wood anatomy not only contributes to the structural integrity of the tree, but it is also critical for transport and storage processes (Hietz, Rosner, Hietz-Seifert, & Wright, 2017; Sperry, 2003). Anatomical structures like vessel size and density are related to cell wall structure and composition and wood density. These traits together affect long-distance axial transport of nutrients and hydraulic conductivity. Lignified cell walls and fibers add strength and living parenchyma cells provide radial transport and storage. There are often trade-offs among vessel properties, wood density and hydraulic conductivity (Preston, Cornwell, & DeNoyer, 2006). Wood traits are generally heritable (Carlquist, 2012) and serve as useful traits in phylogenetic analyses (Hietz et al., 2017). Radial variation in wood anatomical properties in the stem affects wood functional traits such as hydraulic conductivity and often scales with tree size and leaf characteristics (Lachenbruch, Moore, & Evans, 2011). Genome-wide association studies of wood anatomy traits undoubtedly complement the current understanding of genetic architecture of other structural and functional traits in *Populus trichocarpa* and will serve as an important step for optimizing traits suitable for lignocellulosic biofuels. Furthermore, GWAS of morphological traits collected from the same tree and the same time period provide a better understanding of the overall genetic architecture of complex traits.

Nevertheless, the limitation of power due to sample size in detecting the genetic variants associated with complex traits is a major hurdle in trees. Despite the recent revolution in the genomic technology, establishment, management, and intensive phenotyping of large common gardens is expensive and logistically challenging. Furthermore, SNP loci and candidate genes identified thus far explain only a small proportion of the genetic variation in complex traits in general (Visscher et al., 2017). Genes controlling complex traits do not work in isolation, but instead are interconnected in networks of thousands of genes, each of which may contribute incrementally to the variation in complex traits (Boyle, Li, & Pritchard, 2017). Recently, methods such as multitrait GWAS and meta analyses using summary statistics have become increasingly popular due to their role in enhancing the power of GWAS and the identification of potentially pleiotropic loci (Porter & O'Reilly, 2017). While no raw data

is required for summary statistics, using raw data for multitrait GWAS can enhance power and lead to discovery of novel associations (Chhetri et al., 2019).

Here we focus on *Populus trichocarpa*, a targeted species for lignocellulosic biofuel production that has a distribution spanning from northern California to northern British Columbia. Tremendous resources including whole-genome resequencing data, multiple common gardens for association mapping, transcriptome and metabolite data and expression networks are available for this species (Chhetri et al., 2019; Weighill et al., 2019). Here we present a genome-wide association study of wood anatomical traits for the first time for this species. We also present a GWAS for important morphological and wood chemistry traits from the same trees that together with anatomical traits affect overall plant productivity. We complemented the single trait GWAS with multitrait analyses. Since this study is based on data collected from a common garden in Clatskanie, OR this allowed for the direct comparison of GWAS genes identified for the same traits from other plantations (Chhetri et al., 2019; Evans et al., 2014).

4.3 Methods

1.1.1 Phenotypic data collection

Wood anatomical, wood chemistry and morphological trait data were collected from a field trial of 1100 *P. trichocarpa* genotypes that was established in Clatskanie, OR in 2009 (Figure 4.1). These genotypes were previously collected from across the natural range of *P. trichocarpa* from northern California to northern British Columbia and were clonally replicated and planted in a randomized block design with three replicates of each genotype at 2 m x 3 m spacing in the field trial (Evans et al., 2014; Slavov et al., 2012).

In June 2012, 557 trees were sampled for wood anatomical traits. Wood cores of 12 mm diameter in size were taken from the main trunk of the tree using an increment borer. Free hand cross-sections were made from the previous year's growth ring and fixed in 70% alcohol. The tissue sections were stained in 1% Safranin O solution for 30 seconds before preparing the slides for imaging. Images at 100x magnification were taken to sample the early, intermediate and late wood from the growth ring. 100x images were used for measuring vessel density, size and number (Figure 4.2). All images were processed using the software *imageJ* to extract quantitative measurements – vessel count, density and size (Schindelin, Rueden, Hiner, & Eliceiri, 2015).

Leaf characteristics were measured for 676 – 687 trees (Table 4.1). The first and second fully expanded leaf (counting from the apex) were collected from a branch receiving full sunlight. One of the leaves was used for measuring petiole length and minimum and maximum petiole diameter with a caliper and then scanned using a hand-held scanner. The *imageJ* software (Schindelin et al., 2015) was used to estimate leaf area, leaf circularity, leaf length, leaf width and leaf perimeter. Dry and wet weights were measured for the same leaf and leaf area and leaf dry weight were used to estimate the specific leaf area

(SLA). Leaf chlorophyll content (SPAD) was assessed using a SPAD 502 Plus meter (Spectrum Technologies) with an average of 3 replicate measures on a leaf section. The second leaf was used for measuring the abaxial stomatal density. Clear nail polish was applied to the broadest part of the leaf close to the midrib. A clear piece of tape was then used to capture an imprint of the epidermal leaf surface. The slides for the imprints were prepared and the number of stomata in 1 mm² area in four random microscopic fields at 400x magnification were counted.

4.3.1 Phenotyping for lignin content and S/G ratio

Details of the methods for estimating lignin content and the ratio of syringyl:guaiacyl (S/G) lignin monomers are fully described in Muchero et al. (2015). Briefly, 1456 wood samples (919 trees sampled in June and 535 in December of 2012) and 1462 (925 trees sampled in June and 537 in December of 2012) were analyzed for lignin content and S/G ratio, respectively from the plantation in Clatskanie, OR. The increment cores were air-dried before estimating lignin content and S/G ratio using pyMBMS analysis.

4.3.2 Statistical analyses

To estimate the genetic control of quantitative traits, broad-sense heritability (H^2) was estimated for all traits using the genotypes with replicate clonal measurements using the following formula:

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + \sigma_E^2},$$

where σ_G^2 is genotypic variance due to clonal differences and σ_E^2 is environmental variance.

Outliers were removed, and the data were evaluated for normality. Variance components were estimated employing the linear regression model with the *lmer* and *ranef* functions of the *lme4* package implemented in R. Genotype and the position of the tree (i.e. row and column) and year (for lignin and S/G ratio) in the garden was used as a random effect in the model. Error was estimated from the residuals of the model. Genetic correlation between the traits was estimated using the Best Linear Unbiased Predictors (BLUPs) from the same model.

4.3.3 Genotypic data

Methods for obtaining genotypic data were as described previously (Chhetri et al., 2019; Evans et al., 2014; Weighill et al., 2018). Briefly, whole genome re-sequencing data was obtained from 1053 trees using Illumina genetic analyzers at the DOE Joint Genome Institute. After removing trees related more closely than first cousins and highly differentiated California trees, 869 trees were left, which were used for all analyses. A genetic relationship matrix was estimated using GEMMA and used as a covariate in the GWAS analyses. Furthermore, SNPs with minor allele frequency ≤ 0.05 and markers with severe departures from Hardy-Weinberg expectations were removed.

4.3.4 Association analysis

The tests for statistical association were employed using Genome-wide Efficient Mixed Model Association package (GEMMA, Zhou & Stephens, 2012, 2014). Phenotypic BLUPs, genetic relationship matrix and 6,741,160 SNPs were used for the association test. Single trait GWAS was run for 27 phenotypes (Table 4.1). The tested model was:

$$y = W\alpha + x\beta + u + \epsilon,$$

where y is an n -vector of phenotypic BLUP values, where n is the number of individuals tested; W is an $n \times c$ matrix of covariates; α is a c -vector of corresponding coefficients, where c is the number of principal coordinate axes used; x is an n -vector of marker genotypes, β is the effect size of the marker, u is an n -vector of random effects that includes a relatedness matrix and ϵ is an n -vector of errors.

Multitrait GWAS was run for 19 multitrait sets (Table 4.2). The same procedure was used for selecting trait sets as described in Chhetri et al. (2019). Multitrait association was conducted with GEMMA using the same model as for single trait associations, except y is an $n \times d$ matrix of d phenotypes for n individuals.

4.3.5 Analyses of association results

We used a P -value cutoff ($\alpha \leq 0.05$) based on the Bonferroni correction criterion of 7.417×10^{-9} and a more liberal P -value cutoff of 1×10^{-7} to identify suggestive associations. For the purpose of summarizing the results, significant SNPs within 10 kb of one another were merged into peaks. Gene models that were closest to significant SNPs were identified based on v3 of the *P. trichocarpa* genome. Annotation information including gene expression level in different plant tissues and annotation of putative gene function was obtained from Phytozome (Goodstein et al., 2012). Percentage of variance explained (PVE) by SNPs was estimated using the formula in Shim et al. (2015).

4.4 Results

4.4.1 Heritabilities of wood anatomy, chemistry, and morphology traits

Overall the broad-sense heritabilities estimated for wood anatomy, chemistry and morphology related traits in this study were low to moderate (Table 4.1). Broad-sense heritabilities for wood anatomy and wood chemistry traits ranged from 0.114 for late wood vessel area to 0.464 for S/G ratio. Late wood anatomy (late wood vessel count, vessel area and vessel size) and lignin content had low heritabilities compared to other wood traits. For morphology related traits, broad-sense heritabilities ranged from 0.122 for specific leaf area to 0.477 for stomatal density.

Narrow sense heritabilities (SNP chip heritabilities) for wood anatomy and chemistry traits ranged from 0.199 for early wood vessel size to 0.708 for S/G ratio whereas for morphology related traits it ranged from 0.038 for specific leaf area to 0.965 for tree height (Table 4.1). Although there was no correlation between broad-sense and SNP chip heritabilities (r not shown), low SNP chip heritability was

generally reflective of low broad sense heritability and there was a significant correlation between the sample size (number of genotypes used for GWAS) and SNP chip heritability values ($r=0.441$, $P=0.021$) for the traits measured in this study (Table 4.1)

4.4.2 Genetic correlation of phenotypic traits within and between the common gardens

As expected, most morphological traits measured in the Clatskanie common garden were correlated to each other – leaf traits such as leaf area, leaf length, leaf dry weight, leaf wet weight, petiole diameter and length, leaf perimeter and leaf aspect ratio were highly significantly correlated to each other and SPAD and stomatal density had low, but significant correlations with most leaf traits (Table 4.3). Similarly, most wood traits were correlated to each other. S/G ratio was significantly correlated with lignin content. Wood anatomy traits such as vessel area and size and vessel count within each of the growth ring regions (early, intermediate or late wood indicating a different maturation stages) were correlated to each other. Wood anatomy traits were also significantly correlated to each other across growth ring areas (Table 4.3). Furthermore, some morphological traits such as tree height and diameter were significantly (although weakly) correlated with wood traits such as S/G ratio, early wood vessel size and area, intermediate wood vessel size and count, and late wood vessel size and count (Table 4.3).

Genetic correlation of the same phenotypic traits measured between the Clatskanie and Corvallis common gardens showed that most traits were weakly, but significantly correlated to each other (Table 4.4). Pairwise genetic correlations of tree height ($r=0.436$, $P<0.001$) and stomatal density (0.345, $P<0.001$) between the sites showed the strongest correlations among all traits compared (Table 4.4).

4.4.3 Phenotypic trait correlations with climate variables

As seen for the phenotypic traits in Corvallis common garden (Chhetri et al., 2019), most phenotypic traits had significant correlations with latitude, and therefore the correlations between the phenotypic traits and other geoclimate variables cannot easily be discerned. Nevertheless, most morphological traits had significant (although weak) correlations with most geoclimatic variables (Table 4.5). Similarly, wood traits such as S/G ratio and late wood vessel area had significant (although weak) correlations with most geoclimatic variables (Table 4.5).

4.4.4 Genes identified from single trait and multitrait GWAS

We performed single trait GWAS with 6.741 million SNPs for 27 morphological and wood anatomical and wood chemistry traits. Only two SNPs passed the Bonferroni correction threshold of $P<7.417\times 10^{-9}$ (Table 4.1). However, we identified a total of 77 SNPs that passed suggestive association P -value cutoff of 1×10^{-7} (Table 4.1, Figure 4.3, Supplementary Figure S4.1). These associated SNPs belonged to 12 separate SNP peaks and were within or close to 20 *P. trichocarpa* gene models (Table 4.6). PVE for significant SNPs ranged from 3.30% to 5.72%.

Multitrait GWAS performed on 19 sets of traits identified 10 SNPs that passed the Bonferroni correction P -value cutoff and 47 SNPs that passed the suggestive association P -value cutoff of 1×10^{-7} and 9 SNPs passed Bonferroni correction threshold of $P < 7.417 \times 10^{-9}$ (Table 4.2, Figure 4.4, Supplementary Figure S4.2). These SNPs belonged to 31 separate SNP peaks and were within or close to 33 *P. trichocarpa* gene models (Table 4.7). PVE of these SNPs ranged from 0.0003% to 6.32% for the individual traits comprising the multitrait set (large table, data not shown). One gene overlapped between single and multitrait GWAS.

4.4.5 Genes identified for wood anatomical and wood chemistry traits

Out of the single trait GWAS for 11 wood anatomical and wood chemistry traits, we identified a total of 5 gene models belonging to 4 traits. A total of 23 gene models were identified for 13 multitrait sets related to wood traits.

4.5 Discussion

4.5.1 Patterns of genetic variation

Broad-sense heritabilities for most traits in this study ranged from 0.2 – 0.4, suggesting that the traits were under moderate genetic control. Heritability estimates for morphological traits were comparable to other similar studies (Chhetri et al., 2019; Evans et al., 2014; Mckown et al., 2014). However, some wood related traits and SLA had low heritabilities (< 0.2), which may be reflective of relatively small sample size (although this is not the case for lignin content) compared to morphological traits. This might also suggest that the environment had a strong influence on these traits, but it seems unlikely that wood anatomical traits would be more susceptible to environmental effects than morphological traits. One possible reason is the variation in micro-environment in the common garden that might affect the growth ring pattern in trees, so that there is more error in estimating the wood anatomy traits from small wood samples. In any case, the results for these low heritability traits should be interpreted with caution.

As reported in previous studies geography has a major role in shaping adaptive trait variation in *P. trichocarpa*. Correlation of these adaptive traits with latitude obscures the actual between-trait and trait-climate relationships (Chhetri et al., 2019). Most morphological traits such as tree height and leaf characteristics had strong significant correlations with latitude compared to previous studies (Chhetri et al., 2019; Mckown et al., 2014). In contrast to Chhetri et al. (2019), tree height including diameter at breast height had a very low or no correlation with leaf traits as in McKown et al. (2014). However, tree height and diameter had significant negative correlations with wood chemistry (S/G ratio) and intermediate and late wood vessel counts and significant positive correlations with vessel size of all maturation stages (early, intermediate and late woods). However, vessel density was positively correlated with latitude and vessel size was negatively correlated with latitude. It is therefore difficult to deconvolute

the indirect effects of latitudinal variation from the direct effects of wood anatomy on productivity in this study.

Further corroboration for the effects of environment shaping these traits can be gleaned from comparing the common gardens. Genetic correlation of the directly comparable traits between the Clatskanie and Corvallis common garden showed very low or no correlations except for stomatal density and tree height (Table 4.4). Furthermore, different loci seem to be controlling phenotypic traits in the two gardens, based on a complete lack of overlap in significant loci discovered by GWAS in the Corvallis, OR common garden (Chhetri et al., 2019). This might be due to differential effects of local environments on phenotypes in each of the respective common gardens.

As temperature decreases, and moisture and precipitation increase with latitude, relationships of the phenotypic traits with the climate variables corroborates the trait relationships explained above. S/G ratio and leaf characteristics including leaf dry weight and petiole length and diameter had significant positive correlation with latitude, mean annual precipitation and relative humidity, but significant negative correlation with mean annual temperature. Similarly, abaxial stomatal density was negatively correlated with precipitation (Table 4.5). Taken together, southern trees had higher S/G ratio, larger but less dense vessels, smaller leaves with higher abaxial stomatal density and low chlorophyll content, and larger tree height and diameter.

S/G ratio was significantly correlated with lignin content and had significant negative correlations with tree height and diameter. S/G ratio increases with latitude and decreases with temperature, but late wood vessel area (similar to the relationship of height and diameter with latitude and temperature) decreases with latitude and increases with temperature.

4.5.2 GWAS genes

The identification of genomic variants (and associated gene models) controlling morphological and anatomical and wood chemistry traits in this study suggests that these traits are under polygenic control as expected. While we found none to only a few significantly associated or suggestive SNPs for most traits we studied here, we think that this is mainly due to lack of power due to sample size although this is one of the most comprehensive GWAS analyses to date in terms of the number of SNPs used. We did not find any gene model controlling more than one trait from our single trait GWAS analyses, but we identified 33 gene models with potential pleiotropic effects controlling sets of morphological, wood anatomical and wood chemistry traits (Table 4.7).

The GWAS analysis for wood anatomical traits we reported here is the first such study in *Populus*. We identified 4 gene models from single trait GWAS and 7 gene models from multitrait sets that include only wood anatomical traits. We found one additional gene model from single trait and 15 additional gene models from multitrait GWAS that included wood chemistry traits, lignin and S/G ratio

independently or in combination with wood anatomical traits. None of the genes we identified here has been reported in earlier studies.

4.5.3 Gene models detected using single trait and multitrait GWAS

Using single and multitrait GWAS for wood anatomy and chemistry and morphology related traits we identified genes implicated in stress responses, defense mechanisms, growth and development, amino acid and hormone transporters, involvement in RNA interference (RNAi) and micro RNA (miRNA) pathways, signaling pathways, cellular and developmental processes, and enzyme inhibitors. However, we also identified a few genes with unknown functions.

The two GWAS hits from the single trait GWAS that passed the Bonferroni threshold of $P < 7.417 \times 10^{-9}$ were both from the GWAS with stomatal density (Figures 4.3k & l). One of these SNPs with P -value 2.77×10^{-9} was within the gene model, Potri.015G117500 that encodes peptide-O-fucosyltransferase. The gene is highly expressed in the first fully expanded leaf, early and mid-spring stems, normal root, root treated with ammonia and nitrate; low expression in early and late female development organs and late fall stems and moderate expression in apical bud, immature and young leaves, early male development, fully open, predormant, early and late dormant and fully open bud, early winter stem, root tip, mid male development, stem treated with ammonia, nitrate and urea, stem node and internode (Figure 4.5). In *Arabidopsis* O-fucosyltransferase affects DELLA and associated regulators such as phytochrome-interacting-factor3 (PIF3) and PIF4 in the brassinosteroid- and light-signaling pathways (Zentella et al., 2017). DELLA proteins are important integrators of multiple signaling pathways in flowering plants. DELLA are repressors of phytohormone (GA) signaling, master growth repressors that restrict plant growth by affecting genes in cell division, expansion and differentiation (Zentella et al., 2007). In *Arabidopsis* O-fucosyltransferase modifies DELLA protein RGA (repressor of *gal-3*). The GA-deficient mutant *gal-3* is a nongerminating, extreme dwarf that flowers late and produces male-sterile flowers. This protein is also known to be involved in cell-to-cell adhesion (deficiency in pectin biosynthesis pathway leading to loss of cell adhesion) together with *GAUT8* (QUASIMODO1, see below) in *Arabidopsis* (Verger, Chabout, Gineau, & Mouille, 2016). McKown, Guy, et al. (2014) identified a similar gene, Potri.004G059000, encoding BRASSINOSTEROID-INSENSITIVE 2 for stomatal density based on the GWAS on 34K SNP array data for 464 *P. trichocarpa* trees. BRASSINOSTEROID-INSENSITIVE 2 potentially affects the brassinosteroid signaling pathway and the regulation of stomatal development (Gudesblat et al., 2012; Khan et al., 2013). The other top GWAS hit for stomatal density was within a gene model Potri.015G117300 of unknown function. The gene is highly expressed in early and mid-spring stems, stem treated with ammonia, nitrate and urea, root tip, the first fully expanded and young leaves and fully open bud (Figure 4.5)

We identified a total of 8 genes based on the 10 SNPs that passed Bonferroni threshold in the multitrait GWAS, of which 4 genes were detected for the leaf morphology multitrait, (leaf area, leaf dry weight, leaf length and leaf width) and 4 genes were detected for 4 wood anatomy and wood chemistry related multitrait GWASs (Figures 4.4 & 4.7). The gene model near the top GWAS hit (SNP P -value= 3.62×10^{-13}) for leaf morphology multitrait was Potri.010G032600, which encodes a small subunit (40S) ribosomal protein S11. Ribosomal proteins are involved in gene regulation, cellular functions and development mechanisms, apoptosis and aging (Bhavsar, Makley, & Tsonis, 2010). The SNP is 9.5kb downstream of this gene. No expression data is available for this gene (Figure 4.6). There are at least 249 genes encoding 80 ribosomal proteins in *Arabidopsis* (Barakat et al., 2001) and are known to have effects in growth and development (Devis, Firth, Liang, & Byrne, 2015).

With leaf morphology multitrait we also identified the gene Potri.019G067300, 10.5kb downstream of a significant SNP (P -value= 1.9×10^{-8}) that encodes a protein related to galactosyltransferase-8 (*GAUT8*). A gene with a similar function, Potri.004G111000, that encodes *GAUT9* was detected for the same multitrait GWAS from the Corvallis common garden (Chhetri et al., 2019). *GAUT8* and *GAUT9* belonged to the same clade B1 in a phylogenetic tree based on the *GAUT* protein family of *Arabidopsis thaliana* and *P. trichocarpa* (Biswal et al., 2018). *GAUT9* affects leaf size in *Populus deltoides* (Chhetri et al., 2019) whereas *GAUT8* (also known as QUASIMODO1) has high expression in stems in *Arabidopsis* (Caffall, Pattathil, Phillips, Hahn, & Mohnen, 2009) and is thought to be involved in cell wall pectic homogalacturonan (HG) and xylan biosynthesis (Orfila et al., 2005; Verger et al., 2016). Genes encoding glycosyltransferases, a large family of enzymes mainly involved in biosynthesis of polysaccharides and glycoproteins in the plant cell wall (Hansen, Harholt, Oikawa, & Scheller, 2012), were also detected in single trait GWAS for stomatal density and leaf aspect ratio in this study (Table 4.6 & 4.7).

The gene model Potri.006G275800 was found 8.7kb downstream of the top GWAS hit for wood multitrait 1 (vessel area and vessel count for early, intermediate and late wood, and lignin and S/G ratio; SNP P -value= 1.03×10^{-9} , Table 4.4) and for the top two GWAS hits for wood anatomy multitrait 4 (intermediate wood vessel area and count, both SNPs with P -value= 3.99×10^{-8}). The gene encodes leucine rich repeat-containing protein that has a function in defense. A gene with a similar annotation (Potri.012G028700) was detected for leaf morphology multitrait (4th top GWAS hit for leaf morphology multitrait, SNP P -value= 5×10^{-9} , gene found 1.3kb upstream of the SNP) as well (Table 4.4). This suggests functional relationships among wood anatomical, wood chemistry and morphological traits.

The gene model Potri.019G037900 was found 1.2kb upstream of the second best GWAS hit for wood multitrait 5 (intermediate wood vessel size, lignin and S/G ratio; SNP P -value= 1.77×10^{-9}) which encodes for double-stranded-RNA-binding proteins. In *Arabidopsis*, the biogenesis of small RNA

(miRNA and tasiRNA) requires the involvement of DOUBLE-STRANDED RNA BINDING (DRB) proteins (Eamens, Kim, Curtin, & Waterhouse, 2012; Eamens, Smith, Curtin, Wang, & Waterhouse, 2009). Small RNAs are involved in regulating the expression of genes related to growth and development. The same gene Potri.019G037900 (and the SNP) was detected in single trait GWAS for intermediate wood vessel size as well, but the SNP had a weaker association (P -value= 8.44×10^{-9}).

The gene model Potri.004G183900 was found 4.6kb upstream of the top GWAS hit for wood anatomy multitrait 5 (late wood vessel area and count, SNP P -value= 5.91×10^{-9}) which encodes a receptor like kinase (RLK), serine/threonine protein kinase. RLKs play major roles in defense mechanisms (e.g. disease resistance) and development functions in plants (Afzal, Wood, & Lightfoot, 2008). Genes encoding L-type RLKs are involved in resistance to the fungal pathogen *Sphaerulina musiva* in *P. trichocarpa* (Muchero et al., 2018). Wood anatomy is related to lignin composition in plants and lignin biosynthesis genes are involved in defense mechanisms (Moura, Bonine, de Oliveira Fernandes Viana, Dornelas, & Mazzafera, 2010; Xie et al., 2018).

The gene model Potri.017G090200 was found 1.8kb upstream of the 2nd top GWAS hit for wood multitrait 1, (SNP P -value= 7.32×10^{-9}) and encodes calmodulin-binding protein. Calmodulin (CaM) and calmodulin-like proteins (CMLs) are one of the three main families of calcium (Ca^{2+}) sensor proteins in plants (Ranty et al., 2016). Calmodulin (CaM) and calmodulin-like proteins (CMLs) are well characterized in plants and are involved in regulating plant responses to abiotic stresses (Zeng et al., 2015).

One of the calmodulin binding proteins, EF-hand motif that is involved in cell signaling, developmental processes and biotic and abiotic responses was detected in the multitrait GWAS from Corvallis, OR plantation that included carbon isotope, leaf area and stomatal density in the multitrait set (Chhetri et al., 2019). Here we identified a gene with similar role controlling wood anatomy traits. Wood anatomy traits have potential functional relationships with carbon isotope, leaf area and stomatal density.

4.5.4 Comparison with previous studies

We compared our GWAS results with the GWAS from the previous studies for the same traits in *P. trichocarpa* (Chhetri et al., 2019; Evans et al., 2014; McKown, Klápště, et al., 2014; Porth, Ranjan, et al., 2013; Wegrzyn et al., 2010), but we did not find any overlapping gene models. This may be due to a) differential environmental effects on the traits due to plantation sites, b) traits measured in different seasons of the year and different years were subjected to varied environmental pressures, c) variability in the microenvironment within the sites as reflected by the different broad-sense heritability values, d) differences in the developmental stages of the traits across the studies and e) difference in the genotypes and the sequence data types (e.g. SNP array vs whole genome sequence data). Furthermore, we were not able to replicate any significant SNP hits or the gene models for the same traits from another *P.*

trichocarpa common garden in Corvallis, OR in our study, although several single traits such as stomatal density, petiole diameter and tree height and the multitrait set containing leaf area, leaf dry weight, leaf length and leaf width were common between the two common gardens [Clatskanie, OR (this study) and Corvallis, OR] and measurements were performed using the same methods. This might very well reflect the differential influence of the environment on genotypes in the two populations. Nevertheless, we believe that our comprehensive GWAS study with genome-wide sequencing data highlights the underlying genes controlling adaptive traits in *P. trichocarpa* and complements the findings from other similar studies. Moreover, we have reported here genes underlying complex wood anatomy traits in *Populus* for the first time.

4.6 Conclusion

We presented here the first comprehensive GWAS for wood anatomical traits in *Populus* that provides an insight into the type of genes controlling structural and functional properties important for plant development, function and stress tolerance. We complemented this with GWAS for important wood chemistry and morphological traits that have functional relationships with wood anatomical traits. As shown in previous *P. trichocarpa* GWAS study in another common garden (Chhetri et al. 2019), we achieved more power with multitrait GWAS in this study as well. Furthermore, the multitrait sets formed based on the genetic correlations and the functional relationship of the traits within and among the wood anatomical, wood chemistry and morphological traits provided insight into pleiotropic genes controlling these traits. Some of the genes we identified in this study had no known functions which might be interesting to explore further. Genes identified here, especially the genes controlling wood anatomical traits, can be good targets for biotechnology experiment for optimizing wood traits for biofuel production. However, as is the case with most GWAS studies, despite the use of largescale whole genome resequencing data and much broader sampling compared to most of the previous similar studies and with the use of multitrait GWAS, a very small percent of the variation in the traits were explained by the significant SNPs. This warrants a large and homogeneous sampling from across the range of distribution for future studies. Moreover, none of the genetic variants detected in the GWAS from another common garden was duplicated in this study. This may be because of the differential effect of environment between the common gardens, which is somewhat reflected by very low correlation of the traits between the two common gardens. Furthermore, additional variation due to the difference in the timing (different years) of data collection might have influenced the GWAS study.

4.7 Tables and figures

Table 4.1 Broad-sense heritability estimates (H^2) and the number of SNP-trait associations for wood chemistry and anatomical and morphological traits in *P. trichocarpa*.

Trait	H^2	Genotypes	Total trees ^a	SNPs <1×10 ^{-7b}	Chip_ H^2 (+/- CI) ^c
Wood anatomy and chemistry					
Early wood vessel area	0.335	489	548 (59)	0	0.542 (+/- 0.545)
Early wood vessel count	0.38	489	548 (59)	1	0.611 (+/- 0.582)
Early wood vessel size	0.392	489	548 (59)	0	0.199 (+/- 0.325)
Intermediate wood vessel area	0.196	570	633 (63)	0	0.475 (+/- 0.300)
Intermediate wood vessel count	0.233	570	633 (63)	0	0.562 (+/- 0.484)
Intermediate wood vessel size	0.218	570	633 (63)	2	0.301 (+/- 0.406)
Late wood vessel area	0.114	557	636 (79)	0	0.373 (+/- 0.359)
Late wood vessel count	0.146	557	636 (79)	0	0.327 (+/- 0.388)
Late wood vessel size	0.22	557	636 (79)	1	0.373 (+/- 0.359)
Lignin content	0.177	830	1456 (492)	0	0.36 (+/- 0.355)
S/G ratio	0.464	831	1462 (494)	1	0.708 (+/- 0.312)
Morphology					
Diameter (breast height)	0.227	869	2438 (860)	9	0.929 (+/- 0.278)
Height	0.294	869	2438 (860)	0	0.965 (+/- 0.257)
Leaf area	0.434	676	813 (137)	0	0.87 (+/- 0.349)
Leaf aspect ratio	0.251	676	813 (137)	2	0.439 (+/- 0.320)
Leaf circularity	0.285	676	813 (137)	6	0.313 (+/- 0.318)
Leaf dry weight	0.452	685	836 (151)	18	0.99 (+/- 0.325)
Leaf length	0.477	676	813 (137)	0	0.679 (+/- 0.320)
Leaf perimeter	0.459	676	813 (137)	0	0.786 (+/- 0.357)
Leaf wet weight	0.393	687	842 (155)	26	0.985 (+/- 0.351)
Leaf width	0.386	676	813 (137)	0	0.905 (+/- 0.355)

Trait	H^2	Genotypes	Total trees ^a	SNPs <1×10 ^{-7b}	Chip_ H^2 (+/- CI) ^c
Maximum petiole diameter	0.426	687	840 (153)	1	0.928 (+/- 0.323)
Minimum petiole diameter	0.249	683	834 (151)	0	0.843 (+/- 0.345)
Petiole length	0.397	685	839 (154)	0	0.987 (+/- 0.335)
SPAD	0.214	687	843 (156)	0	0.232 (+/- 0.282)
Specific leaf area	0.122	667	797 (130)	0	0.038 (+/- 0.318)
Stomatal density	0.477	721	884 (163)	9 (2)	0.597 (+/- 0.294)

^aNumber of genotypes with replicates in parentheses

^bNumbers in parentheses indicate significant P -value that passed Bonferroni correction threshold of $P < 7.417 \times 10^{-9}$.

^cMean Chip Heritability values for phenotypes with confidence interval (CI)

Note: All RLRTpvalues were highly significant except for late wood vessel area, late wood vessel count and specific leaf area, which was not significant.

Table 4.2 List of traits used for multitrait associations and significant SNPs identified in *P. trichocarpa*.

Trait combination	Abbreviation	Trait name	PopN ^a	SNPs <1×10 ^{-7bc}
Diameter (breast height), height, lignin content, S/G ratio	DB_HT_LI_SG	morphology and wood chemistry trait 1	830	0
Diameter (breast height), lignin content, S/G ratio, early wood vessel area	DB_LI_SG_EWva	morphology and wood multitrait 1	485	0
Early wood vessel area, early wood vessel count	EWva_EWvc	wood anatomy multitrait 1	489	3
Early wood vessel area, early wood vessel count, intermediate wood vessel area, intermediate wood vessel count, lignin content, late wood vessel area, late wood vessel count, S/G ratio	EWva_EWvc_IWva_IWvc_LI_LWva_LWvc_SG	wood multitrait 1	407	7 (2)
Early wood vessel area, early wood vessel count, intermediate wood vessel area, intermediate wood vessel count, late wood vessel area, late wood vessel count	EWva_EWvc_IWva_IWvc_LWva_LWvc	wood anatomy multitrait 1	411	2
Early wood vessel area, intermediate wood vessel area, late wood vessel area	EWva_IWva_LWva	wood anatomy multitrait 2	411	0
Early wood vessel area, leaf area, stomatal density	EWva_LA_SD	morphology and wood anatomy multitrait 1	425	5
Early wood vessel area, lignin content, S/G ratio	EWva_LI_SG	wood multitrait 2	485	0
Early wood vessel count, intermediate wood vessel count, late wood vessel count	EWvc_IWvc_LWvc	wood anatomy multitrait 2	411	1
Early wood vessel count, lignin content, S/G ratio	EWvc_LI_SG	wood multitrait 3	485	0
Early wood vessel size, intermediate wood vessel size, lignin content, S/G ratio, late wood vessel size	EWvs_IWvs_LI_SG_LWvs	wood multitrait 4	407	3
Early wood vessel size, intermediate wood vessel size, late wood vessel size	EWvs_IWvs_LWvs	wood anatomy multitrait 3	411	0
Intermediate wood vessel size, intermediate wood vessel count	IWva_IWvc	wood anatomy multitrait 4	570	3 (1)
Intermediate wood vessel size, lignin content, S/G ratio	IWvs_LI_SG	wood multitrait 5	566	2 (1)
Leaf area, leaf dry weight, leaf length, leaf width	LA_LD_LL_LW	leaf morphology multitrait	674	9 (4)
Lignin content, late wood vessel size, S/G ratio	LI_LWvs_SG	wood multitrait 6	552	4
Lignin content, S/G ratio	LI_SG	wood chemistry multitrait	830	2
Lignin content, specific leaf area, stomatal density, early wood vessel area	LI_SL_SD_EWva	morphology and wood multitrait 2	415	2
Late wood vessel area, late wood vessel count	LWva_LWvc	wood anatomy multitrait 5	557	3 (1)

^aNumber of unique genotypes^bNumber of significant SNPs in multi-trait GWAS^cNumber in parenthesis indicate the number of significant P-values that passed Bonferroni correction threshold of $P < 7.417 \times 10^{-9}$

Table 4.3 Pairwise correlation (r) of *P. trichocarpa* wood chemistry, anatomical and morphological traits in Clatskanie, OR. Values greater than 0.14 or less than -0.14 are significant based on the Bonferroni correction criteria at 5% significance level.

Trait																												
Diameter (breast height)	Diameter (breast height)																											
Height	0.816	Height																										
Leaf area	0.028	-0.039	Leaf area																									
Leaf perimeter	0.042	-0.012	0.952	Leaf perimeter																								
Leaf circularity	-0.046	-0.072	0.064	-0.192	Leaf circularity																							
Leaf length	0.031	-0.016	0.898	0.958	-0.243	Leaf length																						
Leaf width	0.004	-0.057	0.958	0.903	0.179	0.804	Leaf width																					
Leaf aspect ratio	0.060	0.078	-0.244	-0.084	-0.654	0.117	-0.460	Leaf aspect ratio																				
Leaf dry weight	0.006	-0.057	0.894	0.880	-0.039	0.868	0.840	-0.131	Leaf dry weight																			
Leaf wet weight	0.051	-0.022	0.934	0.898	0.014	0.848	0.877	-0.187	0.931	Leaf wet weight																		
Letiole length	-0.064	-0.081	0.601	0.620	-0.054	0.631	0.619	-0.144	0.707	0.566	Letiole length																	
Petiole diameter (max)	0.029	-0.047	0.819	0.793	0.029	0.753	0.782	-0.181	0.837	0.880	0.551	Petiole diameter (max)																
Petiole diameter (min)	0.034	-0.032	0.760	0.735	0.030	0.677	0.726	-0.182	0.725	0.828	0.386	0.875	Petiole diameter (min)															
Specific leaf area	0.060	0.027	-0.007	-0.099	0.304	-0.199	0.021	-0.275	-0.322	-0.104	-0.377	-0.117	-0.039	Specific leaf area														
SPAD	0.124	0.105	0.241	0.307	-0.214	0.375	0.215	0.179	0.398	0.279	0.455	0.253	0.148	-0.499	SPAD													
Stomatal density	-0.112	-0.063	-0.198	-0.174	-0.043	-0.157	-0.175	0.028	-0.187	-0.234	-0.058	-0.220	-0.224	-0.045	-0.047	Stomatal density												
Lignin content	-0.125	-0.096	-0.040	-0.037	-0.038	-0.021	-0.054	0.076	0.002	-0.022	0.032	-0.035	-0.063	-0.025	-0.051	0.020	Lignin content											
S/G ratio	-0.197	-0.215	-0.004	-0.011	0.024	-0.003	-0.004	0.028	0.016	-0.011	0.023	-0.035	-0.036	0.022	-0.059	0.079	0.383	S/G ratio										
Early wood vessel count	-0.065	-0.003	-0.065	-0.058	-0.002	-0.046	-0.060	0.058	-0.081	-0.058	-0.067	-0.062	-0.047	-0.004	-0.057	0.052	0.078	0.037	Early wood vessel count									
Early wood vessel area	0.243	0.266	0.047	0.047	0.030	0.037	0.059	-0.027	0.015	0.041	0.054	0.039	0.013	0.030	0.028	-0.030	-0.020	-0.096	0.557	Early wood vessel area								
Early wood vessel size	0.296	0.246	0.059	0.054	0.035	0.018	0.075	-0.113	0.026	0.026	0.066	0.037	0.003	0.102	0.026	-0.049	-0.080	-0.105	-0.432	0.345	Early wood vessel size							
Intermediate wood vessel count	-0.305	-0.217	-0.028	-0.025	-0.015	-0.007	-0.032	0.049	-0.014	-0.011	-0.057	-0.017	-0.010	-0.089	-0.142	0.049	0.140	0.056	0.449	0.023	-0.366	Intermediate wood vessel count						
Intermediate wood vessel area	-0.052	0.018	0.020	0.036	-0.054	0.042	0.022	0.030	0.020	0.040	0.010	0.031	0.006	-0.057	-0.067	-0.013	0.107	0.029	0.268	0.343	0.043	0.610	Intermediate wood vessel area					
Intermediate wood vessel size	0.301	0.231	0.051	0.070	-0.070	0.068	0.039	0.033	0.036	0.062	0.064	0.053	0.021	0.075	0.109	-0.077	-0.106	-0.073	-0.189	0.249	0.408	-0.496	0.175	Intermediate wood vessel size				
Late wood vessel count	-0.231	-0.188	-0.013	-0.017	0.011	-0.013	-0.017	0.017	-0.016	-0.009	-0.019	0.022	0.019	-0.010	-0.148	0.060	0.157	0.072	0.349	0.117	-0.217	0.586	0.399	-0.296	Late wood vessel count			
Late wood vessel area	-0.038	0.045	-0.077	-0.067	-0.020	-0.072	-0.083	0.043	-0.090	-0.038	-0.118	-0.036	0.001	0.047	-0.142	-0.002	0.167	0.025	0.274	0.250	-0.034	0.403	0.531	0.035	0.633	Late wood vessel area		
Late wood vessel size	0.167	0.191	-0.062	-0.056	-0.025	-0.057	-0.077	0.037	-0.073	-0.040	-0.105	-0.071	-0.051	0.064	-0.025	-0.068	-0.010	-0.006	-0.052	0.073	0.145	-0.176	0.016	0.265	-0.426	0.193	Late wood vessel size	

Table 4.4 Pearson correlation (r) of *P. trichocarpa* wood chemistry, anatomical and morphological traits in Clatskanie, OR with morphological and physiological traits in Corvallis, OR. Values greater than 0.14 or less than -0.14 are significant based on the Bonferroni correction criteria at 5% significance level.

CLATSKANIE	Traits	CORVALLIS																	
		Water	Stomatal	Petiole		Petiole	Specific	Leaf	Leaf	Leaf	Leaf	Leaf	Leaf	Leaf	Carbon	Bud	Bud set		Height
		potential	density	SPAD	length	diameter (max)	leaf area	area	perimeter	length	width	feret	aspect ratio	dry weight	isotope	flush	Bud set	Height	
	Diameter (breast height)	0.09	-0.09	-0.07	0.21	0.17	0.15	0.26	0.28	0.26	0.24	0.25	-0.09	0.18	-0.01	0.23	-0.42	0.38	
	Height	0.13	-0.06	-0.05	0.23	0.15	0.14	0.25	0.25	0.22	0.26	0.22	-0.15	0.17	0.00	0.25	-0.45	0.44	
	Leaf area	-0.01	-0.12	0.04	0.05	0.09	-0.17	0.10	0.08	0.10	0.07	0.09	0.02	0.14	0.03	-0.15	0.26	-0.15	
	Leaf perimeter	-0.01	-0.12	0.07	0.08	0.11	-0.18	0.12	0.11	0.14	0.08	0.13	0.05	0.16	0.05	-0.12	0.26	-0.13	
	Leaf circularity	0.01	0.04	-0.06	-0.10	-0.08	0.04	-0.05	-0.09	-0.10	-0.02	-0.10	-0.05	-0.05	-0.04	-0.13	0.03	-0.09	
	Leaf length	-0.03	-0.12	0.07	0.07	0.11	-0.18	0.11	0.11	0.15	0.06	0.14	0.07	0.15	0.05	-0.12	0.26	-0.12	
	Leaf width	0.00	-0.08	0.04	0.01	0.06	-0.15	0.06	0.04	0.05	0.05	0.04	0.00	0.11	0.02	-0.18	0.29	-0.19	
	Leaf aspect ratio	-0.02	-0.07	0.06	0.08	0.09	-0.01	0.09	0.13	0.17	0.03	0.17	0.11	0.06	0.05	0.15	-0.12	0.16	
	Leaf dry weight	-0.05	-0.10	0.05	0.02	0.10	-0.23	0.10	0.08	0.10	0.07	0.08	0.01	0.15	0.03	-0.14	0.30	-0.15	
	Leaf wet weight	-0.02	-0.14	0.05	0.07	0.15	-0.20	0.17	0.15	0.16	0.13	0.15	-0.02	0.20	0.04	-0.10	0.23	-0.12	
	Petiole length	-0.03	-0.02	0.10	0.02	0.01	-0.20	-0.05	-0.05	-0.03	-0.06	-0.04	0.07	0.01	0.01	-0.19	0.40	-0.26	
	Petiole diameter (max)	-0.02	-0.12	0.03	0.02	0.13	-0.19	0.12	0.10	0.11	0.11	0.10	-0.03	0.15	0.05	-0.08	0.26	-0.12	
	Petiole diameter (min)	-0.01	-0.13	0.04	0.03	0.15	-0.17	0.14	0.13	0.13	0.12	0.12	-0.02	0.16	0.05	-0.05	0.20	-0.10	
	Specific leaf area	0.00	-0.03	-0.09	0.02	-0.04	0.09	0.01	0.01	0.02	-0.01	0.02	0.03	-0.02	0.02	-0.02	-0.10	-0.01	
	SPAD	-0.05	-0.08	0.20	-0.03	0.01	-0.15	-0.05	-0.05	-0.04	-0.04	-0.04	0.02	-0.01	0.08	0.00	0.20	-0.05	
	Stomatal density	-0.02	0.35	-0.09	-0.09	-0.10	0.17	-0.16	-0.16	-0.18	-0.11	-0.18	-0.03	-0.18	-0.09	0.06	-0.01	-0.04	
	Lignin content	0.00	0.00	-0.02	-0.01	-0.03	-0.05	-0.05	-0.04	-0.05	-0.04	-0.05	0.00	-0.02	0.01	-0.01	0.03	-0.07	
	S/G ratio	-0.07	-0.03	0.10	-0.09	-0.07	-0.08	-0.07	-0.05	-0.03	-0.09	-0.03	0.08	-0.04	0.03	-0.11	0.18	-0.17	
	Early wood vessel count	0.07	-0.03	-0.03	0.03	0.04	0.06	0.04	0.03	0.01	0.05	0.02	-0.03	0.05	-0.01	0.09	-0.13	0.12	
	Early wood vessel area	0.08	-0.09	-0.08	0.09	0.09	0.09	0.13	0.13	0.11	0.13	0.11	-0.06	0.13	-0.09	0.07	-0.16	0.15	
	Early wood vessel size	-0.02	-0.06	-0.06	0.05	0.04	0.04	0.05	0.07	0.07	0.05	0.06	-0.01	0.05	-0.01	-0.04	-0.03	0.03	
	Intermediate wood vessel count	0.06	0.07	0.02	-0.01	-0.01	0.00	0.02	0.00	-0.02	0.01	-0.01	-0.03	0.03	0.01	0.07	-0.05	0.06	
	Intermediate wood vessel area	0.06	-0.03	0.01	0.02	0.03	-0.02	0.09	0.09	0.06	0.09	0.07	-0.05	0.09	-0.02	0.10	-0.10	0.12	
	Intermediate wood vessel size	-0.06	-0.10	-0.02	0.03	0.06	-0.01	0.09	0.10	0.10	0.09	0.10	-0.01	0.05	-0.02	0.03	-0.11	0.05	
	Late wood vessel count	0.05	-0.03	-0.07	0.03	-0.01	0.04	0.02	0.01	0.00	0.02	0.01	-0.01	0.04	0.01	0.06	0.01	0.05	
	Late wood vessel area	0.05	-0.05	0.00	0.14	0.10	-0.01	0.13	0.13	0.11	0.13	0.11	-0.06	0.14	-0.05	0.15	-0.16	0.19	
	Late wood vessel size	-0.05	-0.02	0.06	0.06	0.04	-0.03	0.06	0.09	0.07	0.06	0.06	-0.04	0.04	-0.02	0.09	-0.16	0.11	

Table 4.5 Pearson correlation (r) of *P. trichocarpa* wood chemistry, anatomical and morphological traits in Clatskanie, OR with morphological and physiological traits in Corvallis, OR. Values greater than 0.15 or less than -0.15 are significant based on the Bonferroni correction criteria at 5% significance level.

Trait/Geoclimate	Longitude	Latitude	Elevation	MAT	MWMT	MCMT	TD	MAP	MSP	AHM	SHM	DD_0	DD5	DD_18	DD18	NFFD	bFPF	eFPF	FPF	PAS	EMT	EXT	Eref	CMD	MAR	RH	PC1	PC2	PC3	PC4	
MORPHOLOGY	Height	0.346	-0.342	-0.161	0.325	0.119	0.372	-0.386	-0.157	-0.113	0.200	0.057	-0.363	0.266	-0.333	0.135	0.340	-0.261	0.323	0.296	-0.346	0.349	0.153	0.282	0.100	0.116	-0.057	0.320	-0.105	-0.208	-0.153
	Diameter (breast height)	0.288	-0.288	-0.128	0.271	0.110	0.316	-0.325	-0.096	-0.073	0.145	0.029	-0.310	0.220	-0.279	0.107	0.287	-0.224	0.272	0.250	-0.294	0.302	0.128	0.233	0.071	0.103	-0.029	0.265	-0.111	-0.161	-0.143
	Leaf area	-0.049	0.280	0.148	-0.210	-0.126	-0.198	0.176	0.277	0.345	-0.301	-0.307	0.162	-0.212	0.208	-0.165	-0.175	0.124	-0.151	-0.140	0.201	-0.170	-0.232	-0.284	-0.340	-0.074	0.243	-0.244	-0.220	0.160	0.002
	Leaf perimeter	-0.012	0.268	0.119	-0.184	-0.107	-0.180	0.164	0.225	0.306	-0.258	-0.283	0.148	-0.183	0.182	-0.145	-0.154	0.105	-0.133	-0.121	0.164	-0.157	-0.207	-0.255	-0.308	-0.072	0.211	-0.214	-0.195	0.152	0.011
	Leaf circularity	-0.123	0.081	0.120	-0.127	-0.082	-0.118	0.104	0.120	0.081	-0.121	-0.058	0.102	-0.129	0.129	-0.089	-0.127	0.117	-0.119	-0.121	0.138	-0.113	-0.086	-0.113	-0.079	-0.013	0.061	-0.133	-0.011	0.027	-0.012
	Leaf length	-0.004	0.268	0.110	-0.176	-0.114	-0.167	0.145	0.222	0.313	-0.258	-0.294	0.133	-0.179	0.173	-0.145	-0.141	0.095	-0.121	-0.110	0.152	-0.143	-0.216	-0.256	-0.319	-0.065	0.222	-0.208	-0.213	0.139	0.016
	Leaf width	-0.099	0.297	0.166	-0.237	-0.128	-0.232	0.217	0.281	0.331	-0.307	-0.287	0.197	-0.232	0.237	-0.170	-0.210	0.156	-0.185	-0.173	0.233	-0.206	-0.228	-0.296	-0.324	-0.083	0.229	-0.269	-0.184	0.176	0.014
	Leaf aspect ratio	0.158	-0.107	-0.114	0.142	0.037	0.154	-0.168	-0.130	-0.082	0.129	0.037	-0.144	0.124	-0.146	0.060	0.150	-0.124	0.140	0.134	-0.167	0.144	0.054	0.117	0.062	0.049	-0.046	0.145	-0.022	-0.106	-0.005
	Leaf dry weight	-0.024	0.302	0.178	-0.230	-0.130	-0.219	0.200	0.310	0.392	-0.329	-0.347	0.181	-0.228	0.228	-0.168	-0.196	0.137	-0.170	-0.155	0.224	-0.188	-0.243	-0.305	-0.381	-0.051	0.259	-0.268	-0.245	0.199	-0.030
	Leaf wet weight	0.033	0.233	0.130	-0.161	-0.086	-0.149	0.138	0.290	0.371	-0.297	-0.321	0.115	-0.164	0.159	-0.121	-0.128	0.085	-0.102	-0.095	0.164	-0.122	-0.191	-0.236	-0.347	-0.051	0.235	-0.200	-0.256	0.190	-0.062
	Lenticel length	-0.200	0.395	0.163	-0.305	-0.186	-0.312	0.286	0.239	0.326	-0.288	-0.295	0.278	-0.288	0.305	-0.219	-0.280	0.206	-0.267	-0.240	0.278	-0.288	-0.272	-0.354	-0.330	-0.108	0.205	-0.331	-0.138	0.170	0.104
	Petiole diameter (max)	0.032	0.240	0.152	-0.178	-0.076	-0.175	0.174	0.302	0.370	-0.306	-0.312	0.140	-0.172	0.178	-0.108	-0.155	0.099	-0.129	-0.115	0.191	-0.150	-0.178	-0.240	-0.340	-0.033	0.215	-0.215	-0.231	0.225	-0.081
	Petiole diameter (min)	0.045	0.182	0.108	-0.123	-0.054	-0.118	0.115	0.241	0.305	-0.246	-0.257	0.095	-0.120	0.123	-0.070	-0.101	0.055	-0.077	-0.067	0.136	-0.098	-0.141	-0.184	-0.280	-0.048	0.178	-0.155	-0.205	0.178	-0.067
	Specific leaf area	-0.029	-0.057	-0.015	0.032	0.009	0.037	-0.040	0.011	-0.035	0.017	0.054	-0.038	0.021	-0.032	-0.004	0.025	0.006	0.023	0.007	-0.018	0.028	0.034	0.051	0.055	-0.009	-0.039	0.029	0.022	-0.040	-0.029
	SPAD	-0.060	0.152	-0.004	-0.084	-0.035	-0.100	0.101	0.016	0.107	-0.052	-0.106	0.082	-0.072	0.083	-0.053	-0.073	0.020	-0.077	-0.048	0.041	-0.090	-0.088	-0.117	-0.112	-0.077	0.073	-0.088	-0.047	0.072	0.105
	Stomatal density	-0.107	-0.157	-0.016	0.048	0.041	0.035	-0.024	-0.107	-0.182	0.138	0.182	0.000	0.069	-0.044	0.079	0.011	0.024	0.002	-0.011	-0.016	0.020	0.139	0.154	0.216	0.030	-0.223	0.073	0.201	-0.072	-0.043
WC	Lignin content	-0.077	0.052	0.047	-0.068	-0.044	-0.065	0.055	0.030	0.019	-0.027	0.003	0.062	-0.067	0.068	-0.055	-0.065	0.062	-0.066	-0.064	0.058	-0.062	-0.056	-0.059	-0.015	-0.035	0.015	-0.065	0.020	-0.005	0.029
	S/G ratio	-0.192	0.243	0.168	-0.264	-0.155	-0.270	0.247	0.097	0.051	-0.128	-0.041	0.252	-0.248	0.265	-0.190	-0.261	0.218	-0.261	-0.243	0.248	-0.263	-0.179	-0.242	-0.078	-0.111	0.055	-0.255	0.076	0.042	0.096
WOOD ANATOMY	Early wood vessel count	0.020	-0.131	-0.042	0.103	0.059	0.103	-0.100	0.025	-0.011	0.012	0.011	-0.099	0.098	-0.104	0.080	0.090	-0.056	0.103	0.080	-0.057	0.093	0.089	0.121	0.045	0.015	-0.059	0.091	-0.025	-0.005	-0.090
	Early wood vessel area	0.148	-0.102	-0.015	0.103	0.052	0.115	-0.120	0.024	0.046	0.011	-0.068	-0.132	0.079	-0.106	0.044	0.111	-0.087	0.121	0.104	-0.093	0.114	0.035	0.073	-0.040	0.086	0.041	0.087	-0.110	-0.014	-0.090
	Early wood vessel size	0.059	0.031	0.017	-0.007	-0.002	0.002	-0.005	-0.008	0.042	0.006	-0.055	-0.027	-0.025	0.004	-0.043	0.010	-0.021	0.002	0.013	-0.031	0.011	-0.048	-0.045	-0.061	0.039	0.092	-0.009	-0.061	-0.012	0.030
	Intermediate wood vessel count	0.043	-0.116	-0.050	0.102	0.109	0.079	-0.045	-0.019	-0.054	0.040	0.079	-0.072	0.114	-0.098	0.129	0.078	-0.048	0.084	0.069	-0.057	0.078	0.136	0.133	0.091	0.026	-0.090	0.099	0.039	0.053	-0.080
	Intermediate wood vessel area	0.111	-0.122	0.010	0.081	0.064	0.077	-0.065	-0.002	-0.016	0.017	0.009	-0.072	0.078	-0.080	0.072	0.067	-0.029	0.076	0.053	-0.053	0.073	0.082	0.107	0.038	0.044	-0.067	0.075	-0.002	0.004	-0.136
	Intermediate wood vessel size	0.033	-0.012	0.032	-0.001	-0.032	0.021	-0.038	0.002	0.012	0.004	-0.042	-0.011	-0.010	-0.003	-0.031	0.013	-0.017	0.019	0.018	-0.004	0.018	-0.047	-0.013	-0.026	0.011	0.027	-0.001	-0.036	-0.055	-0.012
	Late wood vessel count	0.067	-0.061	-0.057	0.080	0.042	0.072	-0.066	0.007	0.000	-0.021	-0.031	-0.071	0.075	-0.077	0.057	0.076	-0.063	0.084	0.074	-0.056	0.071	0.050	0.065	-0.003	-0.031	-0.020	0.066	-0.047	0.016	-0.053
	Late wood vessel area	0.201	-0.219	-0.058	0.183	0.098	0.191	-0.185	-0.050	-0.081	0.088	0.055	-0.185	0.161	-0.184	0.106	0.171	-0.120	0.170	0.146	-0.159	0.175	0.137	0.193	0.094	0.049	-0.096	0.177	-0.014	-0.067	-0.158
Late wood vessel size	0.108	-0.124	-0.029	0.104	0.059	0.119	-0.117	-0.052	-0.082	0.109	0.088	-0.123	0.086	-0.108	0.048	0.104	-0.075	0.096	0.086	-0.098	0.111	0.069	0.102	0.086	0.073	-0.025	0.110	0.013	-0.073	-0.046	

*Wood Chemistry

Table 4.6 Genes identified from *P. trichocarpa* single trait GWAS.

Gene model ^a	Trait	P-value ^b	Functional annotation
Potri.014G117300	Diameter (breast height)	1.95E-08	Gibberellin 2-beta-dioxygenase 2
Potri.014G117400	Diameter (breast height)	1.88E-08	similar to MYB family transcription factor
Potri.018G141400	Diameter (breast height)	5.67E-08	ABC transporter
Potri.005G247400	Leaf circularity	9.20E-08	similar to prolyl oligopeptidase
Potri.005G247600	Leaf circularity	4.63E-08	similar to maoC-like dehydratase domain-containing protein
Potri.005G247700	Leaf circularity	1.37E-08	multi-copper oxidase type 1 family protein
Potri.005G247900	Leaf circularity	9.01E-08	ubiquitin family protein
Potri.019G042600	Leaf dry weight, leaf wet weight*	1.49E-08	MITOCHONDRIAL OUTER MEMBRANE PROTEIN 25
Potri.019G042700	Leaf dry weight, leaf wet weight	9.73E-09	similar to expressed protein in <i>Arabidopsis thaliana</i> (co-ortholog of At4g27620, At4g27610)
Potri.015G117300	Stomatal density	6.49E-09	NA
Potri.015G117400	Stomatal density	6.11E-08	PROTEIN ARGONAUTE 2-RELATED
Potri.015G117500	Stomatal density	2.77E-09	Peptide-O-fucosyltransferase/GDP-L-fucose:polypeptide fucosyltransferase
Potri.015G117600	Stomatal density	8.31E-08	zinc finger (C3HC4-type RING finger) family protein; similar to C3HC4 type (RING finger)
Potri.002G238700	Leaf weight weight	5.74E-08	similar to hypothetical protein (co-ortholog (1of2) of At5g48890)
Potri.001G311900	Leaf aspect ratio	2.29E-08	Cyanohydrin beta-glucosyltransferase/Uridine diphosphoglucose:aldehyde cyanohydrin beta-glucosyltransferase
Potri.006G119400	S/G ratio	9.15E-08	PLATZ TRANSCRIPTION FACTOR FAMILY PROTEIN
Potri.004G141500	Early wood vessel count	8.11E-08	DNA repair and recombination protein RAD54 and RAD54-like protein
Potri.012G007600	Intermediate wood vessel size	9.58E-08	CARBON CATABOLITE REPRESSOR PROTEIN 4
Potri.019G037900	Intermediate wood vessel size	8.44E-09	Staufen and related double-stranded-RNA-binding proteins
Potri.006G205700	Late wood vessel size	3.17E-08	ubiquitin-conjugating enzyme family protein; similar to DNA-binding protein CROC-1B (<i>Homo sapiens</i>)

^aGene models are annotated using v3 of the *P. trichocarpa* genome

^bSNP p values < 1 × 10⁻⁷

*smallest P-value reported (leaf dry weight)

Table 4.7 Genes identified from *P. trichocarpa* multitrait GWAS.

Gene model ^a	Trait	<i>P</i> -value ^b	Functional annotation
Potri.001G085400	Early wood vessel area, early wood vessel count	3.07E-08	similar to Yippee-like protein (co-ortholog of At4g27740)
Potri.005G038400	Early wood vessel area, early wood vessel count	7.70E-08	PPR repeat family (PPR_2)
Potri.001G140500	Early wood vessel area, early wood vessel count, intermediate wood vessel area, intermediate wood vessel count, lignin content, late wood vessel area, late wood vessel count, S/G ratio	2.29E-08	CARBOXYLATE CLAMP-TETRATRICOPEPTIDE REPEAT PROTEIN
Potri.002G108000	Early wood vessel area, early wood vessel count, intermediate wood vessel area, intermediate wood vessel count, lignin content, late wood vessel area, late wood vessel count, S/G ratio	6.31E-08	Auxin responsive-protein IAA8
Potri.006G275900	Early wood vessel area, early wood vessel count, intermediate wood vessel area, intermediate wood vessel count, lignin content, late wood vessel area, late wood vessel count, S/G ratio	4.16E-08	MYB-LIKE DNA-BINDING PROTEIN
Potri.008G160600	Early wood vessel area, early wood vessel count, intermediate wood vessel area, intermediate wood vessel count, lignin content, late wood vessel area, late wood vessel count, S/G ratio	8.15E-08	acylaminoacyl-peptidase
Potri.017G090200	Early wood vessel area, early wood vessel count, intermediate wood vessel area, intermediate wood vessel count, lignin content, late wood vessel area, late wood vessel count, S/G ratio	7.32E-09	Non-specific serine/threonine protein kinase CALMODULIN-BINDING PROTEIN-LIKE PROTEIN
Potri.017G110300	Early wood vessel area, early wood vessel count, intermediate wood vessel area, intermediate wood vessel count, lignin content, late wood vessel area, late wood vessel count, S/G ratio	7.63E-08	NA
Potri.006G275800	Early wood vessel area, early wood vessel count, intermediate wood vessel area, intermediate wood vessel count, lignin content, late wood vessel area, late wood vessel	2.37E-10	PTHR23155//PTHR23155:SF 609 - LEUCINE-RICH REPEAT-CONTAINING PROTEIN // SUBFAMILY NOT NAMED

Gene model ^a	Trait	P-value ^b	Functional annotation
Potri.014G171300	count, S/G ratio* and Intermediate wood vessel area, intermediate wood vessel count Early wood vessel area, early wood vessel count, intermediate wood vessel area, intermediate wood vessel count, late wood vessel area, late wood vessel count	9.71E-08	similar to heavy-metal-associated domain-containing protein; similar to strong similarity to farnesylated proteins ATPF4
Potri.004G002600	Early wood vessel area, leaf area, stomatal density and early wood vessel area, leaf area, stomatal density	9.57E-09	(1 of 2) PTHR31803:SF10 - UBIQUINOL OXIDASE 4, CHLOROPLASTIC/CHROMOPLASTIC
Potri.016G013400	Early wood vessel area, leaf area, stomatal density	3.20E-08	(1 of 3) K15223 - upstream activation factor subunit UAF30 (UAF30, SPP27)
Potri.015G113800	Early wood vessel area, leaf area, stomatal density*** and lignin content, specific leaf area, stomatal density, early wood vessel area	2.22E-08	Protein of unknown function, DUF594 (DUF594) // Domain of unknown function (DUF4220) (DUF4220)
Potri.011G022200	Early wood vessel count, intermediate wood vessel count, late wood vessel count	2.78E-08	(1 of 3) PTHR33076:SF6 - NON-SPECIFIC LIPID-TRANSFER PROTEIN 15
Potri.009G149900	Early wood vessel size, intermediate wood vessel size, lignin content, S/G ratio, late wood vessel size	8.52E-08	amino acid transporter family protein; similar to low similarity to amino acid permease (<i>Oryza sativa</i>) GI:7415521
Potri.T031400	Early wood vessel size, intermediate wood vessel size, lignin content, S/G ratio, late wood vessel size	9.42E-08	NA
Potri.001G058100	Intermediate wood vessel area, intermediate wood vessel count	4.15E-08	similar to expressed protein in <i>Arabidopsis thaliana</i> (co-ortholog of At1g27290)
Potri.019G037900	Intermediate wood vessel size, lignin content, S/G ratio	1.77E-09	Staufen and related double-stranded-RNA-binding proteins
Potri.001G467800	Intermediate wood vessel size, lignin content, S/G ratio** and Lignin content, late wood vessel size, S/G ratio	3.69E-08	similar to uracil phosphoribosyltransferase
Potri.004G056300	Leaf area, leaf dry weight, leaf length, leaf width	6.88E-12	Fruit bromelain
Potri.007G061600	Leaf area, leaf dry weight, leaf length, leaf width	1.03E-09	vacuolar protein sorting-associated protein 35 (VPS35)
Potri.007G099700	Leaf area, leaf dry weight, leaf length, leaf width	8.07E-08	Leucine rich repeat (LRR_8)

Gene model ^a	Trait	<i>P</i> -value ^b	Functional annotation
Potri.010G031900	Leaf area, leaf dry weight, leaf length, leaf width	5.23E-08	similar to RUB-activating enzyme (Ubiquitin activating enzyme E1 like protein)
Potri.010G032600	Leaf area, leaf dry weight, leaf length, leaf width	3.62E-13	small subunit ribosomal protein S11 (RP-S11, MRPS11, rpsK)
Potri.012G028700	Leaf area, leaf dry weight, leaf length, leaf width	5.90E-09	Ras suppressor protein (contains leucine-rich repeats)
Potri.019G067300	Leaf area, leaf dry weight, leaf length, leaf width	1.90E-08	(1 of 1) PTHR11214:SF125 - BETA-1,3-GALACTOSYLTRANSFERASE 8-RELATED
Potri.005G054400	Lignin content, late wood vessel size, S/G ratio	3.10E-08	RING FINGER DOMAIN-CONTAINING, POLYCOMB GROUP COMPONENT
Potri.006G242100	Lignin content, late wood vessel size, S/G ratio	5.48E-08	similar to expressed protein in <i>Arabidopsis thaliana</i> (ortholog of At2g25737)
Potri.006G242200	Lignin content, late wood vessel size, S/G ratio	8.37E-08	PPR repeat family (PPR_2)
Potri.009G137800	Lignin content, S/G ratio	1.42E-08	similar to vacuolar ATP synthase subunit B
Potri.012G047300	Lignin content, S/G ratio	4.57E-08	METHYLTRANSFERASE
Potri.004G183900	Late wood vessel area, late wood vessel count	5.91E-09	SERINE/THREONINE-PROTEIN KINASE

^aGene models are annotated using v3 of the *P. trichocarpa* genome

^bSNP p values < 1 × 10⁻⁷

*Early wood vessel area, early wood vessel count, intermediate wood vessel area, intermediate wood vessel count, lignin content, late wood vessel area, late wood vessel count, S/G ratio with lowest *P*-value

**Intermediate wood vessel size, lignin content, S/G ratio with lowest *P*-value

***Early wood vessel area, leaf area, stomatal density with lowest *P*-value

Figure 4.1 Source locations of 869 *P. trichocarpa* genotypes sampled in this study (purple dots). The trees were grown in a common garden in Clatskanie, Oregon, USA (black star).

Figure 4.2 Representative *P. trichocarpa* stem cross section at 100x magnification.

(a)

(b)

(c)

(d)

(e)

(f)

(g)

(h)

(i)

(j)

(k)

(l)

(m)

(n)

Figure 4.3 Single trait GWAS – Manhattan (left) and QQ plots (right). Numbers 1 to 19 represent chromosomes; scaffolds are the reads that did not align to any of the 19 chromosomes. (a) and (b) DBH; (c) and (d) Leaf circularity; (e) and (f) Leaf dry weight; (g) and (h) Leaf wet weight; (i) and (j) Petiole maximum diameter; (k) and (l) Abaxial stomatal density; (m) and (n) Intermediate wood vessel size. Red horizontal line indicates Bonferroni correction threshold ($P=7.417 \times 10^{-9}$) and blue horizontal line indicates suggestive association threshold ($P=1 \times 10^{-7}$).

Figure 4.4 Multitrait GWAS – Manhattan (left) and QQ plots (right). Numbers 1 to 19 represent chromosomes; scaffolds are the reads that did not align to any of the 19 chromosomes. (a) and (b) Late wood vessel area and Late wood vessel count; (c) and (d) Leaf area, leaf dry weight, leaf length and leaf wet weight. Red horizontal line indicates Bonferroni correction threshold ($P=7.417 \times 10^{-9}$) and blue horizontal line indicates suggestive association threshold ($P=1 \times 10^{-7}$).

Figure 4.5 Expression level of gene models identified from single trait GWAS in different tissues in *P. trichocarpa*.

Figure 4.6 Expression level of gene models identified from multitrait GWAS in different tissues in *P. trichocarpa*.

4.8 Supplementary figures

(g)

(h)

Supplementary Figure S 4.1 Single trait GWAS – Manhattan (left) and QQ plot (right). Numbers 1 to 19 represent chromosomes; scaffolds are the reads that did not align to any of the 19 chromosomes. (a) and (b) Intermediate wood vessel count; (c) and (d) Late wood vessel size; (e) and (f) S/G ratio; (g) and (h) Leaf aspect ratio. Red horizontal line indicates Bonferroni correction threshold ($P=7.417 \times 10^{-9}$) and blue horizontal line indicates suggestive association threshold ($P=1 \times 10^{-7}$).

(a)

(b)

Supplementary Figure S 4.2 Multitrait GWAS – Manhattan (left) and QQ plot (right). Numbers 1 to 19 represent chromosomes; scaffolds are the reads that did not align to any of the 19 chromosomes. (a) and (b) Early wood vessel area, leaf area and stomatal density; (c) and (d) Early wood vessel size, intermediate wood vessel size, lignin content, S/G ratio and late wood vessel size; (e) and (f) Intermediate wood vessel size, lignin content and S/G ratio; (g) and (h) Lignin content, late wood vessel size and S/G ratio; (i) and (j) Lignin content and S/G ratio; (k) and (l) Lignin content, specific leaf area, stomatal density and early wood vessel area; (m) and (n) Late wood vessel area and late wood vessel count. Red horizontal line indicates Bonferroni correction threshold ($P=7.417 \times 10^{-9}$) and blue horizontal line indicates suggestive association threshold ($P=1 \times 10^{-7}$).

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Chapter 5. Overall conclusion

One of the major goals in population genomics is to understand the role of evolutionary forces such as selection, mutation and gene flow in shaping the spatial and temporal patterns of phenotypic variation. With the revolution in sequencing technology, it is now possible to assess the molecular mechanisms underlying phenotypic variation in adaptive traits. The identified genetic targets have great potential for optimizing traits related to abiotic stress tolerance and improving productivity. The genomic revolution has also enabled the identification of underlying genetic mechanisms affecting local adaptation, leading to better understanding of the biology of the species in the context of rapidly changing climates (Aitken, Yeaman, Holliday, Wang, & Curtis-McLane, 2008). Because of their wide geographical distribution and climatic gradients, large effective population sizes, high genetic variation, forest trees are excellent model systems for understanding local adaptation and genetic architecture of the complex traits (González-Martínez, Ersoz, Brown, Wheeler, & Neale, 2006; Ingvarsson, Hvidsten, & Street, 2016; Neale & Kremer, 2011; Neale & Savolainen, 2004; Street & Ingvarsson, 2011).

Efforts have been made to optimize this ecologically and economically important tree *Populus* for lignocellulosic biofuel production using association genetics by identifying the genomic regions underlying traits of interest (Street & Ingvarsson, 2011). Using natural populations that have undergone many generations of recombination between ancestral haplotypes allows identification of the genomic region affecting a trait at fine scale. To this end, several small and some large scale GWAS studies have been performed to understand the underlying genetic architecture related to morphological, physiological, wood chemistry and disease resistance traits in *Populus* (Allwright et al., 2016; Bdeir et al., 2019; Du et al., 2019; Evans et al., 2014; Fahrenkrog et al., 2017; McKown, Klápště, et al., 2014; Muchero et al., 2015; Porth et al., 2015, 2013; Wegrzyn et al., 2010; Zhang et al., 2018). However, the comprehensive assessment of morphological and physiological traits comparing GWAS from multiple plantations is very rare (Evans et al., 2014). Furthermore, there is no GWAS study on wood anatomical traits such as vessel size and density that is related to wood chemistry and important plant functions. My dissertation provides a comprehensive assessment of GWAS on important wood anatomical, wood chemistry, morphological and physiological traits in *Populus trichocarpa*. I also assess the effect of environment on GWAS for morphological traits measured in two contrasting common gardens. Furthermore, I complemented phenotypic GWAS with the genomics of local adaptation to understand the molecular genetic mechanisms underlying local adaptation in *P. trichocarpa*.

In chapter 2, I performed 14 single and 12 multitrait GWAS on morphological and physiological data collected from a common garden in Corvallis, OR. Using GEMMA software (Zhou & Stephens, 2012, 2014), I tested for the association of these traits with 6.78 million SNPs in the genome from 882

trees. I created multitrait sets based on the correlation and functional relationships of the traits and showed that multitrait GWAS provided substantial increase in power over single trait GWAS for detecting the SNP variants.

Complex adaptive traits are not just affected by single or a few loci, and thousands of loci in the network with non-zero effect sizes affect the trait (Boyle, Li, & Pritchard, 2017). Power analyses indicate that most association studies in forest trees are orders of magnitude too small to detect the effects of alleles of small effect and low frequency (Visscher et al., 2017). Therefore, despite the high heritability of many morphological traits, only a small proportion of heritability is explained by single nucleotide polymorphisms (SNPs) in most GWAS analyses, suggesting insufficient statistical power (Solovieff, Cotsapas, Lee, Purcell, & Smoller, 2013). While increasing sample size of GWAS populations is clearly desirable, it is costly and, in some cases, may not be feasible. Therefore, I used a multivariate framework, to increase the power of my GWAS study. The multitrait approach offers substantial increase in power compared to the standard univariate approach (Porter & O'Reilly, 2017). One of the big advantages of multitrait GWAS is that missing information in one of the phenotypes in the multitrait set can be complemented by the other phenotypes (Ritchie, Holzinger, Li, Pendergrass, & Kim, 2015). Multitrait GWAS also takes advantage of pleiotropic effects of polymorphisms, thereby increasing statistical power even when the traits have low correlation (Broadaway et al., 2016; Hackinger & Zeggini, 2017). Finally, unlike analyses based on principal components, multitrait GWAS effectively captures indirect genetic effects whereby a SNP affects one phenotype through its effects on a functionally-related phenotype (Porter & O'Reilly, 2017; Stephens, 2013).

In chapter 2, I identified 5 SNPs that passed the Bonferroni correction P -value cutoff of 7.37×10^{-9} with multitrait GWAS for a total of 12 sets of traits compared to none with single trait GWAS for 14 traits. Furthermore, a total of 32 SNPs passed the suggestive association P -value cutoff of 1×10^{-7} for multitrait GWAS compared to 4 SNPs for single traits GWAS. With multitrait GWAS I identified 22 *P. trichocarpa* gene models, whereas with single trait GWAS I identified only 4 gene models. For single trait GWAS, no SNP passed Bonferroni correction threshold and only 4 SNPs passed the suggestive association threshold. To gain further insight into possible functions of candidate genes identified by GWAS analysis, I examined the position of the genes in networks constructed from RNA-seq expression data and metabolites profiles for the same population. Functional characterization of one of the genes highlighted by this analysis was Potri.004G111000, identified from the multitrait GWAS for LA-LD-LL-LW. *GAUT9* belongs to the *GAUT* gene family of proven and putative pectin homogalacturonan (HG) galacturonosyltransferases (Atmodjo et al., 2011; Biswal et al., 2018; Sterling et al., 2006; Voiniciuc et al., 2018). Multiple lines of evidence showed that this gene can affect leaf development. *GAUT9* had reciprocal effects on leaf size – leaf size increased on knockdown lines.

Another notable gene I identified was for multitrait GWAS of CI-LA-SD where I identified EF-Hand Calcium Binding Domain. Calcium is an important second messenger in eukaryotes and has important roles in cell signaling and response to biotic and abiotic stresses and developmental cues (Edel, Marchadier, Brownlee, Kudla, & Hetherington, 2017; Ranty et al., 2016; Zhu, 2016). The EF-hand motif is the most common and highly conserved calcium-binding motif (Lewit-Bentley & Réty, 2000; Zeng, Zhang, Zhang, Pi, & Zhu, 2017). The co-expression network further provided the evidence that the gene Potri.001G411800, identified in multitrait GWAS is involved in responses to abiotic stress. It is co-expressed with a late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein (Potri.009G158900), a group that has a major role in responses to drought, salinity and, osmotic and temperature related stresses (Gao & Lan, 2016; Magwanga et al., 2018). Potri.001G411800 is also associated with 10 different metabolites in the same population, including several that are related to plant development and stress responses. These findings have important implications for optimizing traits related to plant productivity and stress tolerance in the biotechnology experiment. Furthermore, the lines of evidence (LOE) approach taken here to identify coexpressed genes and metabolites provided further support to the genes identified by GWAS. In conclusion, using single trait GWAS complemented with a multitrait approach helps identify pleiotropic loci. Furthermore, the LOE approach adds confidence and reduces false positives in GWAS results.

In chapter 3, I tested for the evidence of local adaptation in *P. trichocarpa* using three conceptually different methods. First, I tested for the correlation of phenotypic traits with climate and geography variables. Several traits including bud set, bud flush, tree height and diameter, and leaf traits had significant correlation with climate and geography variables suggesting the role of climate and geography in shaping the variation and local adaptation of this species.

Second, I tested for the association of six environmental variables with 6.741 million SNPs in the genome for 869 genotypes using univariate as well as multivariate genotype-environment association (GEA) approach. Furthermore, I also performed GWAS of the first two PCs of 26 climate and geography variables with 6.741 million SNPs in the genome. I identified a total of 422 significant SNPs in vicinity of 67 genes conferring local adaptation based on a Bonferroni correction threshold of 5% significance level ($P < 7.417 \times 10^{-9}$). Fourteen of these genes were shared across the three genotype-environment association (GEA) detection methods – single and multitrait and PC based associations. Using GEA methods I identified multiple important genes including Potri.010G079500 (a very long chain beta-ketoacyl-CoA synthase, the best match of this gene is KCS11 in Arabidopsis, potential involvement in cuticular wax biosynthesis), Potri.010G080200 (also a very long chain beta-ketoacyl-CoA synthase) and Potri.010G079600 (DNA damage repair protein) genes.

Third, using the multivariate ordination method called redundancy analysis (RDA), I identified several genomic outlier loci conferring local adaptation in *P. trichocarpa*. Decomposing the variance in the SNP response matrix into the matrices of climate and geography, respectively using RDA (climate RDA model), I underscored the relative contribution of climate and geography on neutral population structure. Similarly, I decomposed the variation in phenotypic matrix (11 traits) into the matrices of SNP (113 SNP eigenvectors), climate (5 variables) and geography (space variables selected based on the stepwise regression of 3rd degree polynomials for latitude and longitude) (phenotypic RDA model) and showed that the climate explained the highest amount of variation (21.664%) among the predictor matrices suggesting the impact of climate variables on adaptive traits. Furthermore, I identified a total of 7807 and 16782 outlier genes from climate and phenotypic RDA models, respectively. Comparison of these gene models with 67 gene models identified from GEA methods at *P*-value cutoff of 7.417×10^{-9} showed that 32 of them were shared across climate and phenotypic RDA and the GEA methods. This included a few interesting genes in chromosome 10 including Potri.010G079500. The Potri.010G079500 gene appears to be important for the alkene composition of cuticular wax in *P. trichocarpa* (Gonzales-Vigil, Hefer, von Loessl, La Mantia, & Mansfield, 2017). The gene is downregulated in trees with low alkene composition. Non-alkene producing trees had reduced growth and higher disease susceptibility, emphasizing the adaptive importance of this trait (Gonzales-Vigil et al., 2017).

Because my SNP data set was large (>11.1 million SNPs at MAF cutoff of 0.01), I used an indirect approach for detecting the loci conferring local adaptation. I used significant SNP PCs (eigenvectors) from the PCA analysis as a matrix of genetic data in the RDA analysis. I considered the top 0.1% of the SNPs from the tails of the distribution of the loadings from the significant SNP PCs as outlier loci. A total of 5 and 17 outlier SNP PCs were identified as outliers in the climate and phenotypic RDA models, respectively. Given that the adaptation in forest trees is polygenic such that selection can affect hundreds or thousands of loci, the large number of genes we identified here is not surprising (Boyle et al., 2017; Eckert et al., 2009; Evans et al., 2014; Holliday, Wang, & Aitken, 2013). Furthermore, the use of powerful methods like RDA is important to identify loci especially with weak selection signals. Overall, this study provided important insights on the mechanisms of local adaptation in forest trees. The genomic adaptation signals identified here can have important implications in breeding and optimizing the traits of interest in domestication and natural management of *Populus* and other tree species. This can also aid in predicting the performance of a genotype in a new environment.

In chapter 4, I performed GWAS on phenotypic traits in a different common garden in Clatskanie, OR with contrasting environmental conditions compared to Corvallis, OR. The Clatskanie common garden is located close to the mouth of Columbia river and the Pacific Ocean whereas Corvallis common garden is located in an inland valley, and is not as wet as the Clatskanie site. My objective for

chapter 4 was two-fold: 1) I tested for the effect of environment on the GWAS of comparable morphological traits such as leaf and stomatal density traits from Corvallis common garden; and 2) I performed GWAS on wood anatomy traits such as vessel size and density for the first time in *Populus*.

Genes underlying wood chemistry and functional genomics of wood property traits are fairly well studied (Muchero et al., 2015; Porth et al., 2015, 2013; Wegrzyn et al., 2010), but the genetic architecture underlying wood anatomical traits such as vessel size and density that are functionally related to cell wall composition and overall plant function is unknown. Using GEMMA univariate GWAS for 27 phenotypic traits including wood anatomical, wood chemistry and morphological traits with 6.741 million SNPs in the genome for 869 trees, I detected two SNPs that passed Bonferroni correction threshold of $P < 7.417 \times 10^{-9}$ (both SNPs detected for stomatal density) and 77 SNPs that passed suggestive association threshold of $P < 1 \times 10^{-7}$. These 77 SNPs were within or close to 20 *P. trichocarpa* gene models. As in chapter 2, I also formed 19 multitrait combinations based on the correlation and functional relationships of the traits and identified 9 SNPs that passed the Bonferroni correction P -value cutoff and 47 SNPs that passed the suggestive association P -value cutoff. These SNPs were within or close to 33 *P. trichocarpa* gene models.

Correlation of phenotypic traits with latitude in chapter 3 shows that geography (latitudinal variation in particular) has a major role on adaptive trait variation in *P. trichocarpa*, which similar to other studies, obscures the actual between-trait and trait-climate relationships (Chhetri et al., 2019; Mckown et al., 2014). Low correlations between traits from different common gardens suggest that there is a large effect of environment on phenotypic traits. This is also reflected in the GWAS study such that despite the moderate heritabilities of most traits, the genes controlling phenotypic traits localized to completely different positions in this study compared to the GWAS performed for the phenotypic traits in Corvallis, OR common garden. Furthermore, the correlation of phenotypic traits with climate variables such as temperature and moisture and precipitation supported the evidence of local adaptation in *P. trichocarpa*.

Nevertheless, I was able to identify some related genes for some functionally related traits with the GWAS from two common gardens. The gene model Potri.017G090200 identified from wood anatomy multitrait 5 (late wood vessel area and count, SNP P -value = 7.32×10^{-9}) encodes calmodulin-binding protein-like protein. Calmodulin (CaM) and calmodulin-like proteins (CMLs) are one of the three main families of calcium (Ca^{2+}) sensor proteins in plants (Ranty et al., 2016). Calmodulin (CaM) and calmodulin-like proteins (CMLs) are well characterized in plants and are involved in regulating plant responses to abiotic stresses (Zeng et al., 2017). One of the calmodulin binding proteins, EF-hand motif that is involved in cell signaling, developmental processes and biotic and abiotic responses was detected in the multitrait GWAS from Corvallis, OR plantation that included carbon isotope, leaf area and stomatal

density in the multitrait set (Chhetri et al., 2019). Here I identified a gene with similar role controlling wood anatomy traits. Wood anatomy traits have potential functional relationships with carbon isotope, leaf area and stomatal density.

The gene, Potri.015G117500, identified based on two GWAS hits for stomatal density that passed Bonferroni threshold of $P < 7.417 \times 10^{-9}$, provided another case where a gene with a similar role was identified for the same trait. Potri.015G117500 encodes peptide-O-fucosyltransferase which belongs to the family of glycosyltransferases (Hansen, Harholt, Oikawa, & Scheller, 2012; Vogt & Jones, 2000). In *Arabidopsis* O-fucosyltransferase affects DELLA and associated regulators such as phytochrome-interacting-factor3 (PIF3) and PIF4 in the brassinosteroid- and light-signaling pathways (Zentella et al., 2007). McKown et al. (2014) identified a similar gene, Potri.004G059000, encoding BRASSINOSTEROID-INSENSITIVE 2, for stomatal density based on the GWAS on 34K SNP array data for 464 *P. trichocarpa* trees. BRASSINOSTEROID-INSENSITIVE 2 may affect the brassinosteroid signaling pathway and the regulation of stomatal development (Gudesblat et al., 2012; Khan et al., 2013).

In chapter 4, I detected several interesting genes related to plant growth, development and defense mechanisms, but these genes should be verified with co-expression networks from RNA-seq data and metabolite profiles from the same population. Furthermore, the functional validation of all genes detected in chapter 2, 3 and 4 is necessary in order to target the gene in the biotechnology experiments to optimize important plant growth and development and abiotic stress tolerance traits. This will aid in the domestication of *Populus* trees for feedstock development. Moreover, GWAS methods such as gene and pathway-based methods and insertion deletion (indel) GWAS methods should be used, as the former provide more power and the latter is conceptually different and highlights the importance of indels in the genome for controlling complex traits. SNPs identified in GWAS are useful not only for identifying the genetic variants that can be targeted in the biotechnology experiment to optimize the traits of interest, but they can also be useful, together with other marker information available for the population in a genomic selection program to estimate breeding values from genotypic data.

As I have highlighted, the lack of power in GWAS and local adaptation studies is mainly due to sample size. Increasing sample size by homogeneously sampling from across the range of the distribution is always important for understanding the underlying genetics controlling complex traits. Moreover, powerful methods such as multitrait GWAS coupled with integration of multi-omic data should complement large sample size and compensate for lack of power. Furthermore, given that the local adaptation is believed to be polygenic and confounded by demographic history, multivariate methods like redundancy analysis (RDA) provide more power to detect weak selection signals. Lastly, future local adaptation studies should integrate various data types including phenotypic, genomic and environmental data whenever possible.

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