Genetic variability and drought responses of young and mature aspen in Alberta

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Forest Biology and Management

Department of Renewable Resources
University of Alberta

Abstract

Trembling aspen (*Populus tremuloides* Michx.) is a dioecious clonal tree species with broad distribution in North America. In dioecious species, sexes are predicted to perform differently, based on the principle of allocation and the energy costs associated with reproduction. Aspen reproduce sexually through seeds and asexually through root suckers, forming clonal stands. The diversity of aspen clones was described in the past based on phenotypic assessments. Today, genetic markers have opened up a novel opportunity to explore the genetic diversity of aspen stands and the response of seedlings based on sex. The first breeding strategy for native aspen in Alberta was developed in 1995 with three regions selected based on aspen ecology, genetics, phenotype, and geography. It is imperative to understand the factors affecting clone presence, genetic diversity, and sex distribution in aspen stands, to inform aspen improvement programs in Alberta and to provide insights into the ecological responses of aspen to abiotic stress. This thesis explored sex differential performance in aspen on seedlings (Chapter 2) and mature stands (Chapter 3), including aspects of climate, and the genetic diversity of aspen stands within the AW2 aspen tree improvement region, using microsatellite markers and phenotype (Chapter 4).

Chapter 2 tested the response of aspen seedlings under drought and well-watered conditions to assess if parent tree origin has an influence on seedling performance and if different genotypes and sexes present differential drought performances. Seeds were extracted from 19 female parent trees, from two climactically different regions in Alberta. Sex was determined using the TOZ19 gene sequence. Seedlings were maintained in a greenhouse under well-watered and sustained drought conditions in a split-plot replicated block design experiment. Parkland seedlings showed adaptation to drought conditions. Genotypes showed varied drought strategies, with low and high-productivity families identified. While sexes did not

differ in growth or biomass production, female seedlings had higher intrinsic water-use efficiency ($_i$WUE) under well-watered conditions, and males had higher $_i$WUE under drought.

Chapter 3 described the distribution of mature male and female aspen trees within the southern portion of the AW2 region and examined climate as a potential factor driving the distribution of sex. A minimum of 19 and maximum of 30 trees were sampled in 12 mature aspen stands, in four different areas. Data collected include DNA samples; diameter at breast height; wood density, ring growth, and stand age. Climate data were obtained for each study site using the BioSIM software. An approximate 2:1 female:male sex ratio was observed, and males and females showed similar overall diameter growth and wood density. For the timeframe explored, this study did not find different sex responses to drought in mature aspen trees.

In Chapter 4, aspen clonal structure and ploidy were described in the AW2 (Pembina) region. The same trees from Chapter 3 were studied in addition to DNA collected from trees along transects connecting the stands. Phenotypic assessments were used to delineate putative clones and microsatellite markers were used to confirm clonal identity, diversity, and ploidy. Natural populations within the AW2 region showed high levels of genetic diversity including evidence of occurrence and accumulation of somatic mutations. Mutations introduce more complexity and variability to those populations. Polyploids (i.e., 3n or 4n) had better growth and performance during and post-drought.

This study showed that aspen seedlings show plasticity to drought independent of origin and sex and that genetic variability plays a main role in the maintenance of higher-performing genotypes. In mature aspen stands costs of reproduction may be manifested beyond the production of seeds, with differential allocation of energy in females and males also occurring in root and stem growth. Finally, natural aspen stands exhibited greater diversity and complexity than found previously. The use of trees of different ploidies, sexes, and genotypes in improvement programs and aspens stands would allow for increased growth under varied environmental conditions.

Preface

The research conducted in this thesis is part of Dr. Barb Thomas' Industrial Research Chair in Tree Improvement. Plant materials (catkins) used for Chapter 2 were collected by Dr. Barb Thomas and Morgan Randall before I started the PhD program. The data used in Chapters 3 and 4 were obtained from aspen stands within the Region AW2 aspen controlled parentage program (CPP) in Alberta. Parent tree location information was provided by Weyerhaeuser Pembina Timberlands, courtesy of Mr. Dave Swindlehurst, Silviculture Manager at Pembina Timberlands. DNA extraction was performed by Susan Koziel with my assistance at InnoTech Alberta, Vegreville, Alberta. DNA analyses, including clonal fingerprinting and phylogeny, were performed by Susan Koziel, with data analyses interpretation performed by me. The protocol for aspen cytology was developed and shared by Dr. M. Nurul Islam-Faridi and adjusted at the University of Alberta by Deborah O'Neill. During the COVID-19 pandemic, I was in Brazil from November 2020 until July 2021 due to a cancellation of all flights to Canada. During this time field assistants Stephanie Rudnew and Romy Suliteanu collected aspen root samples in the field in the spring of 2021, Stephanie Rudnew was the field supervisor, following my instructions. Dr. Shakila Ekanayaka Mudiyanselage, a post-doctoral fellow in the Thomas lab, propagated the root suckers in the greenhouse, from field-collected root samples. No part of this thesis has been previously published.

Dedication

I dedicate this thesis to my younger self, who would not believe we got here, and to the ones who supported my decisions unconditionally: my parents, Cristina and Eraldo, and my sister, Janiny.

Acknowledgements

I would like to thank my supervisor Dr. Barb Thomas for giving me the opportunity to work on this project and for the support and guidance throughout the years. I would also like to thank my supervisory committee members Dr. Brad Pinno and Dr. Annie DesRochers for the time you invested in helping make this research possible. I would like to thank the arm's length examiner, Dr. Edward Bork, and the external examiner, Dr. Karen Mock.

Thank you to Mr. Dave Swindlehurst for providing parent tree location information and maps of the AW2 breeding region and for all the support provided during fieldwork. Thank you to Dan McCurdy and all the staff members of the Bonnyville Forest Nursery for housing and taking good care of our seedlings. I would like to thank Susan Koziel and Ralph Lange at InnoTech Alberta for their support with DNA analyses. Thank you to Dr. M. Nurul Islam-Faridi for sharing his methods on aspen cytology and thank you to Deborah O'Neill for guidance during laboratory work. Thank you Robert Matheson.

My thanks are extended to the Tree Improvement lab members, to the lab managers Morgan Randall, Stacy Bergheim, and Lucia Secchia and to the research assistants who supported my project: Stephanie Rudnew, Romy Suliteanu, Jessica Hermary, Michelle Mjolsness, Kennedy Mitchell, Emelie Dykstra, Kayla Frankiw, and Samantha Karpyshin. I leave a special thank you to Stephanie Rudnew and Romy Suliteanu, for the great job done conducting fieldwork without me, under remote supervision. I also appreciate Jana Bockstette, Tara Androschuk, and Dawei Luo for helping me in the field, when field assistants were not available. Thank-you to Dr. Shakila Ekanayaka Mudiyanselage for propagating root suckers in the greenhouse. Thank-you to Kelley Dunfield for all her support during my greenhouse experiment at the Ag-For greenhouse space.

Finally, this research would not be possible without the support of the financial contributors: the Industrial Research Chair in Tree Improvement held by Dr. Barb Thomas (NSERC: IRC461040-13) supported by the Natural Sciences Engineering Research Council (NSERC), Alberta Pacific Forest Industries Inc., ANC Timber Ltd., West Fraser Mills Ltd. (including Hinton Wood Products, Blue Ridge Lumber, Sundre Forest Products, Alberta Plywood), Canfor Ltd. (Grande Prairie Woodlands and Whitecourt Woodlands), Weyerhaeuser Company Ltd. (Grande Prairie and Pembina Timberlands), and HASOC. This research was also supported by the *ACA Grants in Biodiversity* (supported by the Alberta Conservation Association).

I would like to thank the staff members of the faculty of Agricultural, Life and Environmental Sciences, and of the Department of Renewable Resources for their work and kind help in everyday situations at the University of Alberta.

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Glossary of Terms

A	net carbon assimilation
ANOVA	analysis of variances
AW1	Alberta trembling aspen breeding region 1
AW2	Alberta trembling aspen breeding region 2
BA	basal area
BAI	basal area increment
BioSIM	software tool used to obtain climate data
C	carbon
CDM	Canadian Drought Monitor
CMI	Climate Moisture Index
CO_2	carbon dioxide
CPP	controlled parentage program
DBH	diameter at breast height (at 1.3 m $\pm$ 15 cm from the base of the tree)
DDH_2O	distilled water
DIB	diameter inside bark
DNA	deoxyribonucleic acid
DR	drilling resistance
E	transpiration
eRH	equilibrium relative humidity
FGRMS	Alberta Forest Genetic Resource Management and Conservation Standards
FL	few large clones
g_s	stomatal conductance
H	clonal diversity
HCl	hydrogen chloride
iWUE	intrinsic water-use efficiency, calculated as A/g_s
J	clonal evenness
MASL	meters above sea level
MRCs	multi-ramet clones
MS	mostly small clones
N	nitrogen
N_2	dinitrogen

NH_4^+	ammonium cation
NHCl	ammonium chloride
NO_x	nitrogen oxides
PARi	photosynthetically active radiation
PCR	polymerase chain reaction
PET	mean annual evapotranspiration
PPT	mean annual precipitation
PSII	photosystem II
R	genotype richness
R^2	coefficient of determination
RAC	resource acquisition capacity
Rc	recovery
Rfu	relative fluorescence units
RRs	relative resilience
Rs	resilience
Rt	resistance
RUE	resource use efficiency
SD	standard deviation
SE	standard error
SLA	specific leaf area
SRG	single-ramet genotypes
SW	saturation weight
Tmax	mean maximum temperature
Tmin	mean minimum temperature
U	unique genotypes
UPGMA	unweighted pair-group method with arithmetic mean
VPD	vapour pressure deficit
VPDB	Vienna Peedee Belemnite, carbon isotope reference scale
VWC	volumetric water content
WBAC	Western Boreal Aspen Cooperative
$\delta^{13}C$	stable isotope of carbon
%C	concentration of carbon
%N	concentration of nitrogen

Chapter 1. General introduction

1.1. Trembling aspen (*Populus tremuloides* Michx.)

Trembling aspen (*Populus tremuloides* Michx.) distribution in North America ranges from the east to the west coast, from regions as far north as Alaska and south to Mexico (Mitton and Grant 1980; Perala 1990; Schreiber et al. 2013). Aspen trees are also found from sea level to elevations above 3,300 m (Einspahr and Winton 1977; Mitton and Grant 1980). Aspen grow on varied upland soils, with reduced growth on droughty soils (Einspahr and Winton 1977). In western Canada trembling aspen coexist with different tree species, influenced by soil moisture: in drier sites aspen has been associated with paper birch (*Betula papyrifera* Marshall), lodgepole pine (*Pinus contorta* Douglas ex Loudon), and ponderosa pine (*Pinus ponderosa* Lawson & C. Lawson); in moister sites aspen has been associated with balsam poplar (*Populus balsamifera* L.), black cottonwood (*Populus nigra* L.), alder (*Alnus* spp.), white fir (*Abies concolor* (Gord. & Glend.) Lindl. ex Hildebr.), white spruce (*Picea glauca* (Moench) Voss), Sitka spruce (*Picea sitchensis* (Bong.) Carrière), and Engelmann spruce (*Picea engelmannii* Parry ex Engelm.; Einspahr and Winton 1977).

Aspen is a dioecious species, although rarely, some bisexual flowers may occur (Einspahr and Winton 1977; Grant and Mitton 1979; Li 1995). Flowers are arranged along catkins and appear weeks before the leaves (Einspahr and Winton 1977). In Alberta, flowering occurs after a period of six or seven days of warm air temperatures (above 12°C or more; Moss 1960, as cited in Einspahr and Winton 1977; Huybregts et al. 2007). Aspen seed crops vary from year to year, but masting frequency is still not well documented (Landhäusser et al. 2019). Natural stands take about 20 years until the first seed crop (Einspahr and Winton 1977; Perala 1990).

Aspen seed and pollen are very small, seeds contain little to no endosperm and can be carried for several kilometers (Einspahr and Winton 1977; Perala 1990; Jelinski and Cheliak 1992; Rogers et al. 2020). The spread of pollen and seeds in aspen occurs mainly from the top of the trees, as the lower shoots become abscised with age (Yeh et al. 1995), which facilitates dispersal in high wind regions such as in Alberta (Jelinski and Cheliak 1992; Yeh et al. 1995; De Woody et al. 2009). Aspen seedling establishment is considered a rare event in nature, still

aspen seeds potentially have a higher chance of establishment in moist environments and low chances of germinating in arid environments (Einspahr and Winton 1977; Mitton and Grant 1980; Sakai and Burris 1985; Landhäusser et al. 2019).

Aspen is an early successional species (Einspahr and Winton 1977) and colonizes areas by seed where there is little competing vegetation (Landhäusser et al. 2019). The seedlings are more likely to survive and become established in post-disturbance sites, such as post-fire, or cleared areas (Mitton and Grant 1980; Landhäusser et al. 2019). Aspen seedlings need available light and are not shade tolerant (Mitton and Grant 1980; Landhäusser et al. 2019).

Aspen trees can also propagate vegetatively (Barnes 1966). In previous studies, root suckers started to grow from adventitious buds on roots of about 2 to 3 cm in diameter, after the second year of a seedling (Day 1944; Einspahr and Winton 1977). Aspen ramets originated from the parent ortet have the same genotype (Einspahr and Winton 1977). For this reason, ramets belonging to one aspen clone are believed to have the same sex, and similar morphological and phenological characteristics such as time of flowering, leaf flushing and senescence, bark texture and colour, stem form and branching habit, and susceptibility to injury (Baker 1921; Barnes 1966; Kemperman and Barnes 1976; Einspahr and Winton 1977; Jones and DeByle 1985). Even after ramets contained in aspen stands have been replaced through succession, their root systems can remain viable for long periods, and become re-established after disturbance events (Grant and Mitton 1979; Jelínková et al. 2009).

The species distribution can be influenced by climate. Aspen stands tend to occur in areas of low to medium moisture availability (Hogg and Hurdle 1995; Hogg et al. 2008). Moisture can drive spatial variation of aspen, including survival and growth patterns, with high mortality and dieback in drought-affected areas (Hogg et al. 2008; Michaelian et al. 2011). In Alberta, moisture availability limits the southern distribution of the species range (Hogg 1997).

Several characteristics of aspen trees are genetically controlled, such as: growth and form, wood density, fiber length, wood, and pulp properties (Einspahr and Winton 1977). Diploids, triploids, and tetraploids of aspen have been reported and results showed that triploids usually showed good form, faster growth, and improved fiber properties when compared to diploids (Einspahr and Winton 1977). Economically, aspen fibre is used for pulp and paper products and oriented strand board production (Einspahr and Winton 1977; David et al. 2001; Brouard et al. 2017). The economic uses of aspen have started to be explored recently; from the mid 1980s until the 2000s the use of aspen as raw material for the forest industry increased by 800% (David et al. 2001). In Alberta, the annual allowable cut for aspen (hardwoods in general) was set at over 12 M m^3/year in 2020 (Rogers et al. 2020). David et al. (2001) indicated

that additional industrial expansion will depend on future availability of aspen trees. In this sense, the plantation and use of genetically improved aspen trees is an option for stand replacement and better economical return (Li 1995; Thomas et al. 1997; Gylander et al. 2012).

1.2. AW2 aspen controlled parentage program

Aspen plantations are not common in Alberta: most of the plantations are established on reclamation sites after the disturbances caused by oil and gas exploration or on roads disturbed by harvesting operations (Brouard et al. 2017). With the rise in economic uses of aspen fibre (David et al. 2001) there is a need to increase the availability of approved aspen planting stock. In this context, the Alberta aspen breeding program started in 1995, as an initiative of the Western Boreal Aspen Cooperative (WBAC; Gylander et al. 2012). Li (1995) proposed the first breeding strategy for native aspen in Alberta. The objectives of the program were to develop improved aspen for fast rotation forestry, including the improvement of frost resistance and winter hardiness for materials deployed in northern locations, resistance to insects and disease, stem form and fibre quality.

Selection and breeding in Alberta were done within three areas, later reduced to two areas, AW1, and AW2, which now are the aspen improvement programs in the province (Appendix 1; Li 1995; Gylander et al. 2012; Brouard et al. 2017). The breeding regions were delineated based on geographic patterns of genetic variation (Li 1995; Gylander et al. 2012). Naturally occurring aspen populations (i.e., wild stands), adapted to local conditions of Alberta, were used for the original parent material selections (Li 1995). Selection was based on tree form, apparent growth superiority, health, and location (Li 1995; Brouard et al. 2017).

Tree improvement programs apply knowledge obtained from forest genetics to tree breeding (Einspahr and Winton 1977). In terms of productivity, breeding programs of aspen in the Lake States showed a 20-year reduction in rotation time of improved material compared to unimproved (Li et al. 1993). In addition, Gylander et al. (2012) showed that clonal selection alone can result in 15% genetic gain in height and 34% in DBH, prior to the first generation of breeding. In terms of climate change, aspen provenance trials have shown a possible adaptation lag, as clones transferred northward presented better performance (Gray et al. 2011; Gylander et al. 2012; Schreiber et al. 2013). The finding of an adaptation lag has led to studies on assisted migration (Schreiber et al. 2013).

The AW2 breeding program currently includes West Fraser (formerly Norbord Inc.) and Weyerhaeuser Company Ltd. (Pembina) as members of the Western Boreal Aspen Corporation

(WBAC). The objective of the program is to deploy aspen with genetic gain in wood production, generating planting stock to establish 15,000 ha of plantations by 2030 (Brouard et al. 2017). Genetic gains of 11 to 51% are predicted for height, and 19 to 293% for volume, from the selection of the top 18 clones (Brouard et al. 2017). Thomas et al. (1997) predicted 16% genetic gain for height and diameter of native aspen in Alberta (selecting the top 5 clones). Genetic improvement is being achieved in two stages: 1. Clonal testing and selection for rapid short-term gains; and 2. Conventional breeding and progeny testing for long-term gains. Since 2015, 325 clones from the AW2 region have been under test in replicated trials. There are 642 clones in 24 progeny trials in the AW1 and AW2 regions (Brouard et al. 2017). The best-performing clones were scheduled for deployment in 2030 (Brouard et al. 2017) but have started to be operationally deployed in the AW1 and AW2 regions since 2020 (Swindlehurst D., personal communication, 2023).

1.3. Dioecy in plants

A plant species for which an individual exhibits only one sex is known as dioecious (Darwin 1877). Sex can be described as the "*functional sex expression of the plant*" (i.e., male or female) (Sakai and Weller 1999). During reproduction, angiosperms produce unisexual haploid gametes, which are pollen and ovules (Sakai and Weller 1999). In general, male individuals are the ones producing smaller, mobile gametes (i.e., pollen), and females produce larger and less mobile gametes (i.e., ovules; Darwin 1877; Sakai and Weller 1999). Sex determination in dioecious plants is regulated by genetics but can also be influenced by environmental conditions and nutritional status (Bhatla and Lal 2018).

There are several theories to explain the evolution of dioecy in plants. One of the most dominant arguments suggests that having separate sexes promotes outcrossing, reducing inbreeding (Renner and Ricklefs 1995; Charlesworth 1999). Another argument suggests that female plants can produce larger amounts of seed than hermaphrodite (or bisexual) plants. In this case, females would be favoured, since the higher seed production can result in higher fitness (Charlesworth 1999; Webb 1999). The evolution of dioecy has also been correlated with ecological factors, such as dry conditions and wind pollination (Charlesworth 1999; Sakai and Weller 1999). A comprehensive exploration of the evolution of dioecy in plants can be found in Charlesworth (1999).

About 6% of the world's flowering plant species are dioecious, in a total of 157 families (Renner and Ricklefs 1995; Hultine et al. 2016). It has been observed for many dioecious plants that spatial segregation of sexes is linked to resource availability (Dawson and Ehleringer 1993; Hultine et al. 2007; Hultine et al. 2016). Sexes might be segregated as a result of different costs of reproduction (Delph 1999; Hultine et al. 2007; Hultine et al. 2016).

1.4. The costs of reproduction hypothesis

The cost of reproduction hypothesis is based on the premise that energy is a limited resource, so when energy is allocated to reproduction or defence, less energy is available to perform other organism functions, such as growth (Levins 1968; Bazzaz et al. 1987; Delph 1999; Obeso 2002).

According to this hypothesis, female individuals of dioecious plants would incur higher costs of reproduction than males, as females potentially spend more energy on the production of ovules, fruit, and seeds, than males spend on the production of pollen (Darwin 1877; Delph 1999; Obeso 2002; Stevens and Esser 2009). In this sense, the sex that invests more energy on reproduction, that being potentially the female, has trade-offs such as lower growth rates, survival, and total biomass, and delayed reproductive maturity compared to males (Delph 1999).

In dioecious species, the study of costs of reproduction is facilitated, as most individuals will have only one sex (Delph 1999; Sakai and Weller 1999). In woody dioecious plants, females potentially invest more resources in reproduction and less in growth when compared to males, consequently, woody dioecious plants might present differential performance of sexes (Dawson and Ehleringer 1993; Delph 1999; Stevens and Esser 2009).

1.5. Sex differential performance

The differential performance of sexes has been described by many authors. Obeso (2002) described two types of sex differential performance or intersexual dimorphism: somatic, when male and female showed different growth performances; and demographic, when sexes showed different survival and presence on the landscape. Delph (1999) described direct and indirect costs of reproduction. Direct costs refer to allocation of resources at the time of reproduction, and indirect costs refer to the delayed costs of reproduction resulting in effects on the size of populations (Delph 1999; Obeso 2002).

The general expectation that males outperform females might not be applicable for all developmental stages of dioecious species. Literature indicates that when environmental conditions are optimal, female plants have higher photosynthetic rates and resource acquisition capacity, than males (Dawson and Ehleringer 1993; Obeso 2002; Han et al. 2013; Randriamanana et al. 2014; Hultine et al. 2007; Hultine et al. 2016). This difference would give females a performance advantage during early stages of life before the start of reproduction (Dawson and Ehleringer 1993; Delph 1999; Stevens and Esser 2009; Cole et al. 2016). Once reproduction starts, females might allocate the synthesized energy to the production of flowers and fruits, that is when males potentially start to outperform co-occurring females (Stevens and Esser 2009). Additionally, males are known to flower more frequently and reproduce earlier than females (Valentine 1975; Einspahr and Winton 1977; Laporte and Delph 1996). Previous studies on *Populus* spp. indicate that young females present higher growth rates and total biomass when compared to males (Cole et al. 2016; Stromme et al. 2018; Zhang et al. 2018).

In general, aspen sex ratios have been found to be near 1:1 (Einspahr and Winton 1977; Grant and Mitton 1979). In Colorado, this distribution deviated from 1:1 depending on elevation, with female biased ratios in lower elevations and male biased ratios in higher elevations (Grant and Mitton 1979).

1.6. Climate change

Increasing temperatures, more frequent droughts, and extreme climatic events; these changes in climate used to be predicted in studies at the end of the previous century (IPCC 1990; Boer et al. 1992) and are now a reality (Hogg et al. 2008; Allen et al. 2015; Batllori et al. 2020; IPCC 2022). Droughts induced by warmer temperatures have been observed across the planet since the 2000s (Allen et al. 2015; Batllori et al. 2020; Hynes and Hamann 2020). The increase in air temperatures has caused an increase in evaporative demand, which in turn, has resulted in less moisture available in natural forest stands (Gray and Hamann 2015; Keenan 2015; Hynes and Hamann 2020). Less moisture availability combined with longer growing seasons can result in more use of water over the year and consequently higher risk of drought stress, especially during the growing season (Gray and Hamann 2015; Keenan 2015; Hynes and Hamann 2020; Eum et al. 2023).

The occurrence of more frequent and extreme droughts is predicted for the province of Alberta, Canada (Gray and Hamann 2015; Keenan 2015; IPCC 2022; Eum et al. 2023). Future climatic projections indicate that atmospheric temperatures will increase starting in the 2020s

with accelerated warming towards the 2080s (Gray and Hamann 2015). Data from the Canadian Drought Monitor (CDM), released by Agriculture and Agri-Food Canada (2021), identified below-normal precipitation and above-normal temperatures across Canada in their drought assessment for the year 2021. CDM shows that Canadian prairies are under increasing drought conditions caused by short-term and long-term droughts aggravated by below-average precipitation.

The climate moisture index (CMI) indicates the amount of available moisture in the environment and is calculated by subtracting potential evapotranspiration from precipitation (Hogg 1997; Schneider 2013). In Alberta, positive CMI values indicate that conditions are sufficiently wet to sustain a closed canopy forest and negative CMI values indicate that conditions are dry and can only sustain patches of trees, as characteristic of parkland forests (Hogg 1997). Hogg (1994) found that the limits of the boreal forest and parkland regions coincided with the areas where CMI becomes zero or negative. Regions with the lowest CMI, are characterized by dry shortgrass prairies. Increases in CMI (CMI > 0) are reflected in taller vegetation and isolated aspen stands. Closed aspen stands occupy regions where CMI approaches zero, and coniferous trees and mixedwood stands occur where CMI is greater than 1 (Schneider 2013). Areas with negative CMI are characterized by frequent moisture deficits, saline soils, and historically more frequent fires (Hogg 1994).

1.7. The effects of drought on aspen

Environmental stress negatively affects plant growth and establishment, as under optimal environmental conditions, plants can reach their maximal growth and reproductive potential, while under environmental stress, plants cannot express their full genetic potential (Bhatla and Lal 2018). Environmental stress can originate from the interaction with other organisms (i.e., biotic stress), or from the excess or deficit of physical, chemical, and energetic limiting factors (i.e., abiotic stress, such as drought; Bhatla and Lal 2018).

Drought reduces photosynthetic activity on *Populus* spp. and aspen trees, which is reflected in lower growth rates (Hogg and Hurdle 1995; Xu et al. 2008). Drought has been found to significantly decrease gas exchange in *Populus cathayana*, caused by photodamage to PSII reaction centers (Xu et al. 2008). In Hogg and Hurdle (1995), aspen trees showed reduced growth under dry conditions in the Parkland region; productivity was half to two-thirds lower than trees in the boreal forest transect case study. Other studies show that droughts in the parkland

region of Alberta have caused an increase in mortality and a decline in the health of aspen trees (Michaelian et al. 2011; Birch et al. 2019).

Warmer and drier conditions can also promote insects and fungal species (Hogg et al. 2002; Chen et al. 2018). Insect defoliators such as the forest tent caterpillar, as well as fungi infections, can stress aspen, affecting the productivity and survival of trees and whole stands (Hogg and Hurdle 1995; Chen et al. 2018). Lastly, drought stress could drive unbalanced sex ratios of dioecious tree species on the landscape (Hultine et al. 2016).

1.8. Aspen in Alberta: Performance and characteristics of clones and sexes

Aspen clone size has been of interest (Barnes 1966) from an ecological and evolutionary perspective for decades (Mitton and Grant 1996; DeWoody et al. 2008; DeWoody et al. 2009; Long and Mock 2012; Namroud et al. 2005; Latutrie et al. 2019). In the western USA, a small number of ancient large aspen clones have been described, including the famous *Pando* clone (DeWoody et al. 2008; Mock et al. 2008). Kemperman and Barnes (1976) pointed out that in the northern portion of the Rocky Mountains and eastern North America, including Canada, aspen clones are very small when compared to the western USA. Latutrie et al. (2019) did not find a difference in aspen clonal structure across Canada. Nevertheless, the species range and overall persistence on the landscape has been influenced by a decline in aspen health (since the 1990's) across much of North America, due primarily to drought (Michaelian et al. 2011; Worrall et al. 2013).

It is imperative to understand the factors affecting clone presence, genetic diversity, and sex distribution in aspen, to inform the refinement of policy for clonal deployment and associated regulatory standards and to provide insights into the ecological responses of aspen to abiotic stress, including drought. Most of the knowledge pertaining to aspen clone size patterns on the landscape has been acquired under the assumption that phenotypic differences can be used to differentiate clones (Barnes 1969; Kemperman and Barnes 1976). More recently, however, genetic analyses suggest that physiology plays a role in phenotypic expression, which means that stands that look like different clones may have similar genetic origins (Jelínková et al. 2014). Furthermore, the composition of clones identified through phenotypic characteristics (leaf emergence in particular), may be more influenced, than previously thought, by site characteristics, and root grafting than genetics alone (DesRochers

and Tremblay 2013; Jelínková et al. 2014). In other words, genetically distinct clones could have similar phenotypes.

There is a general expectation that male aspen clones will outperform female aspen clones (Obeso 2002). A competing hypothesis is that females maintain their present distribution on the landscape with no added cost to reproduction due to the small cost of producing aspen seed (Miller 1996). Historically, relatively few studies have examined the role of sex in aspen performance (Grant and Mitton 1979; Mitton and Grant 1980). So far, the general expectation that males will outperform females has not been shown consistently. Previous studies found growth-defence trade-offs, where not only females were smaller than males on average, but they also invested in the production of phenolic glycosides and condensed tannins, suggesting a greater allocation to defence vs. growth in females (Stevens and Esser 2009; Randriamanana et al. 2014; Zhang et al. 2018). In other studies, males were predominately found at higher elevations, while females had higher vegetative growth rates independent of elevation (Grant and Mitton 1979).

The results from the studies shown above were obtained before the development of a genetic marker to determine sex in aspen (Pakull et al. 2014). The differentiation of sex in aspen trees based only on flowering was challenging (Kersten et al. 2013, Tuskan et al. 2012). Not all clones in a stand flower in the same year, making field assessments difficult and sex ratios on the landscape hard to quantify. However, the development of a genetic marker for sex (Pakull et al. 2014) opened a novel possibility for exploring sex responses in aspen at any stage of development and within natural stands.

The differential performance of sexes may be complicated by climate change. Early selection (1990s) in Alberta for superior clones based on size (height and DBH) and health, resulted in a phenotypic ratio of approximately 3:1 males:females in parental selections for aspen tree improvement (Thomas B., personal communication, 2019). This suggests that males may show superior growth.

This thesis will contribute to our understanding of the patterns of aspen clone presence and sex distribution on the landscape in south-central Alberta. The results of this study will be especially important to help guide decisions for the future maintenance of aspen forests despite the uncertain conditions resulting from climate change. Such information can be used to support policies for clonal deployment and associated regulatory standards for aspen, on the development of aspen improvement programs, and for the general management of aspen forests by industry and government.

Two approaches were taken to study aspen in Alberta, using both young seedlings and mature stands. In chapter 2, male and female aspen seedlings from two different climatic regions in Alberta were experimentally exposed to well-watered and drought conditions in a greenhouse environment, to: i) test if parent tree origin has an influence on aspen seedling performance; ii) test if different aspen families present differential drought strategies, and; iii) explore if young non-reproductive aspen present different performances between sexes under well-watered and drought conditions. In chapters 3 and 4, mature aspen stands within the AW2 (Pembina) tree improvement region in Alberta were studied to: i) understand the distribution of male and female aspen trees within mature stands and if this distribution is influenced by moisture availability, and explore if male and female aspen trees showed differences in resilience components after a drought period (chapter 3); ii) describe aspen clonal structure using phenotypic assessments and microsatellite markers and explore the influence of polyploidy on clonal structure and growth performance (chapter 4). Chapter 5 summarizes the findings of this thesis and provides recommendations for future management and study.

Chapter 2. Family and sex-related responses of trembling aspen (*Populus tremuloides* Michx.) seedlings to drought stress

2.1. Introduction

Droughts caused by increasingly warmer temperatures have been predicted and observed across the planet since the 2000s, and the potential of these changes in climate to cause shifts in vegetation patterns is now a matter of concern (Allen et al. 2015; Batllori et al. 2020). Drought events are typically observed at local scales, but an increase in frequency and duration of droughts has been predicted for much of the globe in the current century (IPCC 2022; Eum et al. 2023). The Canadian Drought Monitor (AAFC 2021) identified that Canadian prairies are under increasing drought conditions. Warmer temperatures reflect on high evapotranspiration rates, as even small rises in temperature cause large rises in atmospheric moisture demand (also known as vapour pressure deficit; VPD; Allen et al. 2015; Hynes and Hamann 2020). Vapour pressure deficit, consequently, impacts transpiration and photosynthesis; plants lose more water when VPD is higher, and the risk of hydraulic failure is also increased (Allen et al. 2015).

The climate moisture index (CMI) indicates the amount of available moisture in the environment by calculating moisture availability from precipitation minus the potential evapotranspiration (Hogg 1997; Schneider 2013). Positive CMI values indicate that moisture availability is enough to sustain a closed canopy forest and negative CMI values indicate that conditions are dry and can only sustain patches of trees, as characteristic of parkland forests (Hogg 1997). Hogg (1994) found that the limits of the boreal forest and parkland regions coincided with the limits where annual CMI becomes zero or negative.

Aspen (*Populus tremuloides* Michx.; hereafter “aspen”) is a native broadly distributed tree species in North America (Mitton and Grant 1980; Perala 1990). Aspen grow on varied upland soils, with reduced growth on droughty soils (Einspahr and Winton 1977). Aspen is a dioecious tree species with some bisexual flowers (Grant and Mitton 1979). In woody dioecious species, such as *Acer negundo* (Dawson and Ehleringer 1993), *Populus tremuloides* (Stevens

and Esser 2009), and general dioecious plants as described in review papers (Delph 1999; Obeso 2002) females are expected to incur higher energy costs of reproduction than males. Yet, not all dioecious species show sexual dimorphism, as shown for *P. trichocarpa* and *P. balsamifera* (McKown et al. 2017) and for *P. tremula* (Robinson et al. 2014). Additionally, sex differential responses to environmental factors may offset the effects of an increased cost to reproduction (Liu et al. 2021).

Females of different dioecious tree species seem to have higher photosynthetic rates (stomatal conductance, g_s, and net carbon assimilation, A) under optimal environmental conditions when compared to males (Randriamanana et al. 2014; Hultine et al. 2007; Hultine et al. 2016). At the same time, studies found males to have higher survival and vegetative growth under stress, which has been attributed to higher water-use efficiency (A/g_s) when exposed to environmental stress (Dawson and Ehleringer 1993; Hultine et al. 2007; Xu et al. 2008; Han et al. 2013). Zhang et al. (2018) found that under average temperatures of 17.3°C, female European aspen (*Populus tremula* L.) showed greater height increment, leaf, stem, and total shoot biomass than males, while males grew faster than females under conditions of 15.7°C on average. More recently, scientific evidence has shown, for species exposed to different types of environments, for example, *Juniperus thurifera* occurring in mesic versus xeric locations in the Iberian Peninsula (Olano et al. 2017) and *Populus cathayana* exposed to well-watered versus drought conditions (Liu et al. 2022), that females' hydraulic conducting system can be more efficient than males under non-limiting environmental conditions. Therefore, the maximum rate at which water is transported from the soil to the leaves might be higher in females than males (Olano et al. 2017; Liu et al. 2022). Females of *P. cathayana* were found to have thinner xylem cell walls and greater lumen area per vessel and consequently greater hydraulic efficiency and faster growth under well-watered conditions (Liu et al. 2022). However, angiosperm trees have shown a trade-off between hydraulic efficiency and xylem cavitation resistance (Tyree et al. 1994; Pockman and Sperry 2000; Hacke et al. 2006, as cited in Hultine et al. 2007; Liu et al. 2022). Accordingly, the hydraulic conducting system of females may become dysfunctional during drought as there is an elevated risk of cavitation and air embolism, which affects water transport to leaves and carbon acquisition (Hultine et al. 2007; Olano et al. 2017; Liu et al. 2021; Liu et al. 2022). Liu et al. (2022) found that male *P. cathayana* are more tolerant to drought than females due to sex-specific xylem anatomy, carbohydrate dynamics, and osmotic maintenance at the molecular level. Therefore, females and males potentially present different responses to environmental stress (Liu et al. 2021).

Other than sex and environmental effects, genetic variation also plays a role in the performance of plants. Literature has shown that even when most families and individuals perform according to expected results, for example when most males outperform females under drought conditions, some families within the group still present contrasting results, indicating a genotype effect or an interaction of genotype and expected effect, for example *family x sex* (Stevens and Esser 2009; Konatowska et al. 2021). In addition to contrasting results, different genotypes might also vary in the extent of the response, and in drought strategies (Tupker et al. 2003; Larcheveque et al. 2011).

The growth of populations of a species might differ according to climate variability over its range of distribution (Eriksson et al. 2020). In effect, selection pressures favour individuals and phenotypes adapted to the local environment (Levins 1968). Still, selection pressure might now be greater than the ability to adapt (Levins 1968). In Alberta, the Boreal forest is restricted to areas with a surplus of available moisture, where precipitation is greater than evapotranspiration (Hogg 1997) and the Parkland region is influenced by drier conditions (Hogg et al. 2008). Climate projections predict more frequent and extreme droughts for the province of Alberta in this century (Gray and Hamann 2015; Keenan 2015; IPCC 2022; Eum et al. 2023). Considering that Alberta's natural regions are restricted and defined by levels of moisture availability (Hogg 1997), the predicted drier conditions could influence the distribution of natural regions and the adaptability, survival, and health of the species occurring within these fast-changing ecosystems (Hogg et al. 2008; Hynes and Hamann 2020).

The objectives of this study are to: i) Test if parent tree origin (i.e., the parkland region, around Camrose vs. the boreal region, around Peace River) has an influence on seedling performance under well-watered and drought conditions; ii) test if different families (i.e., half-sibling groups) present differential drought strategies; and iii) explore if young non-reproductive aspen show differential performance by sex (considering differences in growth, total biomass, leaf area, and gas exchange) under well-watered and drought conditions. I hypothesized that: i) Seedlings from the boreal region (higher CMI) will present reduced performance under a drought stress treatment when compared to seedlings from the parkland region (lower CMI). Additionally, if seedlings from regions of higher moisture availability have higher photosynthetic rates (Hart et al. 2021; Rudnew et al. 2023), then seedlings from the Peace River region are expected to outperform seedlings from the Camrose region under well-watered conditions; ii) I expect that some families will present contrasting responses to drought stress, and; iii) In general young female aspen seedlings will outperform young male aspen seedlings, in photosynthesis rates (stomatal conductance, g_s, and net carbon assimilation, A), growth, and

total biomass, in the well-watered treatment, as females might uptake resources more efficiently than males under optimal environmental conditions; and males would outperform females under drought conditions, due to higher water-use efficiency (A/g_s) rates in males under environmental stress.

2.2. Methods

Study area

Catkins were collected from 21 female aspen parent trees, ten in the parkland region (from 52.63 ºN to 52.98 ºN latitude and from -112.59 ºW to -112.78 ºW longitude, 720 – 819 meters above sea level; MASL; near Camrose, Alberta; AB) and 11 in the boreal region (from 56.34 ºN to 56.84 ºN and -117.64 ºW to -118.00 ºW, 498 – 820 MASL; near Peace River, AB) during the spring of 2018 (Figure 2-1). When the straight-line distance, generated on ArcMap 10.7.1 (ESRI, Redlands, CA), between selected parent trees was shorter than 8 m, those parent trees (families) were removed from the data. Consequently, 19 families were kept in the study, nine from the parkland region and 10 from the boreal region.

According to Schneider (2013) the parkland region had lower annual climate moisture index (CMI -4.1 cm) when compared to the boreal region (CMI for Dry Mixedwood 0.4 cm and Northern Mixedwood 2.3 cm) for the reference normal period 1961-1990. Climatic data were obtained using the BioSIM (Government of Canada 2022) software for the location of each parent tree for the climate normal period of 1961-1990, to compare with the data reported by Schneider (2013), and for the 1991-2018, to represent recent climate, which was most likely to influence the growth period of the parent trees. However, the growth period of parent trees (more specifically its aboveground parts) might differ from the age of its root system, as aspen root systems can remain alive after the stems have been removed or died (DesRochers and Lieffers 2001). CMI was calculated from the monthly precipitation minus the potential evapotranspiration (Hogg 1997; Figure 2-2).

Mean maximum and minimum temperatures were calculated for the growing season, from May to September, for each parent tree location for the two reference periods, then locations were pooled by region. For the period of 1961-1990, the boreal region had a mean maximum temperature of 18.3°C and a minimum of 5.9°C. The parkland region had a mean maximum temperature of 20°C and a minimum of 7.4°C. For the reference period of 1991-2018, the boreal maximum and minimum temperatures were 19.0°C and 6.1°C, respectively, and the parkland were 20.5°C and 6.6°C, respectively. Mean monthly precipitation for the reference

growth period (i.e., 1991-2018) was 34.7 mm for boreal and 33.3 mm for the parkland region (Table 2-1).

Seed collection, extraction, and storage

Parent trees (i.e., female aspen trees selected for catkin collection) were at least 8 m apart (distance between families F11 and F12), but in most cases parent trees were separated by a minimum of 500 m to avoid collecting catkins from the same clone (Appendix 3, Table A3-1). Female catkins were collected in May 2018 and stored in paper bags for transport until the extraction of seed at the University of Alberta.

Seeds were extracted in June 2018. First, catkins containing cotton and seeds were placed in labelled paper bags. Paper bags were shaken until cotton and seeds were separated from catkins and were further vacuumed to the inside of cotton bags. Cotton bags were then labelled with parent tree information and stored in a refrigerator, if not processed on the same day. To remove seeds from cotton, soil sieves (20, 20, 40, and 60 openings per 2.5 cm^2 of screen; connected in the respective order) were used. Aspen seed cotton was placed into the second '20' sieve, with the first '20' sieve placed on top of it. An air blower (shop vac) was used to blow the seed through the sieves to separate the seed from the cotton. Extracted seeds were then placed in labeled glass vials and stored at -20°C from June 2018 until January 2019 until sowing. To improve seed longevity, seed equilibrium relative humidity (eRH) was measured and determined to be 15-20% eRH prior to storage. The FGRMS (2016) defines eRH as "*the relative humidity of the air in an enclosed environment containing seeds, at the point where seeds are no longer gaining or losing moisture from the surrounding air.*" For eRH measurements, samples were cleaned, to ensure debris contamination consisted of less than 30% of the sample. Seeds were then placed into a portable water activity analyzer (HygroPalm HP23-A / HP23-AW-A hand-held indicator, Rotronic AG), on quick mode for 10 minutes dwell time. If the eRH reading was: >20%, seeds were placed on the counter for 10 minutes to dry and tested again; once seeds reached a reading of 15-20% eRH they were placed in foil bags, sealed with a heat sealer, and stored at -20°C; <15% eRH, seeds were placed in a closed cooler containing a wet towel inside for 10 minutes and tested again. Once the desired eRH was reached, seeds were stored as described above.

Seed vials were removed from the freezer on January 29th, 2019, and left on the counter to thaw overnight. Jars were opened the next day, and 100 seeds were counted for each clone. New eRH measurements were taken prior to germination tests. To determine if seeds were viable for sowing, seeds were plated on moist filter paper in a petri dish, sealed with parafilm,

and then placed in a germination chamber at 25°C with a 12 h photoperiod. The Alberta Seed Testing Standards (2016) categories of germination were followed: germinated, radical visible and at least 4x length of seed; low vigour, radicle visible but not at least 4x length of seed, and; ungerminated seed, no radical visible. Germination results were used to determine if seeds were viable for sowing.

Sowing and first growing season

Seedlings from each of the 19 parent trees were established from seed and grown, for one growing season in 2019 at a commercial nursery (Bonnyville Forest Nursery), in Bonnyville, Alberta.

In August 2019, leaf samples were collected from 48 seedlings per family for DNA extraction using sterilized scissors; scissors were cleaned with water and 5% bleach solution after collection for each family. Leaf samples were placed in a cooler with ice (during collection) and subsequently stored at -80°C until being transported on ice to InnoTech Alberta, in Vegreville, in January 2020, for DNA extractions and analysis.

On November 29th, 2019, dormant trees were lifted at Bonnyville Forest Nursery, bundled into groups of 10, wrapped in plastic and stored at -3°C for six months, prior to transportation to the University of Alberta in May 2020.

DNA analysis: sex screening

Sex was determined on these non-flowering aspen seedlings through DNA screening. DNA was extracted from leaf tissue using a Dneasy Plant Mini Kit according to the manufacturer protocol at InnoTech, in Vegreville, Alberta.

Previously identified female and male aspen trees (identification based on flowering), located near the University of Alberta, were used as controls to verify sex. Control samples were collected on October 31st, 2019, and consisted of four male and two female buds, two male and two female twigs, and three female and one male phloem tissue, for a total of 14 samples. For the sex differentiation, 1008 aspen leaf samples (this included all 21 families initially selected) plus 14 controls of known sex were collected and kept frozen at the University of Alberta at -80°C until DNA extraction.

Control tissue samples were hand ground in liquid nitrogen with a mortar and pestle, while frozen leaf samples (at -80°C) were placed in 2 mL tubes with a mix of 0.5 mm, 2.3 mm zircona/silica beads, and 4 mm stainless steel grinding balls and ground using a Geno/Grinder (SPEX Sample Prep 2010) at 1750 rpm for 1 minute. Next, the Dneasy 96 (Qiagen #69181)

plant kit for frozen tissue was used with the following modifications: after addition of the first buffer (buffer AP1, Rnase A, and reagent DX) the samples were ground on the Geno/Grinder for an additional minute; the final elution was done with 100 µL nuclease free water, the plates were left at 4°C overnight, and heated to 65°C for 10 minutes the next morning before eluting. Samples were all stored at -20°C until all PCR sexing procedures were complete, and thereafter stored at -80°C.

Five microlitres of a 1/10 dilution of leaf sample or control sample were placed in a well of a PCR plate (Biobasic #PCR-960-LP-S2) with 25 µL DreamTaq Hot Start Green DNA Polymerase (Thermofisher scientific #EP1714), 1 uL of each of four primers at 5 µM concentration, and 16 µL nuclease free water. Bands were amplified via PCR (95°C for 2 min; 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min; x 40; 72°C for 5 min, 4°C hold) using an Eppendorf PCR machine (#EP Mastercycler gradient S) and then run on a 1.5% agarose gel (Thermofisher scientific #16500100) and visualized using Ethidium Bromide (Sigma #E1510).

In aspen, sex determination is found on chromosome 19 (Pakull et al. 2014; Hou et al. 2015). The TOZ19 gene is predicted to be involved in the transition from the vegetative to the reproductive phase of a developing bud (Pakull et al. 2014). According to Pakull et al. (2014), the TOZ19 gene sequence is only present in male aspen. We differentiated sex on aspen with the analysis of the TOZ19 locus, using four primers (Table 2-2), following Pakull et al. (2014). The TOZ19-1 primer set is based on gene Potri.019G047300, and the internal control fragment Potri.005G243400 from the *P. trichocarpa* genome (Pakull et al. 2014). The agarose gel was scored for the presence or absence of a ~300 bp band from the TOZ19-1 primer set, where presence was scored a male, and absence scored a female (Figure 2-3). The internal control produced a ~400bp band on all gels. Samples containing no band or only the primer band (occurring under 200bp) were scored as failed, and re-run.

Greenhouse: growing seedlings

After transport of the seedlings from -3°C storage at Bonnyville Forest Nursery to the University of Alberta on May 11th, 2020, seedlings were transplanted into individual 1.67 L round 15 cm (6”) plastic pots containing an artificial soil mixture, Sunshine Mix #4 (SunGro Horticulture, Vancouver, BC, Canada; Ingredients: Canadian Sphagnum Peat Moss, RESiLIENCE®, Perlite, Dolomite Lime, Wetting Agent) and sand (Target products Ltd., Burnaby, BC, Canada; Silica Abrasive; physical properties: colour tan, grain shape sub-rounded, bulk density 1474 – 1522 kg/m3, hardness Moh 6.5 – 7, specific gravity 2.65, moisture content <0.1% weight), at a 1:1 Sunshine mix:sand by volume, and 8.37 g/litre of soil slow-

release fertilizer 14-14-14 (ProHort Fertilizer 14-14-14 Greenhouse and Nursery). 100 mL of liquid fertilizer (Direct Solutions Tree Fertilizer 30-8-8; 50 ppm; 1:40 ratio) was added to all seedlings on days 11 and 25 of the experiment.

Plants were grown in a greenhouse at the University of Alberta, Edmonton, AB, Canada, for a total of nine weeks prior to harvest, from May 11th, 2020 to July 17th, 2020, under an ~18 h photoperiod (6 am – 12 am), in which natural light was supplemented with artificial lights. The artificial lights provided a photosynthetic photon flux density ranging between 200 and 300 $\mu mol.m^{-2}s^{-1}$ at pot level. Humidity and temperature were monitored with three Hobos U12-012 data loggers (Onset Computer Cooperation, Pocasset, MA, USA), located at the south-east, central-west, and central-north locations in the greenhouse. The greenhouse maintained an average temperature of 23.5 ($\pm$0.13)°C in May, 24.4 ($\pm$0.09)°C in June, and 24.5 ($\pm$0.11)°C in July during the day, and 19.8 ($\pm$0.23)°C in May, 21.1 ($\pm$0.03)°C in June, 21.1 ($\pm$0.02)°C in July during the night. Trees were grown at the greenhouse for three weeks before the start of the experiment, and for six weeks after the beginning of the drought treatment.

Treatments

The greenhouse experiment ran for six weeks from June 8th, 2020 (day 0), until July 17th, 2020 (harvest at day 39), with two treatments, well-watered (control) and drought. The treatments were determined based on levels of moisture supply. For that, pots were watered to saturation and then dried out to reach the specific moisture levels. Moisture levels were established based on volumetric water content (VWC) at saturation (i.e., 100% VWC). Appendix 2 contains the pre-experiment trials used to determine the soil mixture used and the reference levels of moisture supply. The level of drought chosen was not based on field conditions, but determined based on achieving a level of drought pre-wilting point. The goal was to maintain seedlings at a sustained drought that would not cause seedling mortality. Once the pre-wilting point was established, seedlings were maintained at that level of drought, to ensure that they were all exposed to drought and to test the effects of drought on the seedlings.

The well-watered (control) treatment pots were maintained at 100 to 90% saturation weight (SW), or 100 to 74% of their VWC. The drought treatment pots were maintained at ~75% SW or 22 to 30% VWC, and rewatered to a maximum of 80% SW or 31% VWC (i.e., maintained a 70 to 78% VWC reduction sustained during the whole experiment). Drought-treated seedlings were bottom watered using an individual tray under each pot, to avoid water loss from evaporation and ensure an even uptake of water. The amount of water needed to maintain the target drought level was determined based on VWC measurements (ProCheck probe logger

and EC-5 soil moisture sensor; Decagon Devices, Inc.) taken three times per week (Table 2-3). See appendix 2 for the determination of volumetric water content levels. All pots in the drought treatment were monitored until harvest (Figure 2-4). Well-watered moisture levels were taken but not recorded except in the pre-experiment trials.

Experimental design

After the sex screening of 48 seedlings per family, the design of the experiment was determined to ensure equal representation of males and females of each family in each treatment and block. A split-plot complete block design was used with eight blocks and the moisture treatment representing the split.

A total of 608 seedlings were included in the experiment, according to the following blocked split-plot design: randomly assigning two seedlings (one male and one female) per family/treatment within a block. As there were 19 families, that is: ((19 families * 2 sexes) * 2 treatments) * 8 blocks = 608 seedlings.

The position of treatments within blocks was randomized (using a random number generator; random.org). Seedlings were randomized every two weeks, with pots close to the centre of the block moved to the edge and vice-versa.

For the blocked split-plot design the main plot factor removed environmental variability (blocks), and the split-plot tested the effects of moisture levels (or treatment; well-watered and drought), within each split-plot family and region levels were included (19 families nested within two regions; Peace River; 10 families; and Camrose; 9 families), and sex (female and male). All eight blocks were used for the analyses of height and diameter. The eighth block was not harvested, as it was used for another experiment, so only seven blocks were harvested and used for the analyses of dry biomass (leaves, stems, roots dry biomass, aboveground biomass, root/shoot mass ratio). Because gas exchange and leaf area data collection are both time-consuming processes, it would not be possible to collect data for these variables from all blocks within the timeframe needed, so four blocks (2, 4, 6, and 8) were used for the collection of gas exchange data (net photosynthesis, stomatal conductance, intrinsic water-use efficiency) and leaf area (total leaf area, and specific leaf area). The four blocks were selected based on their position within the greenhouse space, to capture variability.

Measurements

Sampling in the greenhouse consisted of assessing pot weights and moisture levels, growth, gas exchange, leaf area, biomass, $\delta^{13}C$, and concentration of carbon and nitrogen (%C and %N).

Pot weight and VWC of all seedlings were measured on days -21 and -20 and prior to harvesting. After the beginning of the experiment, starting on day 0, VWC readings were taken every Monday, Wednesday, and Friday, from two opposite spots in each pot, until the end of the experiment.

Height was measured on each seedling with a ruler from the soil surface to the base of the apical bud on the first measurement, and from the soil surface to the base of the shoot apical meristem on subsequent measurements. Height was measured on the main stem, or on the next tallest stem available. Basal diameter measurements were taken with a digital caliper (Mastercraft MD, 150 mm; 6") at pot height; the caliper was placed at a 90 degree angle, perpendicular to the stem of the seedling.

Gas exchange data were collected for the final experimental week (starting on day 36), including net photosynthesis (*A;* $\mu mol.CO^2.m^{-2}.s^{-1}$), transpiration (*E;* $\mu mol.H_2O.m\text{-}^2.s^{-1}$), and stomatal conductance (*gs;* $\mu mol.H_2O.m\text{-}^2.s^{-1}$) to CO_2 using a CIRAS-3, portable photosynthesis system, PP Systems infrared gas analyzer (IRGA, CIRAS-3, PP Systems, Haverhill, Mass.) equipped with a universal leaf cuvette (PLC3, PP Systems; 25 mm x 18 mm, area of 4.5 cm^2). Measurements were taken from 9 am to 3 pm, on the first young fully expanded leaf, from all seedlings in a subsample of four blocks (blocks 2, 4, 6 and 8; total of 304 seedlings). Internal photosynthetically active radiation (PARi) was set to saturating light at 1000 $\mu mol.m^{-2}.s^{-1}$, with RGB (red, green, blue) spectrum set to zero and white set to 100%. The flow rate was set at 300 cc/min^{-1} and CO_2 reference to 390 $\mu mol.m^{-1}$. Humidity ranged between 50 and 75%. Ambient leaf temperature averaged 28.3 $\pm$ 1.1 °C. Intrinsic water-use efficiency (iWUE) was calculated as A/g_s.

Harvesting consisted of removing plants from pots and separating leaves, stems, and roots. Soil was washed out of the roots, and all separated parts (leaves, stems, and roots) were oven-dried at 60°C for 72 h, and weighed (scale Adventurer Pro Precision model AV412, Ohaus Corporation), for dry biomass determination. Prior to drying, all leaves from seedlings included in blocks 2, 4, 6, and 8 were weighed and scanned in a leaf area meter (Li-Cor LI-3100C), for total leaf area determination. Total aboveground dry biomass was calculated by adding stem and leaf biomass. Root/shoot mass ratio, which is the ratio of supportive plant tissues biomass to growth plant tissue biomass (Allaby 1998), was calculated by dividing the root dry biomass by

the aboveground dry biomass. And specific leaf area was calculated as the ratio of leaf area (in cm^3) to leaf dry mass (in g).

For $\delta^{13}C$, % C and % N determination, the first fully developed leaf on the main stem was collected from each aspen seedling in a subsample of four blocks (blocks 2, 4, 6 and 8; total of 304 seedlings). Leaves were then weighed, scanned in a leaf area meter (Li-Cor LI-3100C), dried for 72 h at 60°C in an oven, cooled at room temperature, and finely ground using stainless-steel grinding jars and beads and Qiagen TissueLyser II grinder (QIAGEN Group), at a frequency of 30/s for 45 seconds. Once grinding was complete, ground samples were scraped from metal jars into 2 mL tubes, with a metal spatula. After each sample, grinding jars and spatulas were washed with tap water, rinsed with distilled water, and dried. Tubes containing samples were labelled and stored in sealed zip-loc bags at room temperature until they were shipped to the stable isotope lab for processing (InnoTech Alberta, Vancouver Island Technology Park, Victoria, British Columbia). For the analysis of $\delta^{13}C$, % C and % N by elemental analyzer, samples were run using an established method on a MAT 253 mass spectrometer with Conflo IV interface, and a Fisons NA1500 EA, providing a bulk analysis of $\delta^{13}C$, % C and % N. Solid samples weighed into tin capsules were dropped into a combustion reactor that produces CO_2 and NO_x. A subsequent reactor reduced the NO_x to N_2 before a chromatographic column separated the components. The separated CO_2 and N_2 were transferred to the mass spectrometer for isotopic measurement. Multiple in-house standards, calibrated relative to international standards, were run as samples to allow the results to be normalized and reported vs. Vienna PDB (VPDB; $\delta^{13}C$) and air ($\delta^{15}N$). Results are accurate to $\pm$ 0.1 ‰.

Temperature, light, and relative humidity were monitored using three external data loggers (Hobos U12-012; Onset Computer Corporation) distributed in the greenhouse space at the positions South-East, Central-West, and North.

The software BioSIM was used to extract climate data for the parent tree locations, for the reference periods of 1961-1990 and 1980-2018. The following climate variables were obtained: Tmax – mean maximum temperature (°C); Tmin – mean minimum temperature (°C); PPT – mean annual precipitation (mm); PET – mean annual evapotranspiration (mm); CMI – climate moisture index calculated from monthly precipitation minus evapotranspiration (mm).

Appendix 3 contains data to supplement the methods section of this chapter.

Data analyses

Data was analyzed in R 4.1.0 (R Core Team 2020) with a general linear model procedure (lme4 package, Bates et al. 2018). Growth and gas exchange traits were the response variables, and treatment, region, family, and sex were the explanatory variables. Block was included as a random effect. A significance level of $\alpha < 0.05$ was used. Model assumptions of homogeneity of variances and normality were verified, and data transformations were not needed.

Model structure for analyses including region as a main effect for growth and gas exchange traits is as follows:

$$Y_{ijklm} = \mu + B_i + T_j + (BxT)_{ij} + S_k + (TxS)_{jk} + R_l + (TxS)_{jl} + (GxS)_{kl} + FI_m + (I(R))_{jm} + (SxF(R))_{km} + ISxF(R))_{jkm} + iD + iH + \varepsilon_{jklm} \quad \text{Eq. (1)}$$

Where Y_{ijklm} is the response variable, μ is the overall trait mean, B_i is block (i= 1-4 or 1-8), T_j is the effect due to treatment (j= 1,2, well-watered, drought), $(BxT)_{ij}$ is the interaction effect of treatment and block, S_k is sex (k=1,2, male, female), $(TxS)_{jk}$ is the interaction of treatment and sex, R_l is region (l= 1,2, boreal, parkland), $(TxR)_{jl}$ is the interaction of treatment and region, $(SxR)_{kl}$ is the interaction of sex and region, $F(R_i)_m$ is family nested within region (included as a random effect), $(TxF(R_i))_{jm}$ is the interaction of treatment and family within region, $(SxF(R_i))_{km}$ is the interaction of sex and family within region, $(TxSxF(R_i))_{jkm}$ is the interaction of treatment with sex and family within region and ε_{ijklm} is the residual error. The covariates iD and iH are initial diameter and initial height, which represent the initial size of the seedlings at day 0 of the experiment. The use of covariates was determined by model selection based on Akaike Information Criterion (AIC, AICc for small sample sizes). The model with the smallest AICc value was chosen between the following models: the base model without covariates; the base model plus initial diameter; base model plus initial height. Only one covariate was kept in a model, and to be kept in a model a covariate needed to be significant.

Model structure for analyses including family as a main effect for growth and gas exchange traits is as follows:

$$Y_{ijkl} = \mu + B_i + T_j + (BxT)_{ij} + S_k + (TxS)_{jk} + F_l + (TxF)_{jl} + (SxF)_{kl} + (TxSxF)_{jkl} + iD \text{ or } iH + \varepsilon_{ijklm}$$

Eq. (2)

Where Y_{ijklm} is the response variable, μ is the overall mean, B_i is block, T_j is the effect due to treatment, $(BxT)_{ij}$ is the interaction effect of treatment and block, S_k is sex, $(TxS)_{jk}$ is the interaction of treatment and sex, F_l is family, $(TxF)_{jl}$ is the interaction of treatment and family, $(SxF)_{kl}$ is the interaction of sex and family, $(TxSxF)_{jkl}$ is the interaction of treatment with sex and family and ε_{ijkl} is the residual error. iD and iH are the covariates initial diameter and initial height,

which represent the initial size of the seedlings at day 0 of the experiment. For the analyses of gas exchange data, day of the experiment in which data collection occurred was also included as a covariate.

The R package corrplot (Wei and Simko 2021) was used to investigate correlations between height and diameter growth for the experiment, leaf area, specific leaf area, aboveground dry mass, initial height and diameter, % C, % N, root/shoot mass ratio, net photosynthesis, iWUE, and $\delta^{13}C$.

Height and diameter growth at planting (day -28) and three weeks later at the start of the experiment (day 0), were used as references to control for initial variation (Figure 2-5).

2.3. Results

Region

Seedlings originating from the parkland and boreal regions performed similarly for most variables measured, except for specific leaf area (SLA), as boreal seedlings had 6.6% greater SLA than the parkland seedlings (P = 0.009; Table 2-4). Since differences between regions were generally not significant, the region variable was removed, and model Eq. (2) was used.

Growth

The drought treatment had a strong effect on growth: well-watered seedlings were significantly larger than drought seedlings for all growth variables measured (Table 2-5). Well-watered seedlings had an average height of 36.4 ± 0.8 cm while drought treated seedlings were on average 11 ± 0.8 cm. Aboveground dry mass (stems plus leaves) was three times greater under well-watered conditions than drought (well-watered 14.9 ± 0.2 g; drought 4.9 ± 0.2 g) while root dry mass was two-fold greater under well-watered conditions (well-watered 5.2 ± 0.1 g; drought 2.5 ± 0.1 g; Figure 2-6). There was no difference between males and females for growth variables measured.

All growth variables also showed significant differences among families, and significant interactions between treatment, sex, and family. The growth variables explored showed great variability of performance for seedlings in the control treatment, whereas mean growth under drought conditions was not as variable. Under well-watered conditions, some families showed sexual differential performance. For example, females of families 7, 12, 14 and 17 had a larger diameter, aboveground dry mass and root dry mass growth than males, under well-watered conditions. The same was not observed under drought conditions, in fact, males of families 7

and 14 had greater growth than females for diameter, aboveground dry mass, and root dry mass under drought. On the other hand, males of families 13 and 19 showed greater mean diameter, aboveground dry mass, and root dry mass growth than females under well-watered conditions. However, mean growth of males and females of families 13 and 19 was similar under drought conditions (Figure 2-7). Root/shoot mass ratios were significantly higher for seedlings in the drought treatment, whereas leaf area and specific leaf area were significantly greater under control conditions. Family performance was most variable for leaf area and specific leaf area under well-watered conditions. Different families did not present a trend in performance across the different response variables, that is, there were not better and poorer performers in common for root/shoot mass ratio, leaf area and specific leaf area (Figure 2-8).

Gas exchange and leaf $\delta^{13}C$

Like the pattern observed for biomass growth, treatment had a significant effect on net photosynthesis rates (A) and stomatal conductance (g_s). Seedlings under control conditions showed higher A and higher g_s when compared to seedlings under drought conditions. Overall, net assimilation was 28.9% higher under well-watered conditions, whereas g_s was three times higher under well-watered conditions when compared to drought-treated seedlings. Sexes did not perform differently for A or g_s. In contrast to what was found for biomass growth, the performance of families did not differ significantly for either A or g_s. Treatment was also a significant factor affecting intrinsic water-use efficiency (A/g_s; $_i$WUE), which showed a twofold increase under drought conditions. Treatment interacted with sex for $_i$WUE, where female seedlings had higher $_i$WUE under well-watered conditions and male seedlings had higher $_i$WUE under drought conditions (Figure 2-9).

Seedlings in the drought treatment had higher (less negative) mean values for leaf $\delta^{13}C$ than seedlings in the control treatment. Males and females had similar leaf $\delta^{13}C$ mean values (Figure 2-10a). The performance of families was significantly different. Still, most of the family variation occurred under well-watered conditions (mean -29.2 $\pm$ SD = 1.35, SE = 0.11). Performance under drought conditions (mean -25.7 $\pm$ SD = 0.96, SE = 0.07) was nearly constant amongst families (Figure 2-10b).

Carbon and Nitrogen concentration

Treatment had a significant effect on leaf percent carbon concentration (% C) in the studied seedlings. Percent C was higher on seedlings in the control treatment (mean of 47.8%) than drought (mean of 45.5%). Male and female seedlings did not differ in % C, while families

were significantly different. Significant interactions between treatment and family, and sex and family, indicate that under control conditions, families did not present much variability in % C, however, under drought conditions the performances of families, and performances of males and females were not consistent. Under drought conditions, families 10, 2, and 16, showed the highest means of C concentration, which were not different between males and females. Whereas families 15, 18, and 14, showed the lowest means of % C, and showed different means for males and females. In families 15 and 14, males had a higher mean % C than females, and family 18 had a higher mean % C on female seedlings. In total, % C differed between males and females for seven families under drought: in five families males outperformed females, and in two families, females outperformed males (Figure 2-11).

Treatment was also a significant factor affecting leaf percent nitrogen concentration (% N). Seedlings under drought had a higher mean % N (mean of 3.4%) than seedlings in the control treatment (mean of 3.1%). The performances of sexes and families were not significantly different (Figure 2-12).

Correlations

The correlation analysis showed that *A* was significantly positively correlated, with % C, height and diameter growth during the experiment, aboveground dry mass, and leaf area, (R^2 from 0.1 to 0.2). *A* was negatively correlated with leaf $\delta^{13}C$ (R^2 = -0.3). Specific leaf area was positively correlated with % C, height, diameter, aboveground dry mass growth and leaf area (R^2 from 0.3 to 0.51), and negatively correlated with initial height and diameter, % N, root/shoot mass ratio, $_i$WUE, and leaf $\delta^{13}C$ (R^2 from -0.13 to -0.63). Height growth for the entire experiment was significantly positively correlated to diameter, aboveground dry mass, and leaf area (R^2 > 0.8), and negatively correlated to % N, root/shoot mass ratio, $_i$WUE, and $\delta^{13}C$ (R^2 > -0.4). Diameter growth for the entire experiment was positively correlated to aboveground dry mass and leaf area (R^2 > 0.9), and negatively correlated to % N, root/shoot mass ratio, $_i$WUE, and $\delta^{13}C$ (R^2 > -0.4). Aboveground dry mass was positively correlated with leaf area (R^2 = 0.96) and with initial height (R^2 = 0.2), and negatively correlated to % N, root/shoot mass ratio, $_i$WUE, and $\delta^{13}C$ (R^2 > -0.4). Leaf area was negatively correlated to % N, root/shoot mass ratio, $_i$WUE, and $\delta^{13}C$ (R^2 > -0.4). Initial height and initial diameter were positively correlated with $\delta^{13}C$ (R^2 = 0.18 and 0.22, respectively). % N was positively correlated with root/shoot ratio, $_i$WUE, and $\delta^{13}C$ (R^2 = 0.16, 0.25, and 0.24, respectively). Root/shoot mass ratio was positively correlated with $_i$WUE,

and $\delta^{13}C$ (R^2 = 0.43 and 0.51, respectively). And finally, ¡WUE was positively correlated with $\delta^{13}C$ (R^2 = 0.55; Figure 2-13).

2.4. Discussion

In western Canada the limits of the boreal forest are defined by available moisture: the boundaries between forest, parkland, and grassland regions overlap with the areas where potential evapotranspiration exceeds precipitation (Hogg 1997). Previous studies have shown that moisture directly affected growth and mortality of aspen stands: mortality was greater in most severely drought-affected areas and biomass growth was smaller, in the parkland region (Hogg et al. 2005; Hogg et al. 2008). The current study explored if male and female seedlings of aspen originating from parent trees located in the boreal and parkland regions of Alberta would perform differently under drought conditions, to test if these seedlings show adaptation to local historical climatic conditions or plasticity in the traits studied. While climatic data for the 1961-1990 reference period showed the expected historical difference in CMI between the boreal and parkland regions; where the boreal region had a positive CMI, and the parkland region a negative CMI (Hogg 1997); more recent data, from 1991-2018 showed that the boreal region has been under dry conditions, like the parkland region, over the past 30 years. Hynes and Hamann (2020) showed that the growth of white spruce in the boreal forest in Alberta is limited by moisture deficit and that the forest is susceptible to climate change. At the same time, CMI is not the only factor driving ecosystem change, at local scales other factors such as soil type, topography, disturbances, land use, etc., influence the ecosystem, other than climate (Schneider 2013).

The overall similar response of seedlings from all families in both regions to well-watered and drought conditions indicates that in seedlings, responses to sustained drought might not differ based on different regions of origin. In this study, seedlings originating from the boreal and parkland regions have not shown evidence of adaptation or drought strategies to the level of drought they were exposed to for most of the traits explored. Specific leaf area (SLA) was the only trait that differed. SLA, the ratio of leaf area to leaf dry mass, is a determinant of light interception per gram of leaf and thus plant growth (Shipley 2006). In fact, in the present study, SLA was significantly correlated with height and diameter growth (R^2 = 0.41). Plants with higher SLA have thinner, larger leaves, which capture more light per gram of leaf tissue, and consequently present greater mass-based photosynthesis (Liu et al. 2016). Plants original from

the boreal region had greater SLA when compared to parkland, still growth and photosynthesis rates were not significantly different among the two regions. This suggests that aspen seedlings originating from the two regions present different leaf area strategies to produce similar amounts of carbohydrates. That is, considering that growth and photosynthesis were equal, but SLA was greater in boreal seedlings, seedlings adapted to the environmental conditions of the boreal region produced less carbohydrates per unit leaf area than seedlings adapted to the parkland region.

More importantly, SLA was significantly negatively correlated with instantaneous water-use efficiency $_{i}$WUE and $\delta^{13}C$ ($_{i}$WUE R^2 = -0.49, $\delta^{13}C$ R^2 = -0.63). Leaf $\delta^{13}C$ is related to water-use at the leaf level as $\delta^{13}C$ ratios reflect the balance between *A* and g_s during the entire growth period (Farquhar et al. 1989; Cernusak et al. 2013). Greater $\delta^{13}C$ values indicate greater stomatal closure, as closed stomata accumulate $^{13}CO_2$ beneath intercellular spaces, which increases fixation (Desrochers et al. 2003; Larcheveque et al. 2011). Hamanishi et al. (2012) associated an increase in $_{i}$WUE in *P. balsamifera* under drought stress with a decline in stomatal conductance. In this sense, $_{i}$WUE is expected to be higher in plants exhibiting higher $\delta^{13}C$ (Farquhar et al. 1989). However, $_{i}$WUE is an instantaneous measurement, that tends to respond to environmental conditions at the time of measurement while $\delta^{13}C$ is an integrated measurement and shows long-term responses (DesRochers et al. 2007). In my study $\delta^{13}C$ and $_{i}$WUE were significantly correlated (R^2 = 0.55). Therefore, leaf $\delta^{13}C$ may effectively serve as a surrogate for water-use efficiency (Farquhar et al. 1989). Plants exposed to drought have been shown to increase their WUE, in terms of plasticity, as shown in a short-term experiment on three poplar clones (DesRochers et al. 2007), and in terms of adaptability, as shown in a study of over 31 years, on populations of shrub species and *Pinus ponderosa* stands in the American Southwest that have been exposed to a megadrought (Kannenberg et al. 2021). All things considered, the lower SLA in parkland seedlings is associated with higher iWUE, which in turn, may be an adaptive trait resulting from the selective pressures of the drier conditions experienced by aspen trees in the parkland region.

The ecological structure of the boreal and parkland regions differ as the boreal region has been forested for over 9000 years, whereas the parkland region is marked by a transition between grassland and forest (Schneider 2013). Liu et al. (2016) suggest that plants decrease their SLA in response to higher light availability, as the production of non-structural carbohydrates would exceed meristematic demands if leaves had higher SLA. Therefore, seedlings original from the parkland region, which likely has conditions of higher light availability, potentially have lower SLA. Landhäusser and lieffers (2001) found high plasticity of

P. tremuloides grown in the understory versus open conditions, with seedlings grown in the understory presenting higher SLA, lower light saturation point, and lower assimilation. Increased SLA also indicates higher water content of leaf tissues (Shipley 1995). Historically, the boreal region has had greater available moisture than the parkland region (Schneider 2013), hence seedlings from the boreal region could potentially have higher water content on leaf tissues, as in the past the region had positive moisture availability (this changed in the past 30 years). Freschet et al. (2015) suggests that plants adapted to long-term resource deprivation present greater phenotypic plasticity in biomass allocation to leaves and roots, such that if resources are unavailable, these plants can display traits that avoid nutrient loss in the long term (e.g., lower SLA). Aspen from the boreal region may be more vulnerable to nutrient loss in the long term under future climatic conditions, especially conditions of drought, yet this was not tested in the current study. Further exploration is needed in future studies.

The growth of aspen seedlings in my study was directly affected by water availability where growth was generally reduced under drought stress compared with well-watered conditions. To avoid losing water, aspen seedlings reduce the process of C assimilation under drought stress by closing their stomata, which reflects in reduced stomatal conductance, net photosynthesis, and growth rates (Greitner et al. 1994; Raven 2002; Galvez et al. 2011; Hart et al. 2021). In addition, as nutrients are also transported in water, reduced water availability limits nutrient access (Greitner et al. 1994). Sloan et al. (2020), however, have shown different responses where aspen seedlings conditioned to drought since the beginning of seedling (and xylem) development showed greater growth and photosynthesis rates than highly irrigated seedlings. Lauriks et al. (2022) found that aspen seedling responses to drought varied depending on the time of the growing season (i.e., early or late season), with dry biomass reduced under drought in the early season (spring/summer) but not in the late season (summer/autumn).

In my study the relationship between reduced C assimilation and whole-plant growth was not linear: root/shoot mass ratio of seedlings exposed to drought was higher than control. Similarly, in Galvez et al. (2011) and Hart et al. (2021), roots of aspen seedlings growing under water stress stored more C than well-watered seedlings. Investing energy in root growth and survival is particularly important for aspen, as aspen trees and seedlings can resprout from their root systems after the dieback or removal of aboveground parts (Stout 1929; Long and Mock 2012). This is also an important adaptive physiological mechanism to avoid water loss, by reducing the surface area of transpiring tissues, while allocating biomass to roots to reach for water and nutrients below ground (Greitner et al. 1994; Raven 2002; Poorter and Nagel 2000).

This concept is explained by the theory of a 'functional equilibrium' described by Brower (1963) and expanded by Poorter and Nagel (2000), briefly, plants shift the allocation of biomass to shoots (stems and leaves) or roots, depending on which resources are limiting plant growth, that is, if water and nutrient availability are limited, resources are allocated to the roots, while allocation of biomass to shoots is reduced. This shift in the allocation of biomass was observed in the current study, as seedlings exposed to drought conditions had a higher root/shoot mass ratio, indicating a higher investment in root tissues to reach for water and nutrients when those resources were limited.

Supporting our second hypothesis, the performances of families were variable under different environmental conditions. Family growth performance was highly variable under well-watered conditions, yet performance under drought conditions was less variable. In Monclus et al. (2005) 29 genotypes of a hybrid poplar showed large differences in response under well-watered conditions, yet genotypic effects were reduced under drought stress. Still, the authors stated that the impact of drought on biomass production was genotype-dependent, as less productive genotypes were more tolerant to drought than more productive genotypes (Monclus et al. 2005). Wildhagen et al. (2017) found that cambial activity was reduced in *Populus nigra* under drought stress, and drought-induced variations were independent of genotype. The limited response in family performances under drought stress found in my study may reflect a reduced capacity to acquire resources under drought, such that plants were able to survive and grow but under conditions that limited reaching their genetic potential. Families did not present differences on A, g_s, and $_i$WUE. In Hamanishi et al. (2012) *P. balsamifera* genotypes performed differently in g_s and $_i$WUE under conditions of drought stress, which was attributed to genotypic variation in drought-response strategies, more specifically to differences in gene signalling mechanisms that regulate stomatal development. Such differences were not observed in the present study.

Leaf $\delta^{13}C$ is related to water-use at the leaf level as $\delta^{13}C$ ratios reflect the balance between A and g_s during the entire growth period (Farquhar et al. 1989; Cernusak et al. 2013). Greater $\delta^{13}C$ values indicate greater stomata closure, as closed stomata accumulate $^{13}CO_2$ beneath intercellular spaces, which increases fixation (Desrochers et al. 2003; Larcheveque et al. 2011). Hamanishi et al. (2012) have associated an increase in $_i$WUE in *Populus balsamifera* under drought stress with a decline in stomatal conductance. In this sense, $_i$WUE is expected to be higher in plants exhibiting higher $\delta^{13}C$ (Farquhar et al. 1989). However, $_i$WUE is an instantaneous measurement, that tends to respond to environmental conditions at the time of measurement and $\delta^{13}C$ is integrated and shows long-term responses (DesRochers et al. 2007).

In my study $\delta^{13}C$ and $_{i}WUE$ were significantly correlated ($R^2 = 0.55$). Therefore, family variability in water-use efficiency for the entire growth period might be better captured by leaf $\delta^{13}C$ than gas exchange measurements (Farquhar et al. 1989).

Carbon and nitrogen concentrations were influenced by drought. Plants submitted to well-watered conditions had higher carbon concentration than plants submitted to drought conditions. This reflects the limited growth and starch content of plants under drought stress, as drought inhibits photosynthesis by the closure of stomata and reduction in chlorophyll concentration and Rubisco activity (Cui et al. 2019). Whereas nitrogen concentration was higher in plants submitted to drought stress. This is expected as the uptake of available N is generally increased under drought stress, increasing NH_4^+ production (Cui et al. 2019). Families showed different carbon concentrations under drought stress, suggesting that in some families, drought affected the carbon metabolism more severely than others. Families did not present differential drought strategies for nitrogen assimilation. Different nitrogen assimilation would have been interesting to study as higher nitrogen uptake would indicate an enhanced tolerance to drought (Huan et al. 2017).

Genotypes had different strategies for well-watered and drought conditions. F2, F10, and F21 were generally more productive in terms of biomass, while F7, F11, and F14, showed the lowest biomass production. Under well-watered conditions, the families that showed the lowest biomass production were amongst the ones with the highest $\delta^{13}C$, while the most productive families had average performance in $\delta^{13}C$. In effect, in my study biomass production was negatively correlated with both $\delta^{13}C$ and $_{i}WUE$. This suggests that the low-productivity families might close their stomata more often than other families, even under well-watered conditions.

Low-productivity families might be able to survive severe drought events due to the same traits that reduce their productivity. Taking the low productivity families F7, F11, and F14 as examples, F7 and F11 had high specific leaf areas, which is a direct indicator of greater mass-based photosynthesis (Liu et al. 2016), yet they showed the lower biomass production rates of all families. All three families showed medium to high values of root/shoot mass ratio under well-watered and drought conditions, still, allocation to roots was superior under drought conditions. F7 and F14 had high $\delta^{13}C$ under well-watered and drought conditions, and F11 had high $\delta^{13}C$ only under drought conditions, indicating higher stomata closure (Larcheveque et al. 2011) and water-use efficiency (Farquhar et al. 1989). This is relevant as plants with the genotypic ability to increase WUE have higher drought tolerance (Monclus et al. 2005). These results suggest that although not productive under optimal environmental conditions, those families might present advantageous strategies to survive under extreme drought conditions.

On the other hand, high-productivity genotypes might also be tolerant under drought stress, as those families were generally good performers under well-watered and drought conditions. Taking the high-productivity families F2, F10, and F21, as examples, all three had medium to high specific leaf areas, indicating great mass-based photosynthesis. F2 and F21 had medium $\delta^{13}C$ under well-watered conditions, indicating a lower level of stomata closure when compared to families F7 and F14, but F10 showed high $\delta^{13}C$ under well-watered conditions. In addition to presenting the highest biomass productivity under well-watered and drought conditions, these families also had medium (F10) to high (F2 and F21) allocation of biomass to roots when exposed to sustained drought. This indicates that those families would be good options for genetic selection as they present high productivity under optimal environmental conditions as well as a desirable level of plasticity under drought stress. Families such as F10 may be an interesting option for selection and improvement, as productive and water-use-efficient genotypes may present a solution for future climatic scenarios.

The selection of breeding material for aspen improvement based on family performance in growth or drought strategies can be a novel opportunity to deal with an adaptational lag to climate change, however, the potential conflict between growth and safety should be considered. Kannenberg et al. (2021) surveyed populations of shrub species and *Pinus ponderosa* stands in the American Southwest annually over a 31-year study period to test the effects of a megadrought on iWUE. The authors found a link between drought and increased rates of iWUE, which would be a positive behaviour to conserve water and maintain plant health under drought, however, this shift was also associated with lower growth rates and increased plant mortality (Kannenberg et al. 2021). The tree hydraulics and optimal resource partitioning (THORP) model "*predicts the biomass partitioning of trees as a function of size and in response to water limitations, eCO_2 (without nutrient limitation) and pruning*" (Potkay et al. 2021). THORP's simulation-based predictions indicate that the allocation of tree growth is primarily driven by hydraulic demands, however, nutrient status must also be considered (Potkay et al. 2021). In this scenario, the use of plant materials that have differential responses to drought, as a solution, to risks brought by climate change might not be effective if the biomass loss experienced by selected materials remains the same or similar compared to native plant biomass loss (Anderegg 2021). In addition, *P. tremuloides* from 36 genotypes, propagated from root suckers in a common garden in Wisconsin, showed trade-offs in which fast-growing genotypes had lower resistance to herbivory when compared to genotypes that showed resource-conservative strategies (Morrow et al. 2022). Overall, the selective breeding and planting of aspen with resource conservation strategies under drought conditions will only be a

viable option for adaptation to future climate scenarios if the material selected for drought strategies can perform better in terms of both survival and biomass production rates compared to non-selected trees under drought conditions, and if fast-growing genotypes do not attract herbivores more frequently than non-selected trees.

The varied responses of families, including the identification of high-productivity and low-productivity genotypes, in addition to the lack of differential performance between families of different regions, show a potential for selection of family clonal material for assisted migration, instead of region-based materials. Previous studies have recommended the movement of aspen seed and vegetative propagation of materials from southern provenances to northern regions to obtain greater growth rates and enhanced forest productivity (Gylander et al. 2012; Schreiber et al. 2013; Howe et al. 2020). In my study, seedlings from the parkland and boreal regions did not show differential responses to well-watered and drought conditions, while different families showed varied strategies to drought. These results suggest that programs of assisted migration could source for drought tolerance on different aspen clones, in addition to (or instead of) using materials adapted to different regions. Differences in variability and extent of family performances were more pronounced than sex differences.

The hypothesis of differential performance of sexes was partially supported by the data obtained in the current study. In general growth and biomass production of male and female aspen seedlings did not differ for the growth period of the experiment, yet sexes performed differently under well-watered and drought conditions for some families. Similar results were obtained in studies exploring sexual differences in aspen and *Salix* spp. genotypes, in which less prominent sex differences were observed for main effects, and clear sex differences were observed when genotypes or families were considered (Stevens and Esser 2009; Konatowska et al. 2021).

In my study, females showed greater growth than males under well-watered conditions in four families (F7, F12, F14, and F17). This is following several studies that suggest that female plants tend to have higher photosynthetic rates (stomatal conductance, g_s, and net carbon assimilation, A) and higher resource acquisition capacity under optimal environmental conditions when compared to males (Dawson and Ehleringer 1993; Obeso 2002; Han et al. 2013; Randriamanana et al. 2014; Hultine et al. 2007; Hultine et al. 2016; Zhang et al. 2018). Still, the sexes did not perform differently on A or g_s, at least during gas exchange measurements. Additionally, in two of these families (F7 and F14) males had greater growth performance than females under drought. This is also in accordance with the available literature, as many studies that explored differential performances of sexes under environmental

stress found that females had lower vegetative growth under varying environmental stress factors, including drought when compared to co-occurring males (Dawson and Ehleringer 1993; Delph 1999; Xu et al. 2008; Han et al. 2013; Hultine et al. 2016; Zhang et al. 2018; Liu et al. 2022). On the other hand, two families (F13 and F19) showed opposite results, with males outperforming females in growth under well-watered conditions. Contrasting sex responses of different genotypes have been reported in other studies (Stevens and Esser 2009; Konatowska et al. 2021), showing the potential of natural genetic variability in plant populations to establish individuals and populations under varied conditions, by the success of genotypes that present greater fitness to such conditions (Obeso 2002). Moreover, males of five families (F5, F6, F15, F17, and F14) showed higher carbon concentration than females under drought stress, whereas in two families (F12 and F18), females had higher carbon concentration under drought. The higher carbon concentration of males suggests lower photosynthetic activity of female seedlings compared to males under drought stress (Cui et al. 2019), for the specific families that presented this differential performance. Such differential sex responses found at the family level indicate a potential of selecting aspen families for breeding programs taking into consideration aspects of sex performance and response to drought.

Female seedlings had higher $_{i}$WUE under well-watered conditions, and male seedlings had higher $_{i}$WUE under drought conditions, which supports our third hypothesis. Previous studies found that males had higher iWUE than females when subjected to conditions of low water availability (Dawson and Ehleringer 1993; Hultine et al. 2007; Xu et al. 2008; Han et al. 2013; Liu et al. 2022). Hultine et al. (2016), after reviewing 83 studies on intersexual differences in growth and instantaneous leaf-level gas exchange across plant families of woody dioecious species, concluded that under well-watered conditions, male and female performance did not differ, however, under drought conditions males significantly outperformed females in stomatal conductance and net assimilation. These results are in accordance with the assumption that males may have a capacity for greater resource acquisition than females when exposed to drought stress (Hultine et al. 2007).

Females of most dioecious species invest more energy in the production of flowers, fruits, and seeds, than males invest in the production of pollen (Darwin 1877; Dawson and Ehleringer 1993; Delph 1999; Obeso 2002; Stevens and Esser 2009; Konatowska et al. 2021). In that context, females of dioecious plants seem to have evolved a more efficient hydraulic conducting system when compared to males (Olano et al. 2017; Liu et al. 2022). This hydraulic efficiency allows for increased water acquisition and carbon gain. From an evolutionary standpoint, having a more hydraulic conducting system would compensate females for the

higher demands experienced for carbon and water during reproductive periods (Liu et al. 2021). Such characteristics may give females higher efficiency in acquiring resources. Consequently, the hydraulic system in females may be more vulnerable to xylem cavitation, hydraulic failure, and carbon starvation under drought conditions (Liu et al. 2021; Liu et al. 2022), resulting in females having lower resistance to drought. Franklin et al. (2023) showed that in angiosperms a higher xylem efficiency reflects in narrow hydraulic safety and consequent vulnerability to drought.

The differential responses of males and females to drought stress are relevant in the current climate context, where drought is predicted to increase in the near future (Gray and Hamann 2015). In fact, a prevalence of male vs. female aspen trees has already been reported for drought-prone environments (Goessen et al. 2022). The differential performance of males and females under drought stress could potentially drive unbalanced sex ratios of aspen stands in the future. Biased sex ratios could negatively affect sexual reproduction in aspen populations and although a lack of sexual reproduction alone might not have a direct effect on population growth rates, as aspen mostly reproduces vegetatively (Baker 1921; Barnes 1966; Mock et al. 2008), it could influence rates of adaptation, and a reduction in the effective population size across the landscape (Petry et al. 2016).

2.5. Conclusion

The rise in frequency of drought events driven by warmer atmospheric temperatures, longer growing seasons and higher evapotranspiration rates, is likely to shape future vegetation communities (Allen et al. 2015). In Alberta, the limits between the boreal and parkland regions have been associated with a gradient of moisture availability, captured by measurements of climate moisture index (CMI; Hogg 1997). In this gradient, the boreal region has historically had a positive CMI, while the parkland region has had a negative CMI (Hogg 1997). This study showed that the CMI values in the boreal and parkland regions were as expected for the reference period of 1961-1990, but for the period of 1991-2018 both regions had negative CMI. Such sustained shifts in patterns of moisture availability will likely influence vegetation communities.

While using aspen (*Populus tremuloides* Michx.) to explore if seedlings that come from a region of lower CMI would be more tolerant to drought conditions when compared to seedlings that come from a region of higher CMI, this study found that seedlings from both regions

showed the same level of plasticity to drought under our study conditions for most traits, except specific leaf area (SLA). The lower SLA of seedlings from the parkland region, associated with a negative correlation between SLA and water-use efficiency (WUE), indicates that seedlings from the parkland region have higher WUE under drought, showing adaptation to the dry conditions of the parkland. At the same time, boreal and parkland seedlings seem to be adapted to the levels of light and resource availability of their place of origin. This could have implications under future climatic conditions, in which aspen communities in the boreal forest might become exposed to long-term water deprivation and greater light incidence, different from the historical weather conditions to which they were adapted. Future studies could test if adaptation to historical light and resource availability makes aspen from the boreal region more vulnerable to nutrient loss in the long term when compared to aspen from the parkland region.

The maintenance of sustained drought has proven to reduce the growth of aspen seedlings when compared to well-watered conditions. Moreover, drought seemed to reduce the variability in the response of different genotypes to growth conditions. Such findings highlight the negative impacts of drought on aspen seedling performance, independently of genetic variability. Still, even at this young age, drought strategies were observed, and high-productivity and low-productivity genotypes were identified. High-productivity genotypes showed a greater level of plasticity to environmental conditions (in both well-watered and drought treatments), whereas low-productivity genotypes showed potential for greater drought resistance. The exploration of drought strategies and genetic variation is relevant for the selection of clones for tree improvement and planting. In this sense, aiming to obtain greater survival and productivity on forest plantations, families can be selected according to their potential plasticity to present and future environmental conditions of the deployment area. On the other hand, trade-offs between growth and hydraulic safety, and growth and defense must be taken into consideration before establishing the potential success of the selected materials. Similarly, aspen clones and families can be selected for based on their drought response as a potential solution for programs considering assisted migration, as drought resistant families can be planted in areas predicted to be exposed to more frequent droughts in the future. Aspen families can also be selected for in breeding programs based on their differential sex responses.

The exploration of sexual differences in young non-reproductive aspen showed that biomass production and growth differences between males and females were not evident. This lack of response could have been due to the duration of the experiment, especially since female seedlings had higher $_{i}$WUE under well-watered conditions, and male seedlings had higher $_{i}$WUE under drought conditions. The lack of differences could also indicate that female and male

aspen seedlings do not allocate energy differently before reproduction. Furthermore, for some families, females outperformed males under well-watered conditions and males outperformed females under drought conditions. A possible explanation for this differential performance of males and females is that females may have evolved a more effective hydraulic system to meet higher carbohydrate demands during reproductive periods. This strategy may provide females with a greater resource acquisition capacity under optimal environmental conditions, but lower resistance to environmental stress, including drought. Still, a few genotypes showed the opposite behaviour. This variability in response highlights the ability that natural genetic variation has to maintain genotypes that can thrive under variable environmental conditions. These results suggest that in the context of drier and warmer climates, females of some genotypes may continue to persist with relatively high fitness and grow and reproduce. Still, only a small number of genotypes showed the opposite performance of sexes to that hypothesized. In summary, the variability shown by different genotypes can present an evolutionary advantage to aspen populations under climate change, however, it might not be enough to prevent biased sex ratios and their subsequent effect on the adaptation of the species on the landscape.

Tables

Table 2-1. Climatic data for parent tree collection sites pooled by region (boreal and parkland), for two reference periods. Growing season (GS; May until September) mean maximum (GS Tmax, °C) and minimum (GS Tmin, °C) temperatures were calculated, as well as annual mean temperatures (Annual Tmax and Tmin, °C). Monthly means were calculated including the months of January to December for precipitation (PPT, mm), potential evapotranspiration (PET, mm) and climate moisture index (CMI, mm). Data were obtained with BioSIM software for the reference periods of 1961-1990 and 1991-2018.

Reference period	Region	GS Tmax (°C)	GS Tmin (°C)	Annual Tmax (°C)	Annual Tmin (°C)	PPT (mm)	PET (mm)	CMI (mm)
1961-1990	Boreal	18.3	5.9	6.1	-5.7	35.2	34.1	1.1
1961-1990	Parkland	20.0	7.4	8.4	-3.2	37.7	40.4	-2.6
1991-2018	Boreal	19.0	6.1	6.9	-4.8	34.7	35.8	-1.1
1991-2018	Parkland	20.5	6.6	9.1	-3.4	33.3	42.5	-9.2

Table 2-2. Sequences of primers used for sex differentiation in *P. tremuloides* (Pakull et al. 2014) including primer name and sequence (5’ to 3’).

Primer name	Sequence (5' to 3')
TOZ19-1_for	5’-TTAGGTGCTGATGGTTTGGTAAAGCAG
TOZ19-1_rev	5’-CTTGCATGCAGATAGCCAACACAAGAATT
Control_for	5’-CTACCATGCTGAGTTTGAATTCTGGGTC
Control_rev	5’-AATGAGCAGCTTCACGTTCCAACTCAACT

Table 2-3. Moisture levels observed (in sensor raw readings and volumetric water content; VWC; $m^3.m^{-3}$) before and one hour after watering of drought-treated seedlings. The amount of water added (mL) was relative to moisture levels. For example, if the mean VWC reading of a pot was lower than 0.087 $m^3.m^{-3}$ that pot would receive 100 mL of water.

Before watering			One hour after watering	
Sensor (raw)	VWC ($m^3.m^{-3}$)	Water added (mL)	Sensor (raw)	VWC ($m^3.m^{-3}$)
<650	<0.087	100	717	0.127
650-680	0.087-0.105	80	709	0.122
680-700	0.105-0.117	60	701	0.118
>700	>0.117	0	>700	>0.117

Table 2-4. Estimated marginal means ± SE of variables measured and *P*-values between regions for seedlings originating from the two regions: Parkland and boreal. Including: height and diameter growth (cm) from day 0 to day 39, at harvest (*n* = 608); dry biomass (stems, leaves, aboveground dry mass, roots dry mass, root/shoot mass ratio; g) and nutrients (i.e., carbon and nitrogen, %) after 39 days of experiment (*n* = 532); leaf area (cm^2) and specific leaf area ($cm^2\ g^{-1}$), measured post-harvesting (*n* = 225); and gas exchange data (Net photosynthesis, *A*, stomatal conductance, g_s, intrinsic water-use efficiency, iWUE) collected in the final week of the experiment (*n* = 294). Value in bold indicates significance at $\alpha = 0.05$.

	Region		
Measurement	Parkland	Boreal	*P*-value
Height growth (day 39 - day 0; cm)	23.1 ± 1.1	24.2 ± 1.0	0.437
Diameter growth (day 39 - day 0; cm)	2.2 ± 0.1	2.5 ± 0.1	0.091
Stems dry mass (g)	4.5 ± 0.2	4.6 ± 0.2	0.587
Leaves dry mass (g)	5.5 ± 0.3	5.3 ± 0.3	0.637
Aboveground dry mass (g)	9.9 ± 0.5	9.9 ± 0.5	0.988
Roots dry mass (g)	3.8 ± 0.3	3.9 ± 0.2	0.839
Root/shoot mass ratio	0.4 ± 0.0	0.4 ± 0.0	0.354
Leaf area (cm^2)	944 ± 65.5	987 ± 62.4	0.631
Specific leaf area ($cm^2\ g^{-1}$)	171 ± 3.5	183 ± 3.3	**0.009**
Carbon isotope ratio $\delta^{13}C$ (‰)	-27.5 ± 0.2	-27.5 ± 0.2	0.755
% Carbon	0.5 ± 0.0	0.5 ± 0.0	0.142
% Nitrogen	0.03 ± 0.0	0.03 ± 0.0	0.705
Net photosynthesis (*A*)	8.5 ± 0.5	8.8 ± 0.5	0.600
Stomatal conductance (g_s)	146 ± 13.5	166 ± 12.5	0.131
Intrinsic water-use efficiency (iWUE)	0.06 ± 0.0	0.06 ± 0.0	0.981

Table 2-5. Estimated marginal means ± SE and *P*-values of variables measured for treatment (control and drought), sex (female and male), and family. Including: height and diameter growth (cm) for the entire experiment, excluding growth before the start of the experiment (n = 608); dry biomass (stems, leaves, aboveground dry mass, roots dry mass, g) and root/shoot mass ratio (n = 532); and leaf area (cm^2) and specific leaf area ($cm^2\ g^{-1}$), measured post-harvesting (n = 225). *P*-value across the two treatments, two sexes, and the 19 families (values in bold indicate significance at α = 0.05).

	Treatment			Sex			Family
Measurement	Control	Drought	*P*-value	Female	Male	*P*-value	*P*-value
Height growth experiment (day 39 - day 0; cm)	36.4 ± 0.8	11 ± 0.8	**< 0.001**	23.3 ± 0.7	24.1 ± 0.7	0.408	**0.001**
Diameter growth experiment (day 39 - day 0; cm)	3.5 ± 0.1	1.2 ± 0.1	**< 0.001**	2.4 ± 0.05	2.3 ± 0.05	0.148	**< 0.001**
Stems dry mass (g)	7.1 ± 0.1	2.0 ± 0.1	**< 0.001**	4.6 ± 0.1	4.5 ± 0.1	0.801	**< 0.001**
Leaves dry mass (g)	7.8 ± 0.1	2.9 ± 0.1	**< 0.001**	5.4 ± 0.1	5.3 ± 0.1	0.466	**< 0.001**
Aboveground dry mass (g)	14.9 ± 0.2	4.9 ± 0.2	**< 0.001**	10.0 ± 0.2	9.8 ± 0.2	0.606	**< 0.001**
Roots dry mass (g)	5.2 ± 0.1	2.5 ± 0.1	**< 0.001**	3.9 ± 0.1	3.8 ± 0.1	0.543	**< 0.001**
Root/shoot mass ratio	0.3 ± 0.01	0.5 ± 0.01	**< 0.001**	0.4 ± 0.01	0.4 ± 0.01	0.777	**0.018**
Leaf area (cm^2)	1472 ± 43.5	456 ± 43.7	**< 0.001**	965 ± 38.7	963 ± 38.9	0.967	**< 0.001**
Specific leaf area ($cm^2\ g^{-1}$)	196 ± 2.5	159 ± 2.6	**< 0.001**	176 ± 2.3	180 ± 2.3	0.169	**< 0.001**

Figures

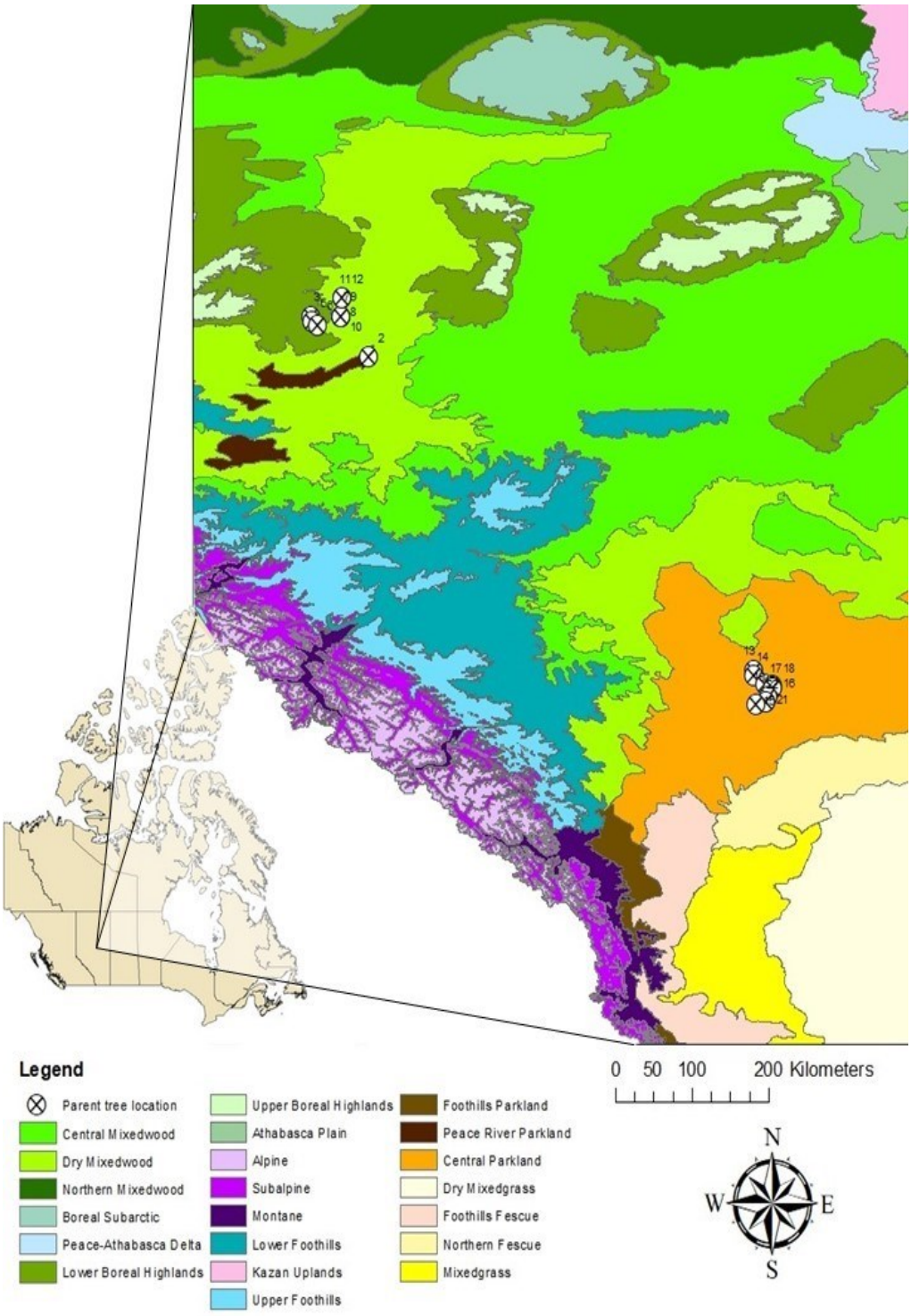

Figure 2-1. Collection sites where parent trees were selected (white circles with a black cross) in Alberta. Position of collection sites relative to Alberta's Natural Subregions, and position of Alberta relative to Canada.

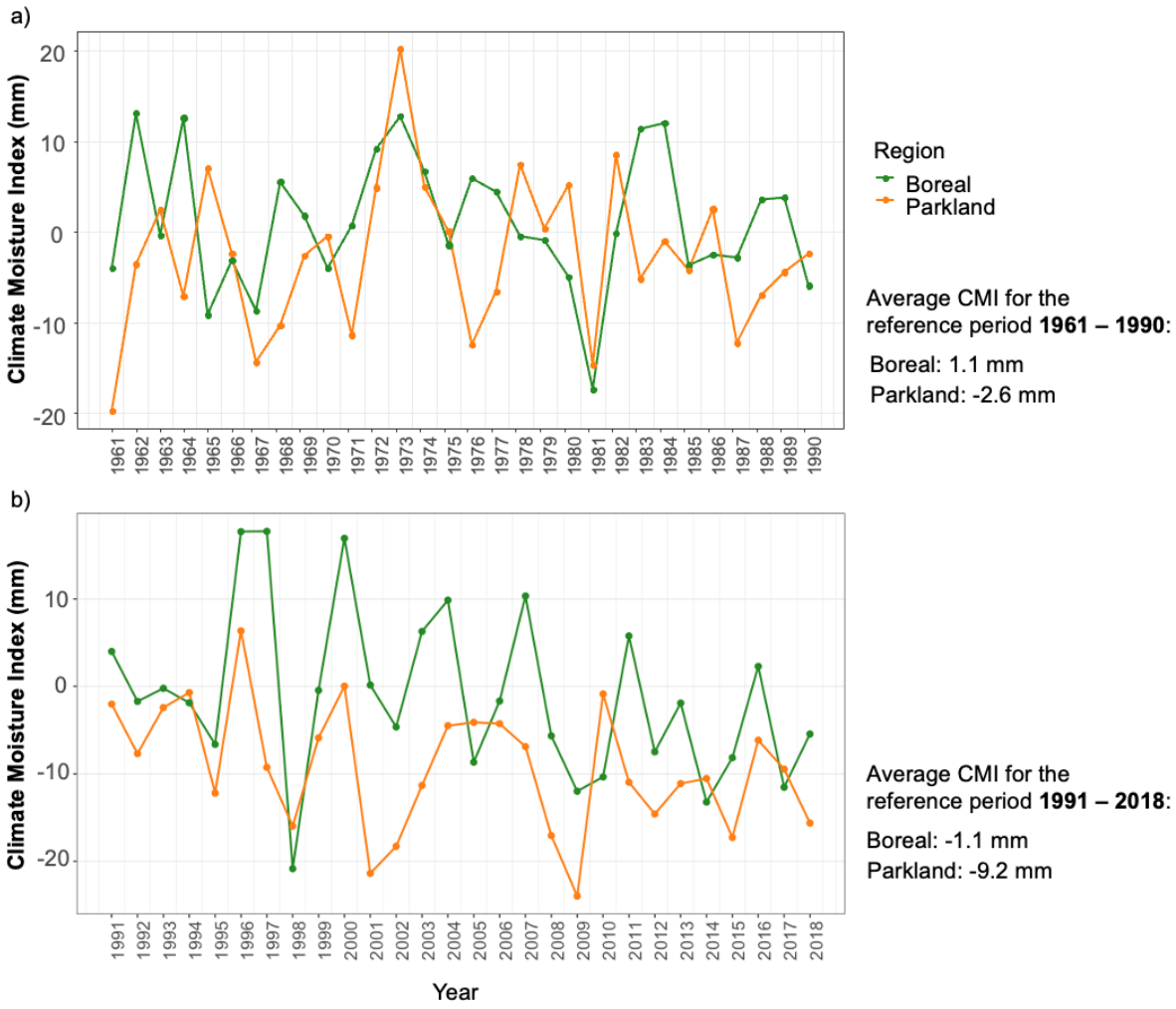

Figure 2-2. Climate moisture index (CMI) values for the location of each parent tree for the reference periods (a) 1961-1990, and (b) 1991-2018, including mean annual values for each year and one averaged CMI value (average across all years in the period) for each of the two reference periods by region (boreal and parkland).

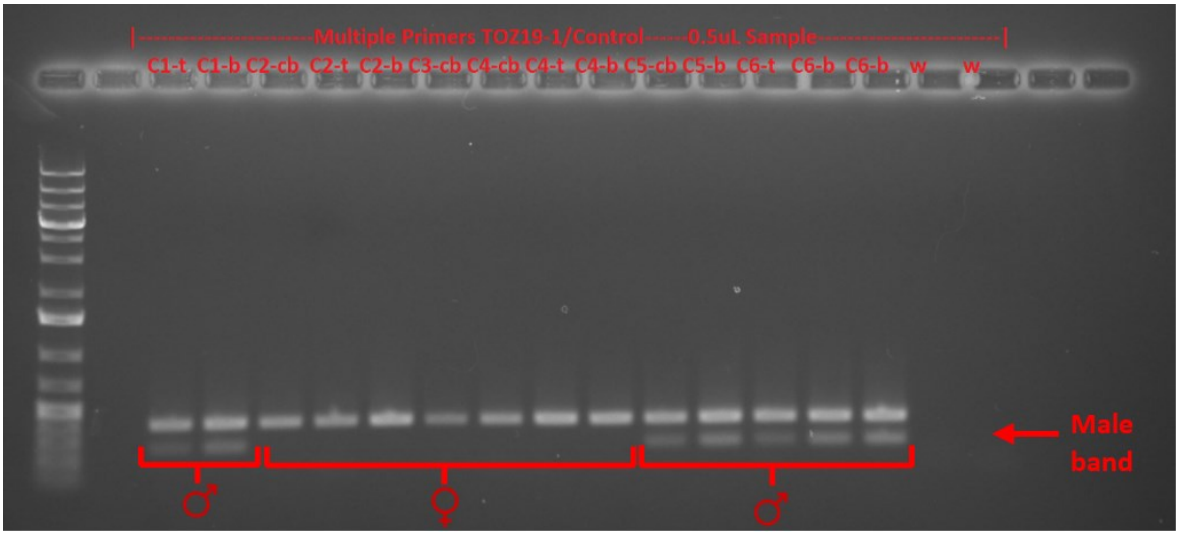

Figure 2-3. Banding patterns identifying male and female trembling aspen using the TOZ19-1 primer (Pakull et al. 2014), using twig (t), vegetative bud (b), and phloem (cb) tissue types, and water (w) PCR blank controls showing no contamination of the solutions on an agarose gel. A control ladder (Invitrogen 1Kb Plus DNA ladder) on the left demarks the band size in base pairs (bp), and females (♀) are identified by a single band (at approximately 414 bp) and males (♂) identified with an additional band (bands at 414 bp and at 260 bp).

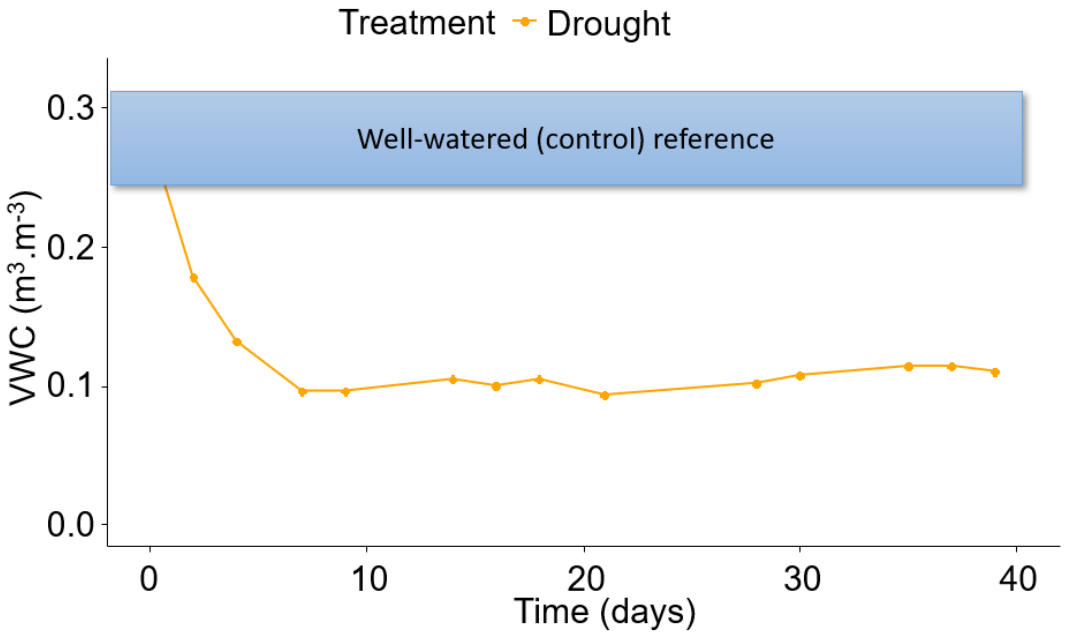

Figure 2-4. Mean ± SE volumetric water content (VWC) of drought-treated seedlings. Seedlings in the drought treatment (pre-wilting point) were monitored three times a week for the duration of the experiment, until Day 39. Well-watered controls were maintained in the range of 100 to 90% saturation weight (or 100 to 74% VWC) based on pre-experiment trials.

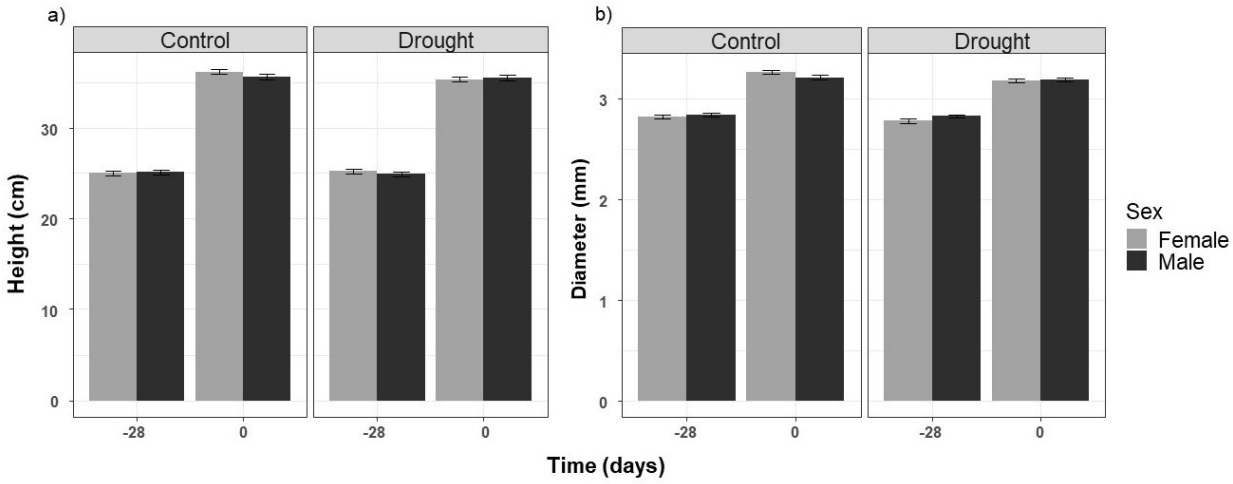

Figure 2-5. Mean ± SE a) height (cm), and b) diameter (mm) by sex at planting (-28; 28 days before the start of the experiment) and on day 0 of the experiment.

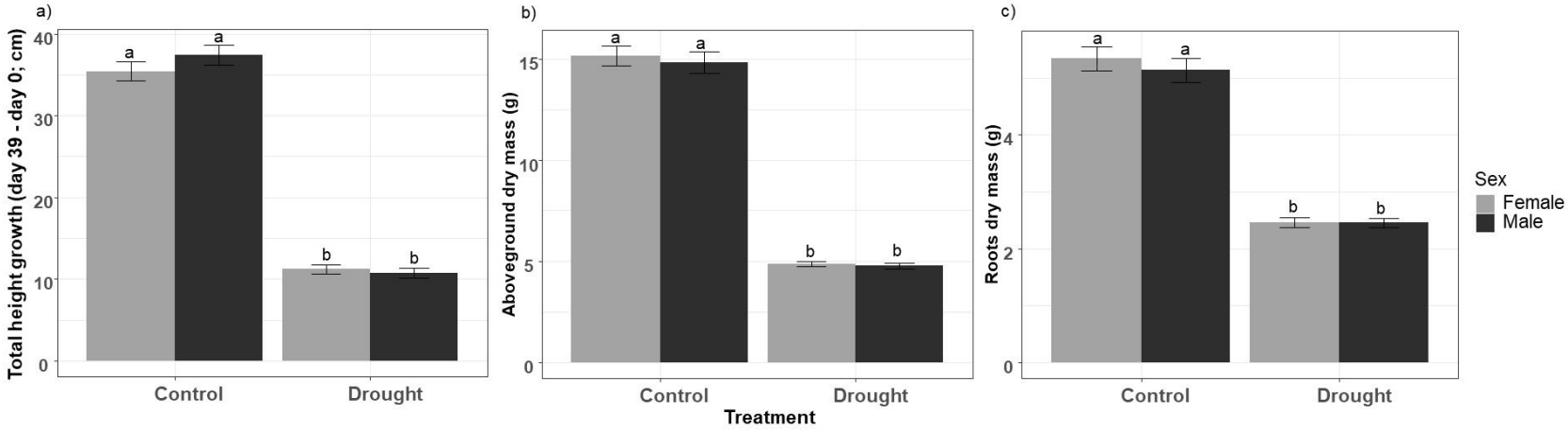

Figure 2-6. Mean ± SE of a) total height growth (cm) for the duration of the experiment; b) aboveground dry mass (g), and c) roots dry mass (g) for male and female aspen seedlings under well-watered (control) and drought conditions. Different letters indicate significant differences at $\alpha = 0.05$.

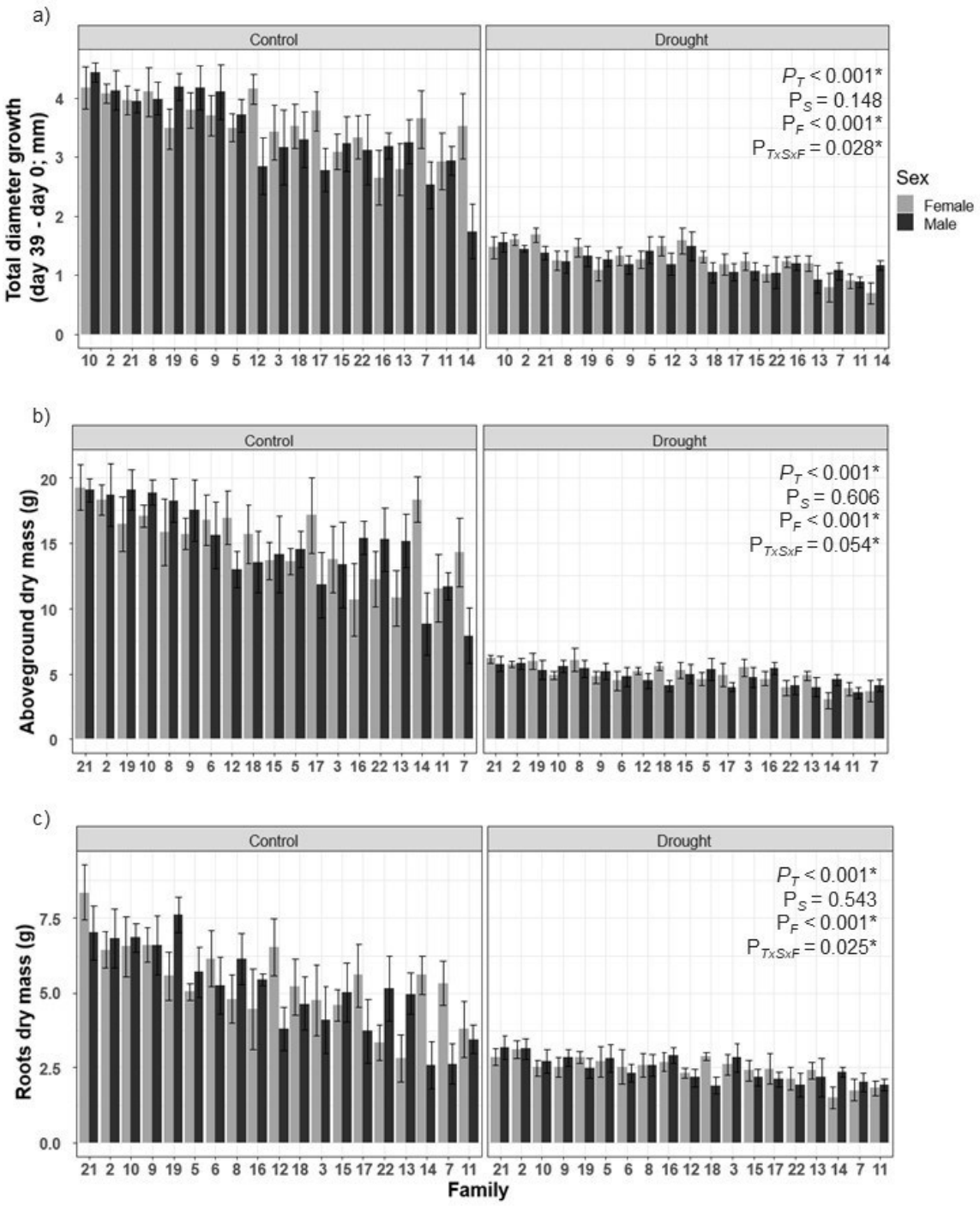

Figure 2-7. Mean ± SE of a) total diameter growth (mm); b) aboveground dry mass (g), and c) roots dry mass (g) for male and female aspen seedlings under well-watered (control) and drought conditions, for all 19 aspen families. The *P*-values correspond to treatment (*T*), sex (*S*), family (*F*), and the treatment x sex x family interaction. Levels of family are ordered from largest to smallest mean Y values. Asterisk indicates significance at $\alpha = 0.05$.

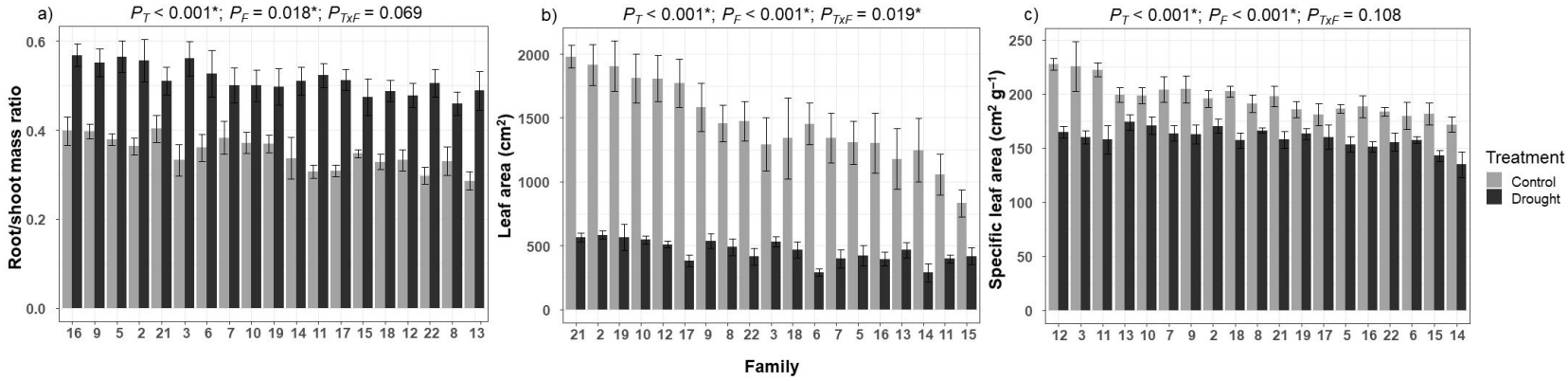

Figure 2-8. Mean ± SE of a) root/shoot mass ratio; b) leaf area (cm^2), and c) specific leaf area (cm^2 g^{-1}) for seedlings under well-watered (control) and drought conditions, for all 19 aspen families. The *P*-values correspond to treatment (*T*), family (*F*), and the treatment x family interaction (*TxF*). Levels of family are ordered from largest to smallest mean Y values. Asterisk indicates significance at α = 0.05.

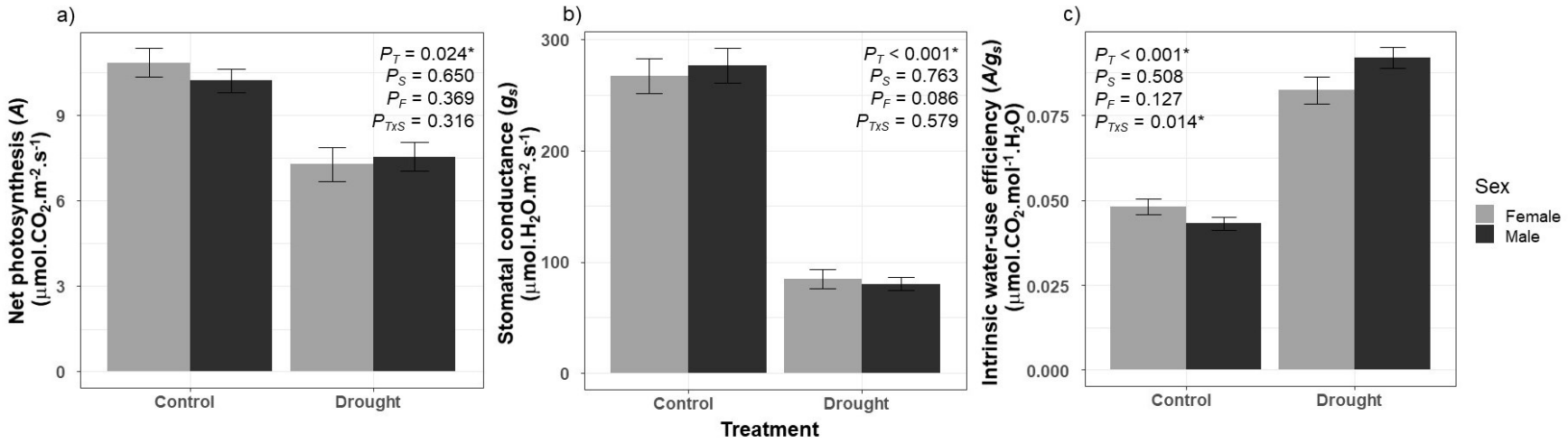

Figure 2-9. Mean ± SE of a) net photosynthesis (*A*, μmol.CO_2.m^{-2}.s^{-1}); b) stomatal conductance (g_s, μmol.H_2O.m^{-2}.s^{-1}), and c) intrinsic water-use efficiency (A/g_s; $_i$WUE, μmol.CO_2. mol^{-1}.H_2O) for female and male seedlings under well-watered (control) and drought conditions during the last week of the experiment. The *P*-values correspond to treatment (*T*), sex (*S*), family (*F*), and the treatment x sex interaction (*TxS*). Families are not shown in the figure. Asterisk indicates significance at α = 0.05.

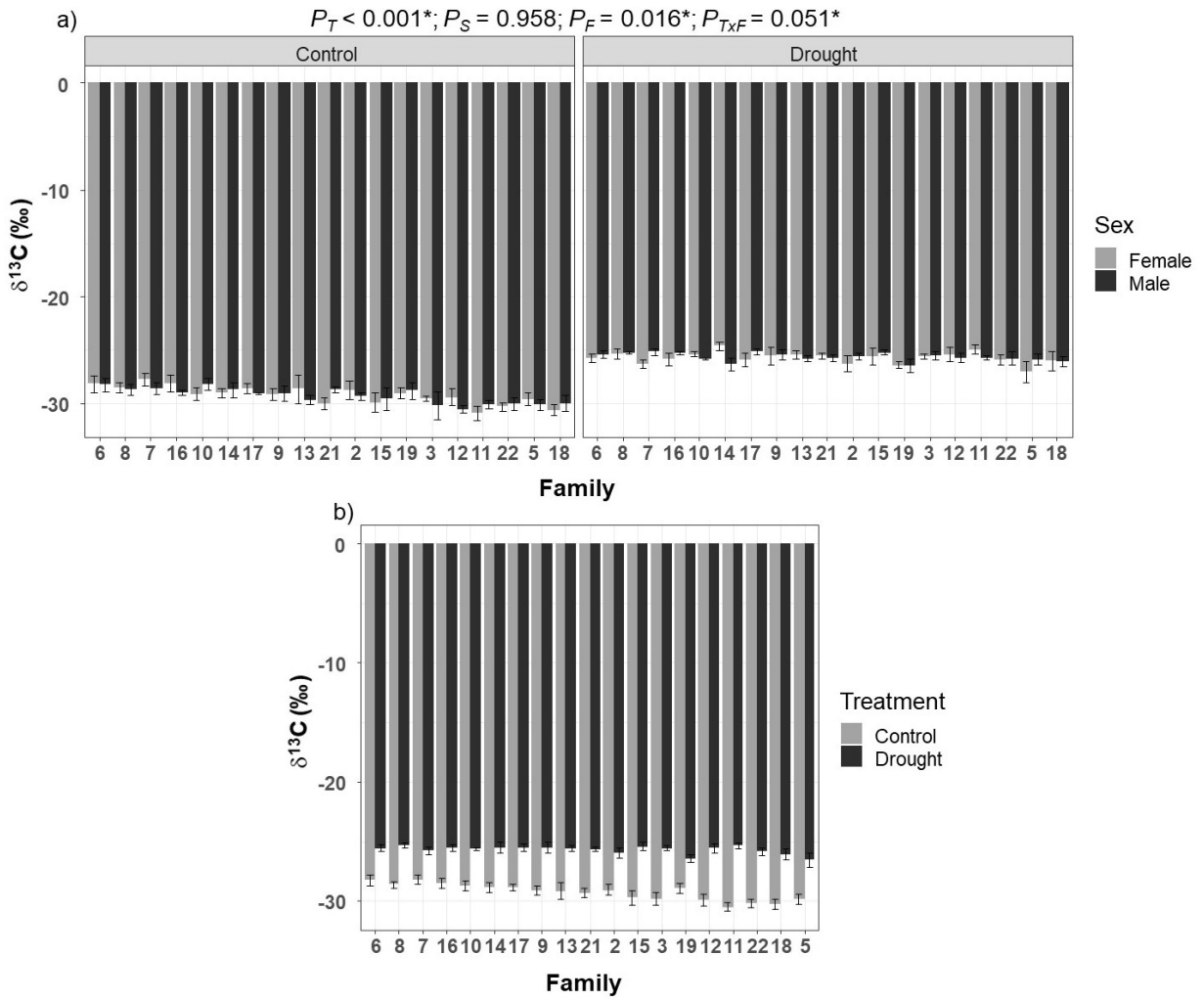

Figure 2-10. Mean ± SE of leaf δ^{13}C (‰) for a) female and male seedlings under well-watered (control) and drought conditions, for all 19 aspen families, and b) seedlings under well-watered (control) and drought conditions, for all 19 aspen families, without the sex variable. Levels of family are ordered from largest to smallest mean δ^{13}C values. The *P*-values correspond to treatment (*T*), sex (*S*), family (*F*), and the treatment x family interaction (*TxF*). Asterisk indicates significance at α = 0.05.

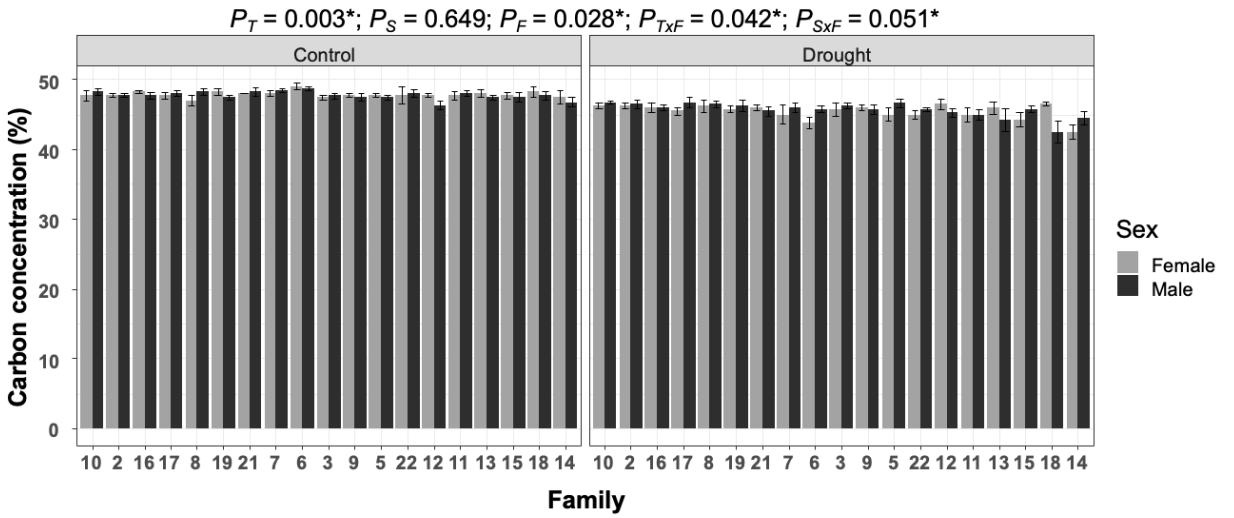

Figure 2-11. Mean ± SE of leaf carbon concentration (%) for female and male seedlings under well-watered (control) and drought conditions, for all 19 aspen families. The *P*-values correspond to treatment (*T*), sex (*S*), family (*F*), the treatment x family interaction (*TxF*), and the sex x family interaction (*SxF*). Levels of family are ordered from largest to smallest mean % C values. Asterisk indicates significance at α = 0.05.

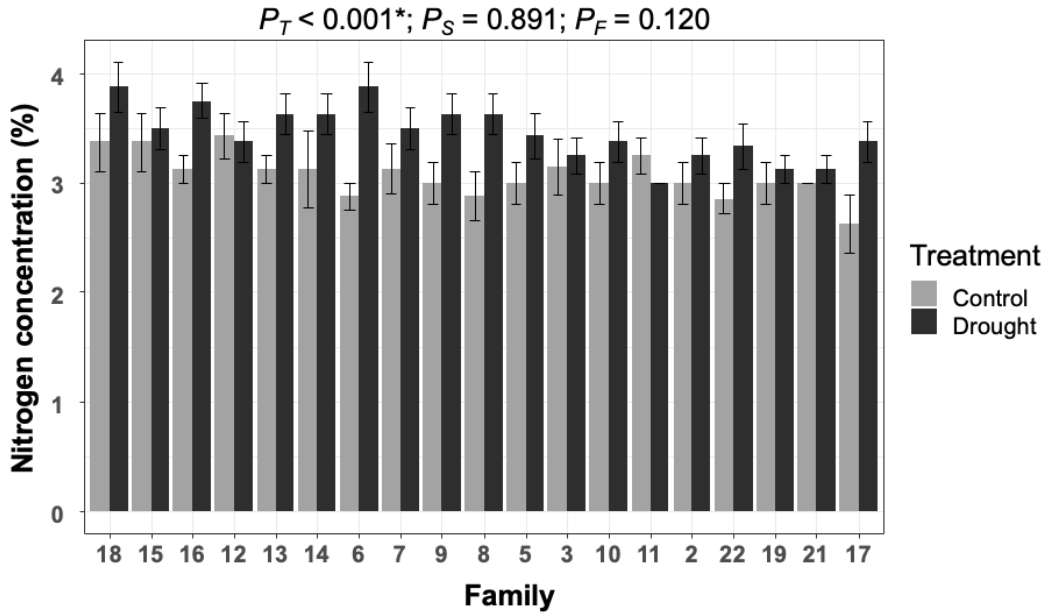

Figure 2-12. Mean ± SE of leaf nitrogen concentration (%) for seedlings under well-watered (control) and drought conditions, for all 19 aspen families. The *P*-values correspond to treatment (*T*), sex (*S*), and family (*F*). Levels of family are ordered from largest to smallest mean % N values. Asterisk indicates significance at α = 0.05.

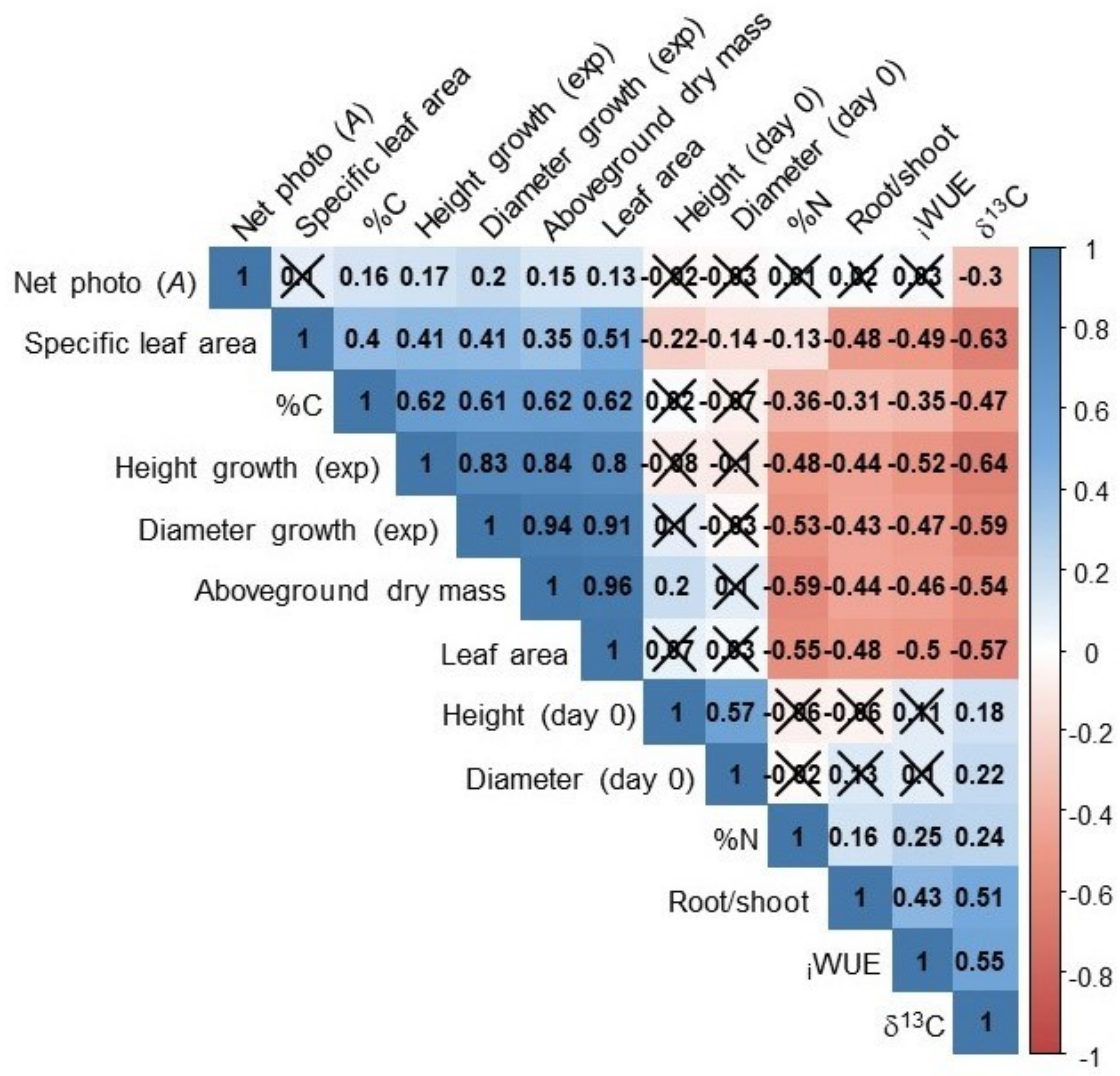

Figure 2-13. Correlation matrix including the variables: net photosynthesis (Net photo; *A*), specific leaf area, carbon concentration (%C), height growth for the entire experiment (Height growth; exp), diameter growth for the entire experiment (Diameter growth; exp), aboveground dry mass, leaf area, initial height at the start of the experiment (Height; day 0), initial diameter at the start of the experiment (Diameter; day 0), nitrogen concentration (%N), root/shoot mass ratio (Root/shoot), intrinsic water-use efficiency (¡WUE), and leaf $\delta^{13}C$. Numbers are R^2 values for correlations. Boxes containing an "X" represent non-significant correlations and boxes without an "X" on top represent significant correlations at $\alpha = 0.05$.

Chapter 3. Ratios and differences in growth and drought responses of female and male aspen trees

3.1. Introduction

Aspen (*Populus tremuloides* Michx.) is an economically important tree species in North America, its fibre is commercialized for pulp and paper products and oriented strand board for construction (Einspahr and Winton 1977; David et al. 2001). In Alberta, aspen fibre accounts for almost half of the annual forest harvest (Schreiber et al. 2013). The AW2 aspen breeding program was proposed, along with two other aspen improvement regions, by Li (1995), with the main objective of enhancing forest productivity under present and future environmental conditions. Clonal selection of aspen can grant 15% genetic gain in height and 34% in diameter (Thomas et al. 1997; Gylander et al. 2012; Brouard et al. 2017). Additionally, provenance trials have shown that assisted migration may be an option to compensate for adaptation lags caused by the fast-changing climate in Alberta (Gray et al. 2011; Gylander et al. 2012; Schreiber et al. 2013).

Aspen is a dioecious tree species (Grant and Mitton 1979). In general, dioecious trees are expected to have sex ratios close to 1:1, although deviations from this ratio occur towards males (Fisher 1930; Liu et al. 2021). Unbalanced distributions of aspen sex ratios have been observed at different elevations (Grant and Mitton 1979), and natural populations of European aspen (*P. tremula* L.) and trembling aspen (*P. tremuloides* Michx.) have been found to be generally male dominated (Valentine 1975; Myking et al. 2011). Understanding the causes of sex ratio biases is needed for predicting sex responses to environmental stresses and climate change, especially since highly skewed sex ratios may cause population declines if there is a reduction in the effective population size within populations (Petry et al. 2016; Liu et al. 2021).

In dioecious species, females are predicted to invest more resources in reproduction than co-occurring males (Darwin 1877; Dawson and Ehleringer 1993; Delph 1999; Obeso 2002; Stevens and Esser 2009; Liu et al. 2021). Because of reproduction costs typically associated with seed formation, females would have potentially lower growth rates and lower total biomass

than males (Delph 1999; Liu et al. 2021). At the population level, costs can be expressed as different survival rates and presence on the landscape (e.g., differences in vegetative propagation, survival, and frequency of reproduction; Delph 1999; Obeso 2002).

Climatic factors, the amount of plant-available water, and site and soil characteristics can drive the productivity of aspen stands (Hogg et al. 2008). Males of dioecious plant species have shown greater survival, root biomass, root-to-shoot ratio, and vegetative growth under drought stress (Dawson and Ehleringer 1993; Delph 1999 and references therein; Han et al. 2013). The better performance under stress has been attributed to higher water-use efficiency (WUE, A/g_s) in males (Dawson and Ehleringer 1993; Hultine et al. 2007; Xu et al. 2008; Han et al. 2013). With greater WUE under limited resource availability, males may be able to use resources more efficiently than females (Hultine et al. 2007). Female plants of different dioecious species have been found to present lower fitness (i.e., lower growth, survival, and reproductive success) when resource availability and habitat conditions were not optimal, whereas males were able to use resources more efficiently and withstand sub-optimal environmental conditions (Hultine et al. 2016).

Differences in resilience to drought between males and females might further influence the distribution of sexes on the landscape (Han et al. 2013; Hultine et al. 2007; Hultine et al. 2016; Liu et al. 2021). Studies focused on the impacts of drought in mature stands of dioecious tree species found a trend of females outperforming males in sites of moderate water availability (Dawson and Ehleringer 1993; Hultine et al. 2007; Hultine et al. 2016). Males, in contrast, were found to outperform females in dry habitats (Dawson and Ehleringer 1993; Han et al. 2013; Hultine et al. 2016). Liu et al. (2022) revealed differences in wood anatomical traits between males and females of *Populus cathayana*, which may contribute to the differential performances of sexes under drought stress. Furthermore, males had a lower starch content in wood than females in all treatments (Liu et al. 2022); starch content is related to growth performance and physiological traits (Bellasio et al. 2014). If segregation of sexes occurs as a response to drought, climate change might pose a risk for dioecious tree species, since the drastic reduction of one sex on the landscape could reduce genetic diversity and effective population size (Hultine et al. 2016; Petry et al. 2016).

Differences between sexes, however, have not always been observed for dioecious tree species (Hultine et al. 2016). In fact, studies have found contradictory results for differences between sexes in aspen. In Tupker et al. (2003) there was a trend of female rooted aspen cuttings outperforming males under experimentally elevated CO_2 levels, whereas in Wang and Curtis (2001) males outperformed females under both ambient and elevated CO_2 levels. Grand

and Mitton (1979) found varying proportions of males and females distributed across different elevation ranges, with female biased ratios at lower elevations and male biased ratios at higher elevations, interestingly, females had higher growth rates at all elevations. Relatively few studies have explored sex differential performance of *P. tremuloides* in natural stands, in fact, most of the studies found on the topic are performed in controlled environments and are dated from at least 20 years ago. Prior to molecular tools to identify sex, the sex of aspen ramets was described based on morphological observations made on catkins and flowers (Mitton and Grant 1980). Today, genetic markers have been developed to determine sex in aspen (Pakull et al. 2014), providing a novel opportunity for the exploration of sex characteristics and responses in aspen in both seedlings and non-flowering mature trees.

This chapter explored naturally occurring aspen populations (i.e., wild stands), adapted to the local conditions of Alberta. All study populations were comprised of naturally occurring aspen trees within the AW2 (Pembina) tree improvement region. This study aims to: i) explore the patterns of female and male aspen distribution, growth, and wood resistance in mature aspen stands in the AW2 (Pembina) tree improvement region; ii) test if patterns of water availability (precipitation, PET, and CMI) influenced the distribution of sexes on the landscape; and iii) explore if female and male aspen trees respond differently to a past drought event, considering basal area increment, resistance, recovery, resilience, and relative resilience. I hypothesize that: i) there will be male biased sex ratios in the studied areas within the AW2 (Pembina) region, as well as that males will present greater diameter growth than females; I expect wood resistance to differ between males and females, with greater resistance on males, due to potentially lower starch content (Liu et al. 2022); ii) I expect to find more males in areas of lower moisture availability, and similar distributions of males and females in areas of higher moisture availability; and, iii) I expect males and females to respond differently to a past drought period, with males presenting higher indices of recovery, resilience, and relative resilience post-drought.

3.2. Methods

Study area

Four areas were selected within the trembling aspen AW2 (Pembina) controlled parentage program (CPP) region, in Alberta, Canada (Figure 3-1). Naturally occurring aspen populations (i.e., wild stands), adapted to the local conditions of Alberta were sampled. Aspen

trees occurring within each selected study site were considered an aspen population, since they occupy the same area and are separated from the other populations by distance (at least 500 m) or physical barriers, such as roads, relief, spruce stands, or water bodies. Site selection was based on the presence of AW2 CPP parent trees, where plus tree selections based on health and growth found a sex ratio of approximately 1:3 female:male mature aspen trees (Thomas B., personal communication, 2019). Areas were selected along the southern range of the AW2 region, to represent a broader landscape within such range. While field sex ratios of 1:3 female:male were found in the AW2 region, in Chapter 2 seed crops from the boreal and parkland regions in Alberta had sex ratios close to 1:1 (greenhouse experiment supplemental data, Table A3-4). Parent tree location information was provided by Weyerhaeuser Pembina Timberlands, courtesy of Mr. Dave Swindlehurst, Silviculture Manager at Pembina Timberlands. The study area range stretches from 52.65°N to 53.26°N latitude and from -114.81°W to -115.32°W longitude, 856 - 1023 meters above sea level (MASL). Within each of the four areas, three mature aspen populations were selected as study sites (in a total of four study areas and 12 study sites). In three areas (Eta Lake, Dominion, and Tower), sites were chosen based on the presence of an AW2 selected parent tree. The study sites were separated by a minimum of 500 m, to avoid sampling from the same clones, but trees sampled within each site could belong to a same clone. The study areas, study sites, and sampled trees used for Chapter 3 are the same trees used for Chapter 4. See Chapter 4 for a detailed description of clonal structure within the study sites.

The AW2 CPP region has 72% of its land located within the Foothills Natural Region, and the remaining area in the Boreal Forest Natural Region. The land cover is also characterized by four subregions: 70% in the Lower Foothills, 28% in the Central Mixedwood, 1% in the Upper Foothills and 1% in the Dry Mixedwood (Brouard et al. 2017). The four study areas are in the Lower Foothills subregion.

BioSIM was used to obtain data on the environmental characteristics of each study site. For the climate normal period of 1960 to 1990 annual average precipitation on all 12 study sites ranged from 570 mm to 608 mm, annual average potential evapotranspiration ranged from 435 mm to 472 mm, and annual climate moisture index (CMI) ranged from 111 mm to 173 mm (Table 3-1).

Sampling

Sampling in the field occurred in the summers of 2019 and 2021. Within each study site, stems were sampled at random from the plot centre, moving towards north, then northeast,

east, southeast, south, southwest, west, and northwest. The total number of stems sampled on each site varied from 19 to 30, depending on the size of the study area and stand density. In 2019, GPS coordinates were obtained, to perform spatial analyses, from each stem using the Avenza Maps (Avenza Systems Inc.) application on an iPad (6th generation, model MR722CL/A) and phloem tissue was collected for DNA extraction, using a hole puncher (2.5 cm diameter) at 1.3 m ± 15 cm from the base of the tree, or in areas where the bark of the tree was soft enough to collect a sample. In the field, phloem tissue was placed on individualized plastic sandwich bags and kept cool in a cooler with ice packs then refrigerated within ten hours until samples were transferred to a -20°C freezer at the University of Alberta. In 2021, diameter at breast height (DBH, at 1.3 m ± 15 cm from the base of the tree) was measured, to explore differences in diameter growth, using a digital caliper (Haglöf Digitech DP II Computer Caliper).

Increment core sampling and processing

In the summer of 2021, a 5 mm bark-to-bark increment corer was used to sample a subset of 12 trees within each site: 6 males and 6 females, whenever possible, for a total of 132 increment cores across the 12 study sites. Cores were collected in a north-to-south direction, at approximately breast height (1.3 m ± 15 cm from the base of the tree). The north half of each core was used for ring width analysis and the south half of the core was used for wood specific gravity measurements. Tree cores were stored in plastic straws in a cooler containing ice until being transported to the University of Alberta and stored at -20°C until processing.

For processing, cores were removed from the freezer, air-dried for one week, and then glued (Elmer's Carpenter's Wood Glue Max) to wooden mounting blocks holding five cores each. Cores were then sanded with progressively finer sandpaper starting with 80, 180, 400, 600, and finally 1000 grit to obtain a surface with clearly visible cell and fiber structures. Sanded cores were scanned at a resolution of 3200 dpi (Epson Perfection V800 Photo) and tree ring width was measured using WinDENDRO (version 2017a, Regent Instruments Canada Inc.). Ring width was confirmed under the microscope for some cores that had weak ring contrast. Broken or unreadable (not enough contrast at the point of transition from one year to the next) cores or core sections were discarded from the analysis. Ring width measurements were cross-dated visually and statistically, using WinDENDRO (version 2017a, Regent Instruments Canada Inc.).

Stand density was measured, to account for the effects of stand density on diameter growth, following the temporary sample plot manual for the Lesser Slave Lake Regional Forest Management Plan (2018). In brief, one circular plot of 5.64 m radius (100 m^2) was established

within each study site. Stand density was measured for 100 m^2 and the value obtained was then extrapolated to one hectare. Within each plot, live trees greater than 1.3 m in height and 7.0 cm DBH were counted and species and DBH were recorded. Stand age was determined from increment cores by counting the number of growth rings on each core, for each studied aspen population, and then averaged across all trees within the population. For the determination of stand age, only complete cores (from bark to pith) were included. The area of each study site was calculated using ArcMap 10.7.1 (ESRI, Redlands, CA) software: a polygon was created using tree coordinate information and the area was extracted from each polygon using the 'calculate geometry' tool (Table 3-1).

DNA analysis: extraction

Phloem samples were removed from the -20°C freezer and transported to InnoTech Alberta in Vegreville, for DNA extraction of 372 phloem samples, and 7 control samples (see Chapter 2 for description). The outer bark was cut off the samples and phloem tissue hand-ground in liquid nitrogen with a mortar and pestle. Ground phloem tissue was then frozen at -80°C placed in 2 mL tubes (Bio Spec #522S) with a mix of 0.5 mm, 2.3 mm Zircona/Silica beads (Bio Spec #11079105z, and #11079125z), and 4 mm Stainless Steel Grinding Balls (Spex #2150) and further ground using a Genogrinder (Spex Model# 2010) at 1750 rpm for 3 minutes. The Qiagen 96 well DNeasy (Qiagen #69181) kit for frozen tissue was then followed with the following modifications: after addition of the first buffer (Buffer AP1, RNase A, and Reagent DX) the samples were ground on the Genogrinder for an additional minute; the final elution was done with 100 µL nuclease free water, the plates were left at 4°C overnight, and heated to 65°C for 10 minutes the next morning before eluting. Samples were stored at -20°C until all PCR and sexing procedures were complete, and thereafter stored at -80°C.

DNA analysis: sex screening

Sex was determined for the phloem samples using the method described in Chapter 2.

Spatial distribution of sexes

The position of each sampled tree within a population was extracted using the GPS coordinates described above. After obtaining the results from sex screening, sex was assigned to each sampled tree within a population and plotted on the maps to determine the spatial distribution of sexes.

Climate analysis

All climate data were obtained with the BioSIM software using the GPS coordinates of each study site.

To test if patterns of moisture availability influenced the distribution of sexes on the landscape, climate patterns were explored for the periods of establishment and post-establishment of each stand. Annual (January to December) climate data for the climate normal period of 1960-1990 was used, as all stands are older than 50 years. For annual climate data, precipitation (Ppt), potential evapotranspiration (Pet), and climate moisture index (CMI) were summed from January to December of each year and then averaged across years. The climate averages were tested statistically to explore differences between areas.

To explore if female and male aspen trees respond differently to a past drought event the selected climate normal period was 1991-2020. This period was selected to compare the most recent climate with the most recently formed tree rings; as recent rings had better quality, and all trees had reached maturity by 1991 (i.e., all trees reached mature height size and started reproduction by then, as the youngest of the sampled stands would be 20 years old in 1991). Ppt, Pet, and CMI were summed for the growing season months (May to September) of each year and compared with annual growth ring data from the increment cores. Overall averages (for growing season months) of Ppt, Pet, and CMI were calculated across years within the climate normal period (1991-2020). The climate averages were tested statistically to explore differences between areas.

Wood specific gravity

The south halves of increment cores were used to determine wood specific gravity as a measure of wood density. Cores were processed following the AGTIP (2000) protocol for the water displacement method to obtain wood relative density, with the following modifications: oven drying temperature was decreased from 103°C to 71°C; oven drying period was extended from 24h to 72h.

For the water displacement method increment cores were removed from the -20°C freezer and left on the bench to thaw for 24h. Outer and inner bark layers were removed from each core sample, then cores were labelled, and placed in a vacuum chamber, where they were kept submerged in distilled water for approximately 24h until the moisture content of the cores reached their fibre saturation point (i.e., >30% moisture content). Moisture content was confirmed by surface contact with the use of a digital moisture meter (MM4DE General Tools & Instruments).

Volume of each saturated core was determined using the water displacement method (AGTIP 2000). A 250 mL gravimetric cylinder containing distilled water was placed on top of a scale (Fisher Science Education™ Portable Balances, readability 0.001 g) which was then zeroed, and each core was individually submerged into the cylinder. The increase in weight on the scale after the immersion of the saturated wood is equivalent to the volume of the saturated wood sample in cm^3, which in turn is equal to its volume in millilitres. Samples were also weighed after the wetting process (before drying), to confirm that the moisture content of the wet wood samples was higher than 30%, by calculating percent moisture as (dry weight*100)/wet weight. Cores were air dried for 24h, then dried to constant weight at 71°C for a period of 72h. After drying, cores were weighed using the same scale used for the displacement measurements. Wood specific gravity (or relative density) was then determined as the ratio of oven-dried weight in grams to wet volume in cubic centimetres (g/cm^3).

Drilling resistance

Drilling resistance (DR) and diameter inside bark (DIB) were measured with the use of a Resistograph Series 6 Scientific (Rinntech), on the same trees, and at the same time as increment cores were collected.

The Resistograph drives a 3 mm brad point drill bit through the stem. The force applied varies to maintain a pre-selected speed, which means that in wood with higher density the equipment needs to apply more torque (Rinn et al. 1996; Rinn 2012). The Resistograph's standard output is given as percent DR. DR can be used as an indirect measure of wood density (Matheson 2019).

Bark-to-bark Resistograph profiles were taken at breast height (1.3 m $\pm$ 15 cm) using a drilling speed of 20 mm/second (equipment default) for a total of 132 sampled trees. Two measurements (or profiles) were taken per sampled tree, one in a north-to-south direction and the second one in an east-to-west direction, to reduce error and ensure an entire profile was captured. Values of DR and DIB from the two directions were averaged, and the final mean value was used to perform further analysis. Measurements were taken from stem areas visually free of branches and other defects (e.g., knots, rots, flat spots, crooks) to obtain as clear and well-delineated profiles as possible. Some samples, however, had internal wood knots or heart rot, and these profiles were discarded from the data. The drill bits were replaced after 200 measurements, following Rinntech's instructions.

Basal area increment and resilience components

Basal area increment was calculated using ring width values from the bark until 1991. The DIB was used to calculate the radius of each tree, using the formula radius = diameter/2. Next, the cumulative radius was calculated using the radius from the last year subtracted by the ring width of the next year, for example, radius at 2019 = radius 2020 – ring width 2020. After obtaining a cumulative radius for each year, the basal area (BA) was calculated for each radius, using the formula BA = π * (radiusYear^2). Finally, with the BA values of each year, the basal area increment (BAI) was calculated from BAI(Year) = BA(Year) – BA(Year-1) e.g. BAI2000 = BA(2000) – BA(1999).

The resilience indices resistance, recovery, resilience, and relative resilience were calculated following Lloret et al. (2011), with the formulas:

Resistance (Rt) = Dr/PreDr;

Recovery (Rc) = PostDr/Dr;

Resilience (Rs) = PostDr/PreDr;

Relative resilience (RRs) = ((PostDr-Dr)/(PreDr-Dr))*(1-(Dr/PreDr)), where:

Dr is the average of BAI values for the drought period. PreDr is the average of BAI values for the pre-drought period, and PostDr is the average of BAI values for the post-drought period (Lloret et al. 2011).

Selection of drought period

The drought period used to calculate resilience components was determined by a period of lower precipitation, that was preceded and followed by regular average precipitation during the period of 1991 to 2020. A similar pattern was also observed for CMI for the same period. Therefore, the drought period chosen, based on climate data, was 2002 and 2003. The pre-drought period was five years before the drought (1997 to 2001), and the post-drought period used was four years after the drought (2004 to 2007; not 5 years to avoid lower than usual Ppt and CMI in 2008, this avoided an overlap with a subsequent drought period in 2008 and 2009; Figure 3-2). Drought period selection followed the methods of Lloret et al. (2011), thus including the pre-drought, drought, and post-drought periods, but did not consider delayed effects of drought.

Data analyses

To analyze potential differences between areas, three study sites were grouped within each area. Data were analyzed in R 4.3.0 (R Core Team 2023). A significance level of $\alpha < 0.05$ was used. Model assumptions of homogeneity of variances and normality were verified, and

data transformations were applied when needed. The variables resistance and recovery were log-transformed for data analyses.

To analyze patterns of sex distribution, a chi-squared test of independence was used to see if there is an association between the general distribution of males and females, and between sex distribution across different areas (Eta Lake, Dominion, Tower, and Cathedral). The chi-squared test compares the expected and obtained frequencies of each sex. When significant differences were found, a Bonferroni-adjusted pairwise nominal independence test (Mangiafico 2016) was applied as a post hoc test to explore the differences in sex distributions between areas.

Analyses of variances (ANOVA) with pooled variances were used to test if wood resistance (wood specific gravity) differed between males and females. Wood specific gravity (g/mL) was the response variable and sex was the explanatory variable. A linear mixed-effect model was applied to test if there were differences in DBH growth between sexes. Sex was included as an explanatory variable, stand age, and stand density were included as covariates.

To test climate differences between areas for the climate normal period of 1960-1990, analyses of variances (ANOVA) were used. To test for differences in precipitation (Ppt) and climate moisture index (CMI) between areas Welsch's ANOVA for unequal variances was applied, and to explore potential evapotranspiration (Pet) an ANOVA with pooled variance was applied. Post hoc multiple comparisons were performed using the pairwise.t.test function (R Core Team 2023), with a Bonferroni correction to adjust the *P*-values for multiple comparisons. Welch's test for unequal variances was used for Ppt and CMI.

To test if sex distribution was influenced by climate moisture availability, first correlations were calculated between Ppt, Pet, and CMI. Ppt and Pet were not correlated, but CMI was highly correlated with both, so only CMI was used. A generalized linear mixed-effect model fit by maximum likelihood (Laplace approximation) was used to test if sex distribution was influenced by CMI, where sex (binary, coded as 0 for male and 1 for female) was the response variable, CMI (mm) was the explanatory variable, and area (Eta Lake, Dominion, Tower, and Cathedral) was included as a random effect. Including area as a random effect accounts for variation caused by site characteristics, knowing that individuals within the same area may be more similar to each other than individuals from different areas. The glmer() function on the lme4 package (Bates et al. 2015) was used to fit a logistic regression model as sex is a binary response variable.

Linear mixed-effect models were applied to test if there were differences in mean basal area increment (BAI) pre-drought, during drought, and post-drought, and in resistance,

recovery, resilience, and relative resilience indexes between males and females. The lmer function on the lme4 package (Bates et al. 2015) was used to fit the linear model. Pre-drought BAI, drought BAI, post-drought BAI, resistance, recovery, resilience, and relative resilience were included as response variables, sex was included as an explanatory variable, stand age was included as a covariate, and area was included as a random effect.

3.3. Results

Patterns of sex distribution, growth, and wood specific gravity

The studied sites within the AW2 (Pembina) CPP region showed sex proportions of 64% females and 36% males, with an approximate sex ratio of 2:1 females:males. A chi-square test of independence showed that the total proportion of females to males differed ($\chi^2 = 713.67$, df = 1, $P < 0.001$; Figure 3-3a). Three of the four areas had higher female:male sex ratios and one area had a 1:1 ratio. A second chi-square test of independence showed that the association between sex and area was also significant ($\chi^2 = 350.28$, df = 3, $P < 0.001$). The sex proportions found were 50% females and males in Cathedral, 63% females and 37% males in Dominion, 70% females and 30% males in Eta Lake, and 76% females and 24% males in Tower (Figure 3-3b). There was no clear pattern of distribution by sex at any of the four study sites (Figure 3-4).

Males had higher mean DBH than females by an average of 2.6 cm (males: 32.5 $\pm$ 0.97 cm; females: 29.9 $\pm$ 0.70 cm; Figure 3-5a), however, differences were not significant when taking into consideration the effects of stand age and density. The differences in wood specific gravity between males and females were not significant ($F_{1, 117} = 3.75$, $P = 0.055$), although, males' wood specific gravity appeared slightly higher at 0.369 $\pm$ 0.005 g/mL, whereas females' was 0.357 $\pm$ 0.004 (Figure 3-5b).

Moisture availability and sex distribution

An exploration of the annual historical climate from 1960 to 1990 showed differences between the four study areas. Precipitation (Ppt) was significantly different between the four areas ($F_{3, 202} = 2.72$, $P = 0.045$; Figure 3-6a). Potential evapotranspiration (Pet) was significantly different between the four areas, with the highest Pet in Cathedral and lowest in Tower ($F_{3, 368} = 12.36$, $P < 0.001$; Figure 3-6b), while climate moisture index (CMI) was highest in Tower, followed by Eta Lake. Although differences in CMI between areas were significant, none of the areas had negative CMI during the period of 1960-1990 ($F_{3, 202} = 4.56$, $P = 0.004$; Figure 3-6c).

Ppt and Pet were not correlated, but CMI was highly correlated with both, so only CMI was used to test sex distribution as a function of moisture availability. When exploring sex distribution as a function of CMI the generalized linear mixed-effect model results showed no differences in predicting the sex distribution (β = -0.009, SE = 0.02, P = 0.699). Nonetheless, there appeared to be a pattern of a higher ratio of females:males in areas of higher CMI as found at the Eta Lake and Tower locations, while Cathedral and Dominion had similarly lower CMI values, and the sex ratios were 1:1 at Cathedral and approximately 2:1 female:male in Dominion (Figure 3-7).

Basal area increment and resilience components

The average basal area increment growth during the periods of pre-drought, drought, and post-drought was not significantly different between the female and male aspen trees sampled in this study ($\beta_{Pre\text{-}drought\sim sex}$ = -26.558, $SE_{Pre\text{-}drought\sim sex}$ = 70.11, $P_{Pre\text{-}drought\sim sex}$ = 0.705; $\beta_{Drought\sim sex}$ = 6.563, $SE_{Drought\sim sex}$ = 88.25, $P_{Drought\sim sex}$ = 0.941; $\beta_{Post\text{-}drought\sim sex}$ = -13.605, $SE_{Post\text{-}drought\sim sex}$ = 84.67, $P_{Post\text{-}drought\sim sex}$ = 0.873; Figure 3-8). Similarly, resistance, recovery, resilience, and relative resilience indexes did not differ between females and males ($\beta_{Resistance}$ = -0.047, $SE_{Resistance}$= 0.13, $P_{Resistance}$= 0.721; $\beta_{Recovery}$ = -0.054, $SE_{Recovery}$ = 0.11, $P_{Recovery}$ = 0.617; $\beta_{Resilience}$ = 0.016, $SE_{Resilience}$= 0.13, $P_{Resilience}$= 0.901; $\beta_{RelResilience}$ = -0.102, $SE_{RelResilience}$= 0.13, $P_{RelResilience}$= 0.424; Figure 3-9).

3.4. Discussion

Patterns of sex distribution, growth, and wood specific gravity

The current study explored if female and male mature aspen (*P. tremuloides* Michx.) trees within the AW2 (Pembina) CPP region, in Alberta, Canada, showed differential performance in growth and dominance on the landscape. Overall, there was a female-biased sex ratio of approximately 2:1 across the study region, suggesting that factors other than the cost of reproduction are driving this distribution, although males were found to be larger. The sex ratios determined by testing 48 seedlings per seedlot in the aspen seed crops are close to 1:1 (greenhouse experiment supplemental data, Table A3-4), therefore deviations from this ratio in wild aspen stands suggests that sex-biased mortality or differential environmental pressures have occurred as the trees have matured.

Reproductive costs have been shown to favour males over females in dioecious species (Dawson and Ehleringer 1993; Delph 1999; Obeso 2002; Cole et al. 2016; Liu et al. 2021), still the general expectation that males outperform females might not be applicable for all developmental stages and environments (Cole et al. 2016). More recent studies indicate that for dioecious tree species, including different *Populus* spp., females may have a greater ability to uptake resources available in the environment (resource acquisition capacity; RAC) when resources are non-limiting (Hultine et al. 2007; Liu et al. 2021), while males, have been shown to have greater resource use efficiency (RUE; Hultine et al. 2007; Liu et al. 2021), which is the capacity of turning obtained resources into biomass (Hodapp et al. 2019).

Differences in RAC and RUE could potentially give females a performance advantage during the early stages of life before the start of reproduction (Dawson and Ehleringer 1993; Delph 1999; Stevens and Esser 2009; Cole et al. 2016). Once reproduction starts, females may allocate the synthesized energy (e.g., carbon) and absorbed resources (e.g., water) to the production of flowers and fruits (Stevens and Esser 2009), thus giving males a competitive advantage when allocating resources. Interestingly, males have been found to flower more frequently and reproduce earlier than females (Valentine 1975; Einspahr and Winton 1977; Laporte and Delph 1996) suggesting that if males incurred costs to reproduction, those energy allocation costs would occur earlier in males than females. Previous studies on *Populus* spp. indicate that young females show higher growth rates and total biomass when compared to males (Cole et al. 2016; Stromme et al. 2018; Zhang et al. 2018). Altogether, females would potentially outperform males in early developmental stages and males would outperform females after the reproductive maturity of females (Delph 1999; Cole et al. 2016).

If previous work that has shown that young female dioecious trees outperform young males due to higher growth rates and total biomass (Cole et al. 2016; Stromme et al. 2018; Zhang et al. 2018) holds for aspen, then during the stage of stand establishment and development females may be able to expand their root systems faster than males, acquiring greater resources. Furthermore, since males have been shown to flower more frequently and reproduce earlier than females (Valentine 1975; Einspahr and Winton 1977; Laporte and Delph 1996) this may give females a competitive advantage over males prior to the onset of flowering. In Wang and Curtis (2001) female aspen seedlings had significantly greater height, stem, and root biomass growth than males at ambient CO_2 levels. Given that aspen can also reproduce asexually, through root suckers (Day 1944; Einspahr and Winton 1977; Landhäusser et al. 2019), the faster development of female root systems would result in more root biomass to acquire resources and to produce root suckers from those female clones. In effect, Sakai and

Burris (1985) found that female aspen clones had a greater number of ramets than male clones. Altogether, greater growth rates of females during early stages of stand development may explain the female biased sex ratios in the studied areas. Females might also allocate resources into asexual reproduction, spreading root systems and producing more ramets, instead of investing in flowering and seed production. This theory is based only on previous literature and current knowledge of aspen and dioecious species, as it is not possible to explore how the studied stands were formed. Future studies could investigate young aspen stands established from seed to determine if young female aspen outperform males in the field in traits such as height, diameter, and most importantly root growth and suckering, as sexual dimorphism has not been consistently shown in *Populus* (Robinson et al. 2014; Mckown et al. 2017).

This study showed that within the studied areas, diameter growth did not differ between males and females. These results indicate that there might not be a higher allocation of energy to reproduction in females. Only a few studies have compared sex performance in aspen by sampling mature trees, as most studies that explored differences between males and females did so in greenhouse environments using either seedlings or rooted suckers. It is important to compare the obtained growth results with studies that were conducted in mature stands, as the trees included in those stands would have been through several reproductive events, from which reproduction costs have accumulated. In Cole et al. (2016), a study that explored aspen sex differences in young seedlings propagated from root material of 12 aspen clones and on mature trees from 51 aspen clones in wild stands in south-central Wisconsin, USA, the authors reported 12% greater volume in females versus males, where females were grown from root material in a common garden for three years; and 10% greater volume of males than females, for trees grown in wild populations, where tree ages ranged from 5 to 51 years. Stevens and Esser (2009) conducted a study on a 40-year-old aspen stand from an experimental trial in Oneida County, Wisconsin, where they sampled 54 females and 32 males of four different aspen families, and found that the diameter of males was 9% larger than females. Other field studies on natural aspen stands either did not find differences in diameter growth between sexes (Sakai and Burris 1985, study located in northern lower Michigan, USA, 31 aspen clones sampled) or found that females had greater diameter growth than males (Grant and Mitton 1979, but the authors did not mention the average age of the sampled trees; study sites located northwest of Boulder, Colorado, USA, sampled 304 aspen clones). In my study, the lack of differences in mean diameter growth between males and females of mature aspen populations indicates that even after going through several reproductive events, those aspen trees did not incur differential growth costs to reproduction as found by McKown et al. (2017) in other poplar

species. Overall, costs of reproduction in aspen may be more complex than just differences in energy allocation costs associated with seed production. The reproduction of aspen occurs mainly clonally, through the root systems (DeWoody et al. 2008). While the production of seeds and pollen still occurs, aspen seeds have little or no endosperm and are very small in size (Einspahr and Winton 1977; Jelinski and Cheliak 1992), consequently the energy costs of producing seed and pollen might not be much different, and aspen might not have reproduction costs associated with the production of seeds (Delph 1999). Instead, sexual differences might be related to the allocation of energy to the growth of aboveground parts (e.g., stem, branches, and leaves) or root systems.

Wood specific gravity is commonly used as a proxy for wood density, as they both measure the mass of wood per unit volume which can also be used as an indicator of wood quality (Rao 1966). Wood with higher density or specific gravity is generally heavier and stronger (Pande et al. 2012). In the present study, wood specific gravity appeared to be higher in males of mature aspen trees compared to female trees, albeit the results were not significant. Pande et al. (2012) found similar results with 6-year-old *P. deltoides* Bartr. ex Marsh, where the wood specific gravity of males was significantly greater than females, yet the authors found that females had greater fibre length and diameter; the authors concluded that the wood of female *P. deltoides* produced good fibre quality while males produced wood with higher density. The results partially support our hypothesis that males would have higher wood specific gravity than females, as the wood specific gravity of males is higher on average, but not significantly. Different wood specific gravity values between sexes point to a potential difference in wood anatomy between male and female aspen. Sex differential dimorphism in wood characteristics has been shown for other dioecious and *Populus* spp. and has been considered as a factor driving differential performances of sexes under drought stress (Hultine et al. 2007; Olano et al. 2017; Liu et al. 2021; Liu et al. 2022). In my greenhouse experiment (Chapter 2), sexes did not perform differently on carbon assimilation (A) or stomatal conductance (g_s), yet female seedlings had higher $_i$WUE (A/g_s) under well-watered conditions and male seedlings had higher $_i$WUE under drought conditions, which aligns with the theory that the hydraulic systems of females and males may be different.

Moisture availability and sex distribution

We hypothesized that male aspen clones would be more prevalent in areas of lower moisture availability, compared to females, and similar distributions of males and females would be expected in areas of higher moisture availability. The results did not show a consistent

pattern of sex distribution and CMI values in the four study sites, however, the two areas with the highest CMI values were also the areas with the highest female:male ratios. While not significant, these results might symbolise a trend of higher female presence in areas of higher water availability, as suggested by Grant and Mitton (1979) and Goessen et al. (2022).

We did not find evidence of the differential performance of male and female mature aspen trees under varied CMI values, nevertheless, all areas included in this study had enough plant-available water during the climate normal period of 1960-1990 (CMI > 0; Hogg 1997). It is complex to determine when the studied populations were established due to the clonal nature of aspen, yet they could have been established under optimal climate and growth conditions from which differential performances would not emerge. Future studies could compare aspen sex distribution in wild populations from historically climatically different regions, such as the parkland (CMI ≤ 0) and the boreal region (CMI > 0) in Alberta, Canada. The present study was focused on aspen populations within the AW2 (Pembina) tree improvement region of Alberta, so the objective was to explore population differences within the boundaries of the region.

Basal area increment and resilience components

Lloret et al. (2011) developed indices to estimate components of resilience based on tree ring growth data. The four components are resistance, recovery, resilience, and relative resilience, where: resistance compares performance during and before the drought event; recovery considers the post-disturbance performance as a function of the changes caused by the event; resilience is the ratio between post-drought and pre-drought growth, and is used to determine the ability of the tree to have pre-disturbance performance levels after drought; and relative resilience is the ratio of resilience:resistance, so that it is the ability of the tree to reach pre-disturbance performance weighed by the damage experienced during the drought (Lloret et al. 2011).

In this study we hypothesized that males and females would respond differently to a past drought period, with males showing higher indices of recovery, resilience, and relative resilience post-drought. Our results showed that sexes did not differ significantly in either performance before, during, or after drought, or for resilience components. Similarly, Sakai and Burris (1985) did not find differences in annual ring width between sexes in field-grown aspen with stem ages of approximately 30 years. In Hynes and Hamann (2020) climatic factors, including CMI, explained 46% of the variance in tree ring growth of white spruce trees, with the remaining variance in response coefficients being attributed to site factors, such as soil characteristics and groundwater access. Hynes and Hamann (2020) also point out that radial increments do not

reflect whole tree growth, as trees may allocate growth to height or roots, depending on environmental resource limitations. Other site conditions and disturbance events could also influence radial growth, such as pest outbreaks and fire, as well as clone-specific characteristics such as physiology and genetics (Lloret et al. 2011). Altogether, components of resilience did not confirm the hypothesis that male and female aspen trees respond differently to drought, which could mean that, in aspen, sexes do not perform differently under drought, or are similarly affected after drought events; or other factors may have offset sex responses to drought, such as site and soil characteristics, or allocation of growth to other parts of the tree, such as roots.

3.5. Conclusion

The region AW2 trembling aspen breeding program was developed with the goal of deploying aspen with genetic gain in height and diameter for wood production (Brouard et al. 2017). The initial selection (in the 1990s) of superior clones was based on the size and health of aspen trees and resulted in a sex ratio of approximately 3:1 males:females (Thomas B., personal communication, 2019), which suggests that phenotypically there was greater growth and health of male aspen clones within the AW2 region. Literature suggests that dioecious tree species show potential sex differential growth performance and different responses to stress (Grant and Mitton 1979; Dawson and Ehleringer 1993; Delph 1999; Hultine et al. 2007; Liu et al. 2021). However, most previous studies focused on sex differences in aspen were performed on seedlings in a greenhouse environment, before reproductive maturity. In addition, most studies focused on sex differential performance under stress conditions were conducted on other dioecious and *Populus* spp., but to the author's knowledge, there are no recent studies on aspen sex differential performance under stress in natural stands.

Overall the results showed a higher female:male (approximately 2:1) sex ratio in the southern portion of the AW2 (Pembina) tree improvement region. The greater proportion of females vs. males on the landscape may be due to a possible advantage in growth and biomass production that females have before reproduction, added to the earlier reproductive maturity of males. In this case, males may start to incur costs of reproduction earlier than females, while females continue to allocate energy to growth. The greater root system growth of females during the non-reproductive stage of stand development could impact the female:male sex ratios even after females reach sexual maturity. Males and females showed similar diameter growth and while males had a a strong trend toward greater wood specific gravity. The lack of sex

differences in diameter growth in mature aspen trees does not agree with the results of early selections of superior clones for the tree improvement program, which found male aspen to have greater diameter growth than females. Our results suggest that the production of aspen seeds may not be more costly than the production of pollen, such that the energy allocation on diameter growth is similar between the two sexes. A potentially higher wood specific gravity in males would mean that males have heavier wood, in addition to greater growth, which are desired traits of superior trees for clonal selection. Furthermore, the potential difference in wood specific gravity between sexes could indicate that female and male aspen trees have different wood anatomy, which could have further implications in performance under different environmental conditions.

The exploration of sex distribution as a function of moisture availability showed that differences in the distribution of the sexes in aspen within the study areas under different climate moisture indexes were not evident. Similarly, tree ring growth of aspen before, during and after a drought event, as well as resilience components did not differ between sexes. Overall, this study did not find different sex responses to varied moisture availabilities during either the historical establishment phase of growth, nor the more recent 20-years of growth or drought. Future studies can explore sex responses of mature aspen trees under drought and optimal conditions, as it is unclear if the sex differences found on other *Populus* spp. could also be found in aspen.

Understanding the patterns of aspen sex distribution, growth, and responses to drought is important to help guide decisions for the deployment of improved materials from the aspen breeding programs that were created in the 1990s in Alberta. Both existing programs (AW1 and AW2) are starting to operationally deploy improved material in these regions. Further studies are needed to investigate if male and female aspen trees respond differently to drought, as Alberta continues to become drier each decade. Such information would be essential for guiding climate-based deployment or programs of assisted migration.

Tables

Table 3-1. Environmental characteristics of study sites and aspen populations sampled including area where site is located, latitude (lat, °N), longitude (long, °W), elevation (m), area of study site (m^2), stand density (trees/ha, measured on 100 m^2 plot and extrapolated to ha), average age (obtained from increment cores and averaged across trees within a stand) and DBH of sampled trees (± standard error, SE), mean maximum temperature (Tmax; °C), mean minimum temperature (Tmin; °C); average annual precipitation (Ppt; mm), potential evapotranspiration (Pet; mm), and climate moisture index (CMI; mm). Climate data were obtained with BioSIM software with the coordinates of each study site (annual data for the climate normal period 1960-1990).

Study site	Area name	Lat (°N)	Long (°W)	Elevation (m)	Area (m^2)	Density (trees/ha)	Age ± SE	DBH ± SE (cm)	Tmax (°C)	Tmin (°C)	Ppt (mm)	Pet (mm)	CMI (mm)
1	Eta Lake	53.27	-115.32	937	2038	2000	68 ± 3	27 ± 1	8.5	-4.5	608.4	450.4	158.0
2	Eta Lake	53.27	-115.32	919	293	2100	55 ± 8	24 ± 1	8.6	-4.5	605.2	455.1	150.1
3	Eta Lake	53.27	-115.33	924	1493	2600	52 ± 1	22 ± 2	8.5	-4.5	606.4	453.8	152.7
4	Dominion	52.67	-114.82	1021	3073	1100	59 ± 2	32 ± 3	8.7	-3.6	571.9	457.3	114.7
5	Dominion	52.66	-114.81	1023	9503	500	87 ± 2	29 ± 1	8.7	-3.6	571.8	457.3	114.5
6	Dominion	52.65	-114.82	1018	1226	2100	86 ± 11	31 ± 2	8.7	-3.6	570.0	459.2	110.7
7	Cathedral	53.00	-115.23	856	7542	500	113 ± 7	37 ± 2	9.3	-3.2	584.4	472.0	112.4
8	Cathedral	53.00	-115.24	872	10496	500	na	41 ± 2	9.3	-3.1	586.1	468.5	117.6
9	Cathedral	53.00	-115.23	864	6726	1100	110 ± 4	39 ± 2	9.3	-3.1	585.0	470.5	114.5
10	Tower	53.54	-115.87	958	2131	800	103 ± 11	35 ± 2	8.3	-5.0	606.0	436.6	169.4
11	Tower	53.53	-115.87	949	6432	1700	75 ± 4	25 ± 1	8.3	-4.9	604.8	438.9	165.9

Study site	Area name	Lat (°N)	Long (°W)	Elevation (m)	Area (m^2)	Density (trees/ha)	Age ± SE	DBH ± SE (cm)	Tmax (°C)	Tmin (°C)	Ppt (mm)	Pet (mm)	CMI (mm)
12	Tower	53.52	-115.87	960	5142	1600	81 ± 4	28 ± 2	8.3	-4.9	607.7	434.7	172.9

Figures

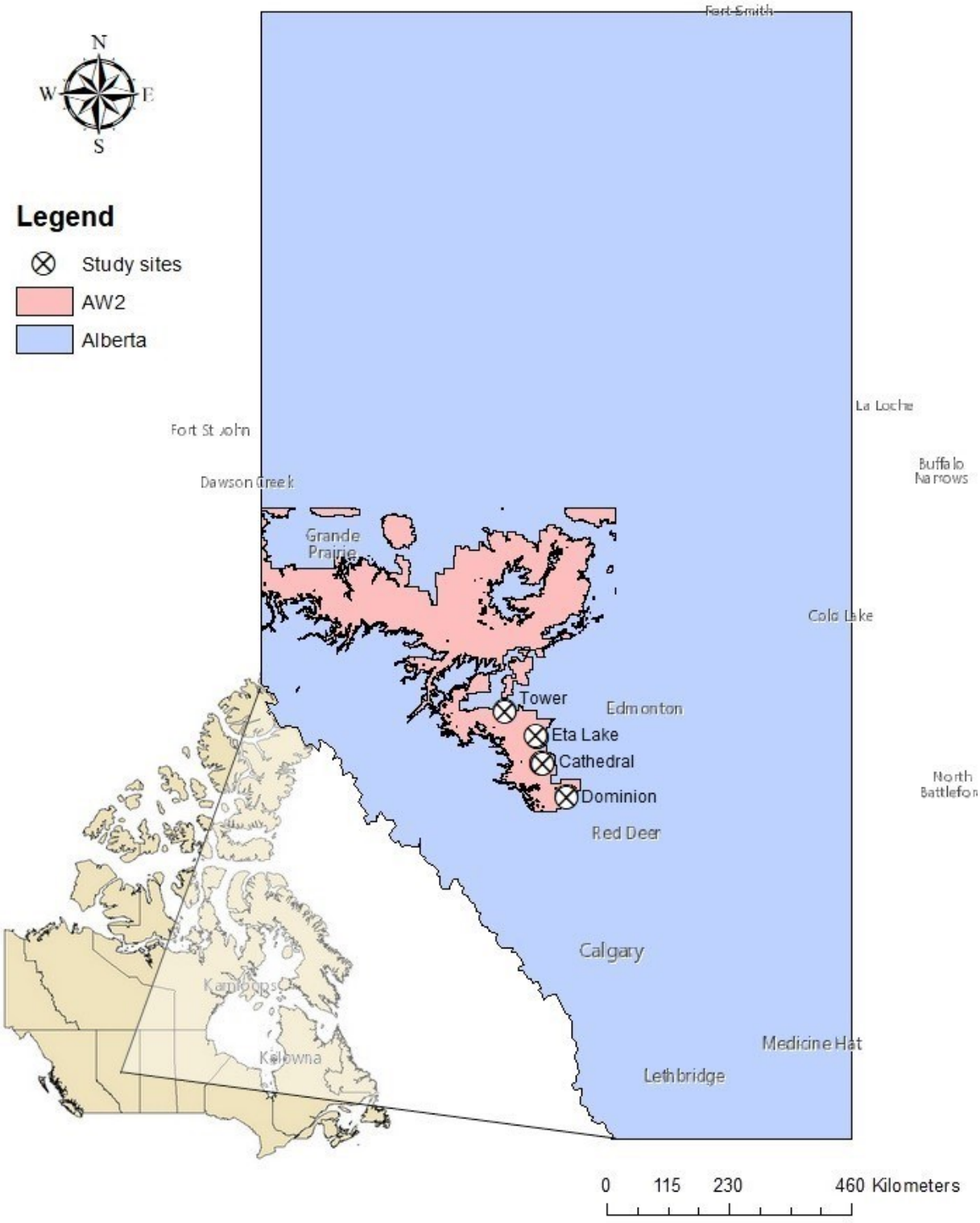

Figure 3-1. Study sites (white circles with black crosses) relative to the AW2 CPP region in Alberta, and the position of Alberta relative to Canada.

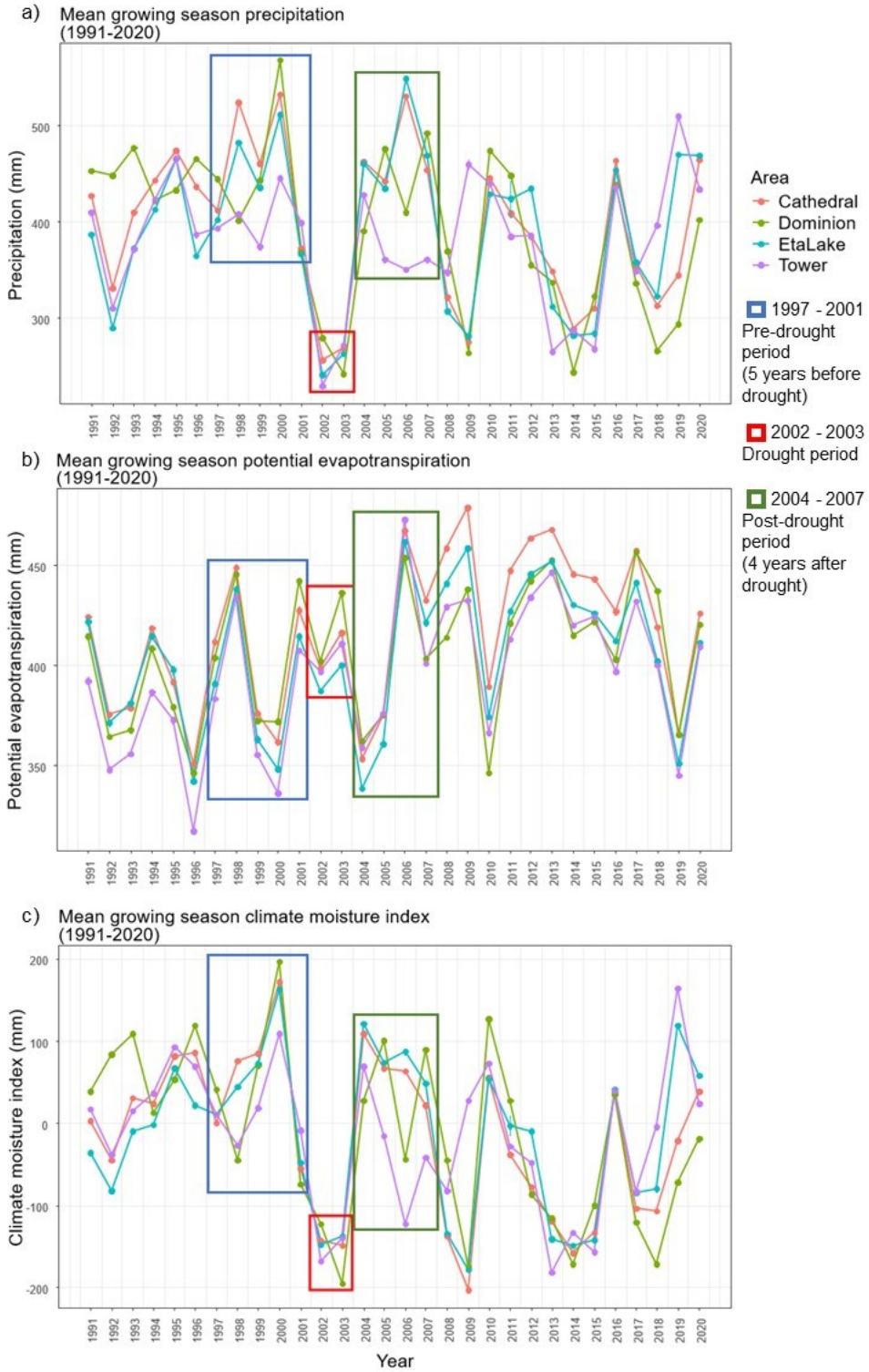

Figure 3-2. Mean growing season a) precipitation, b) potential evapotranspiration, and c) climate moisture index, by area (Cathedral, Dominion, Eta Lake, and Tower) for the climate normal period of 1991-2020. Pre-drought (blue), drought (red), and post-drought (green) periods are highlighted.

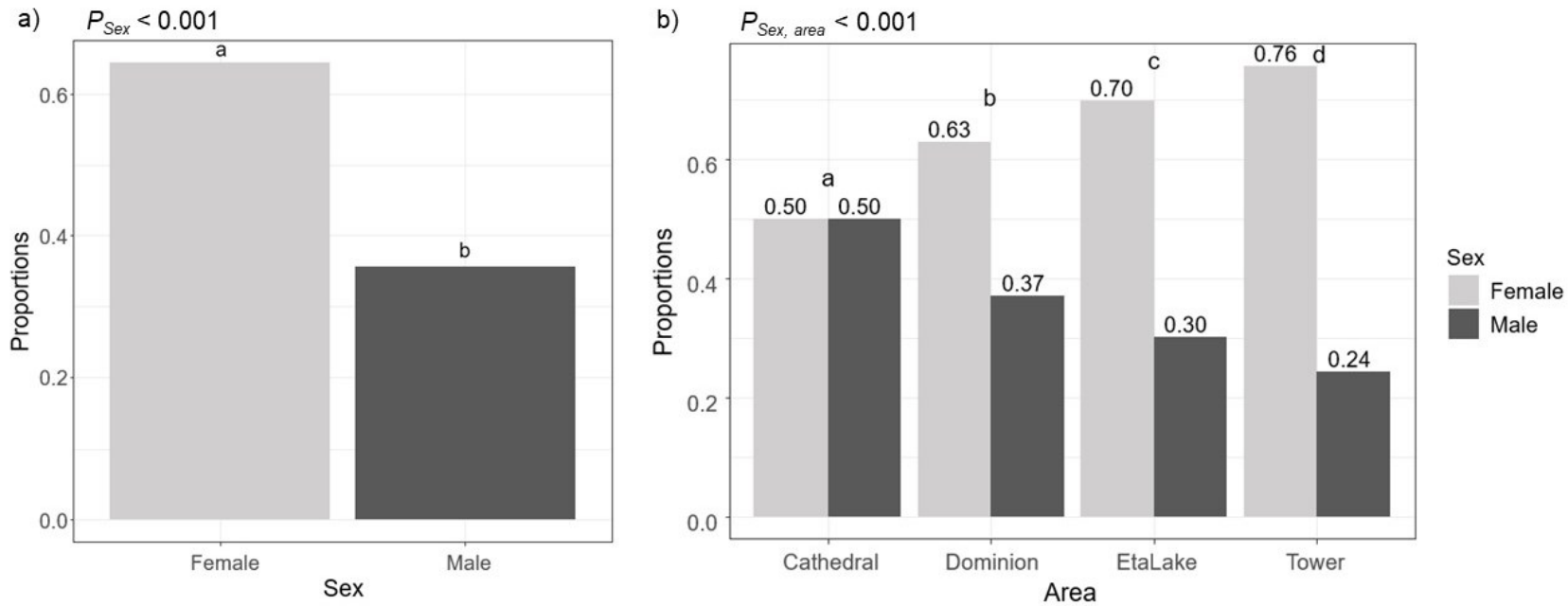

Figure 3-3. Sex proportions of mature aspen populations within the AW2 (Pembina) CPP region: a) total sex proportions, and; b) sex proportions by study area. The *P*-values correspond to results of chi-squared tests on sex and sex and area. Different letters indicate significant differences at $\alpha = 0.05$.

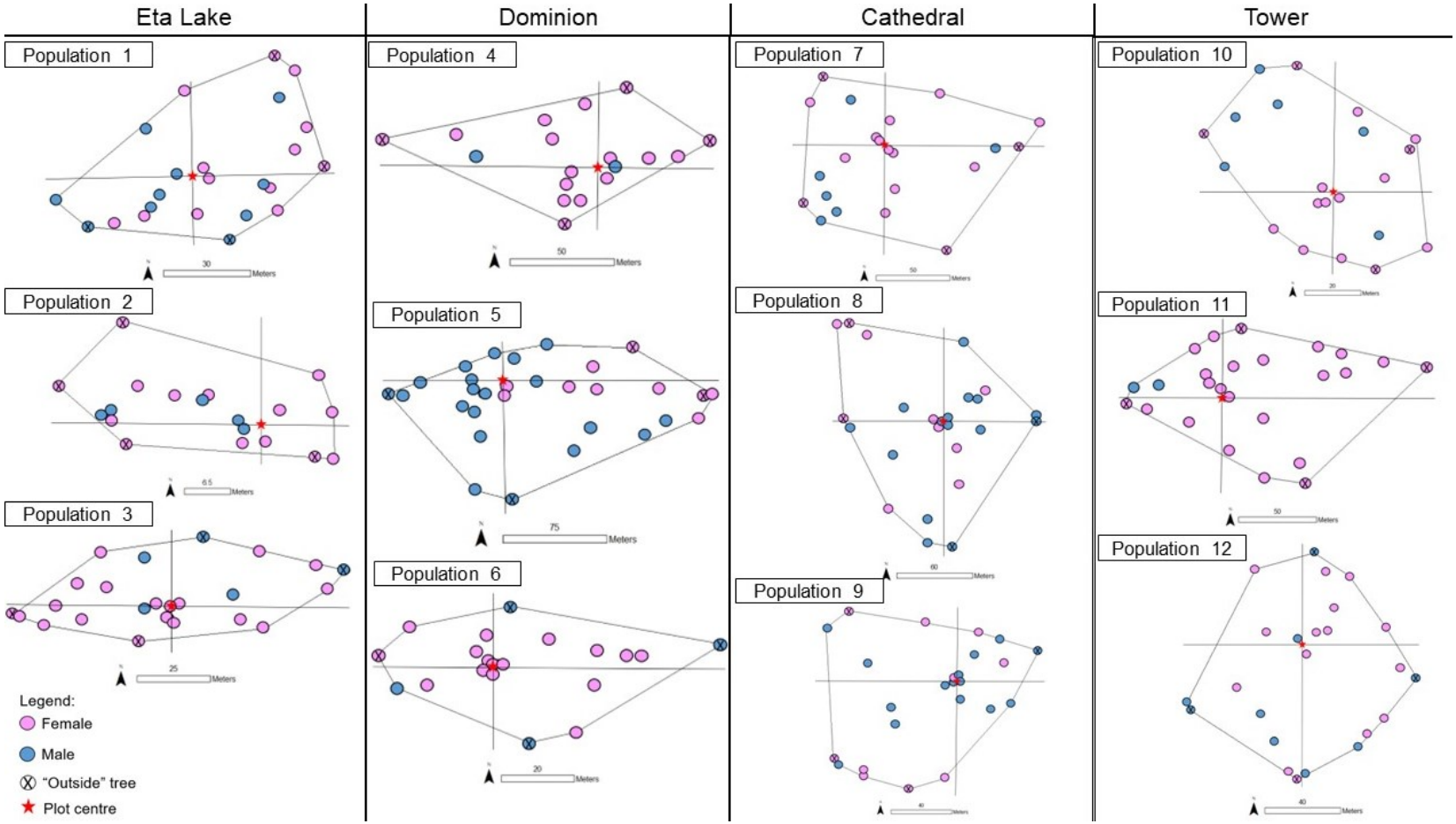

Figure 3-4. Spatial distribution of sexes on each of the 12 studied aspen populations within the AW2 (Pembina) CPP region. Each study area included three populations. Pink circles represent female trees, blue circles represent male trees, circles with a cross on top represent "outside" trees (used to delineate putative clones based on phenotype, described on chapter 4), and the red star represents the referential plot centre.

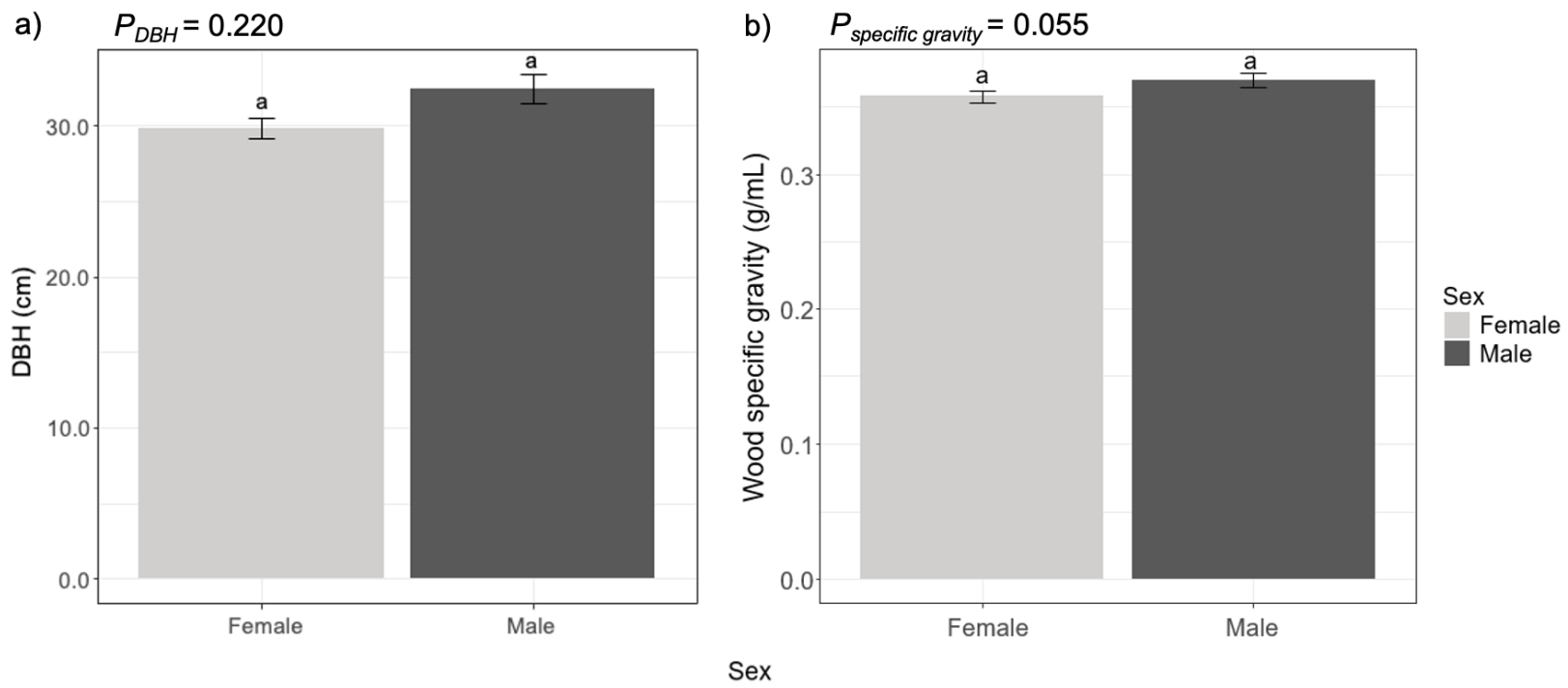

Figure 3-5. Mean ± SE of a) diameter at breast height (DBH, cm), and; b) wood specific gravity (g/mL; c) of mature aspen trees within the AW2 (Pembina) CPP region, in Alberta, Canada. The *P*-values correspond to results of ANOVAs with pooled variances where DBH or wood specific gravity were the response variables, and sex was the explanatory variable. Different letters indicate significant differences at α = 0.05.

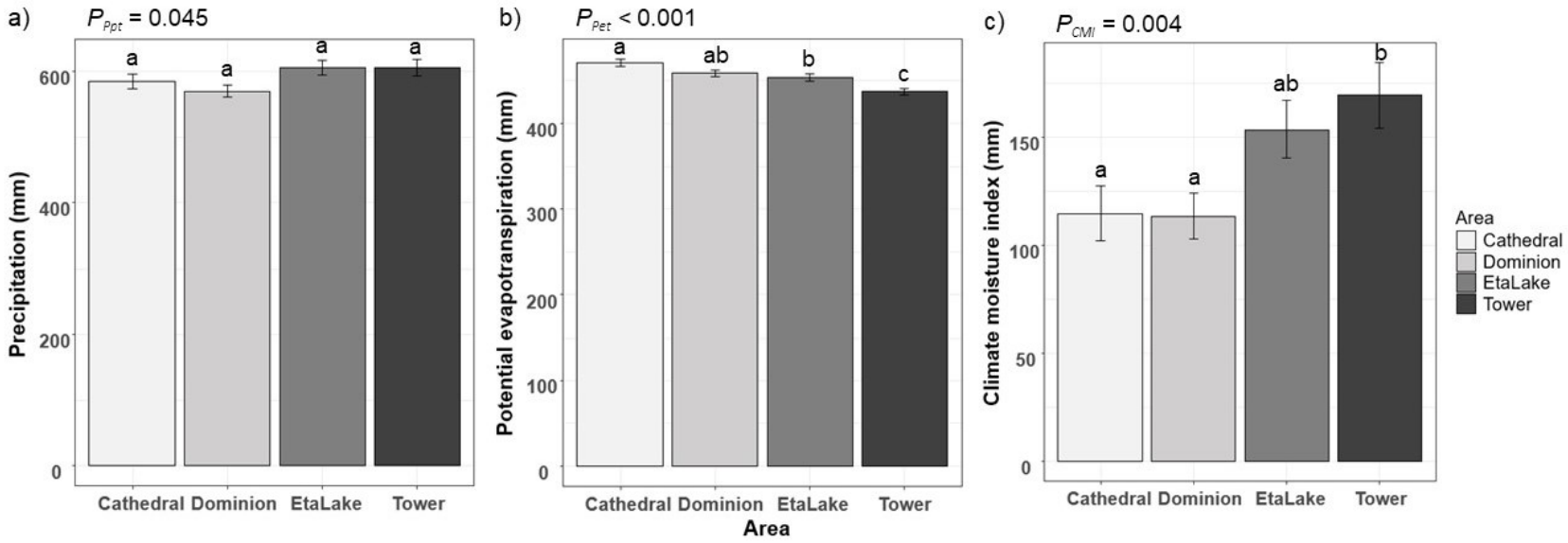

Figure 3-6. Mean annual ± SE of a) precipitation (Ppt, mm); b) potential evapotranspiration (Pet, mm), and; c) climate moisture index (CMI, mm) of study areas within the AW2 (Pembina) CPP region, in Alberta, Canada. The *P*-values correspond to results of ANOVAs where Ppt, Pet, and CMI were the response variables, and area was the explanatory variable. Climate normal period of 1960-1990. Different letters indicate significant differences at α = 0.05.

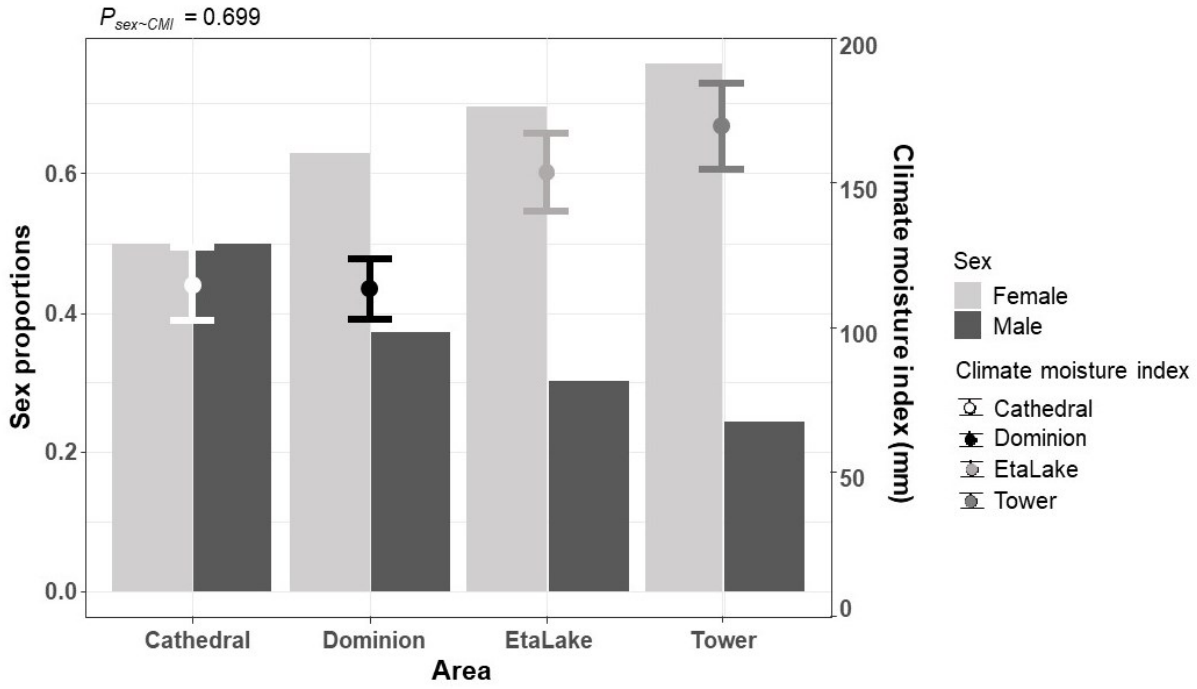

Figure 3-7. Sex proportions (left Y axis) and annual climate moisture index (CMI, mm, right Y axis) of aspen populations in study areas within the AW2 (Pembina) CPP region, in Alberta, Canada. The *P*-value corresponds to results of a generalized linear mixed-effect model where sex was the response variable, and CMI was the explanatory variable. Climate normal period of 1960-1990. Significant differences at α = 0.05.

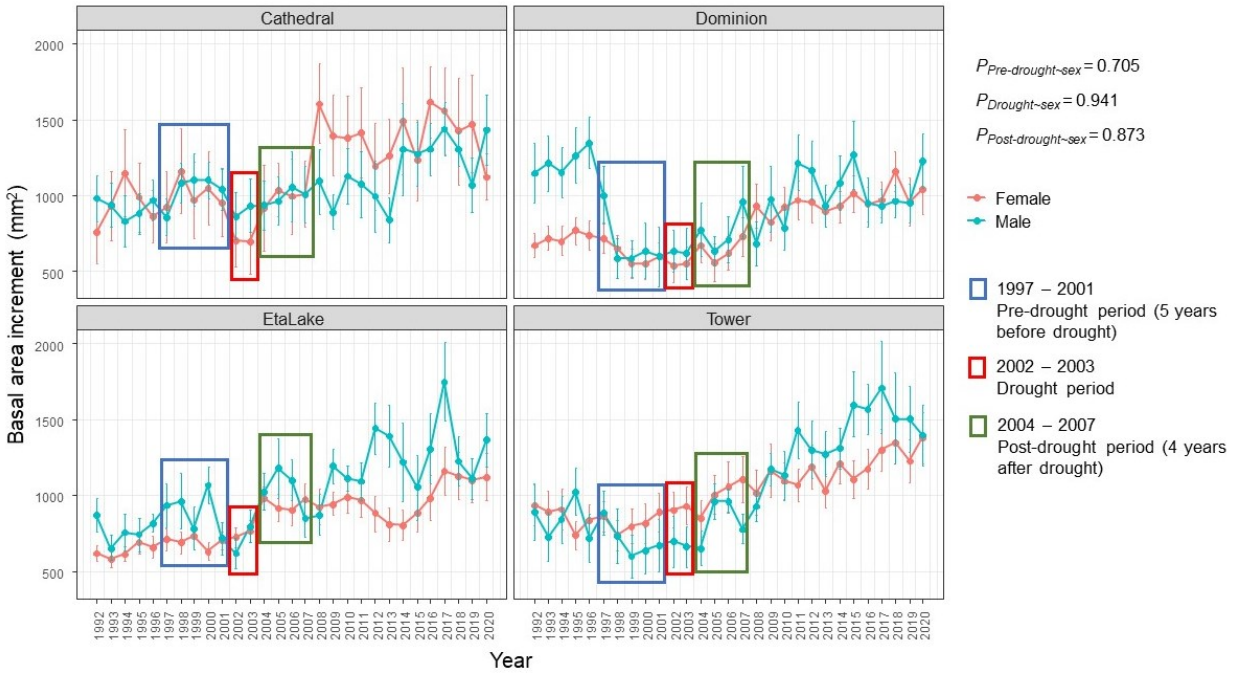

Figure 3-8. Mean ± SE of basal area increments (BAI, mm^2) from 1992 to 2020 with pre-drought (1997-2001, blue), drought (2002-2003, red), and post-drought (2004-2007, green) periods highlighted. Sampled trees are in study areas within the AW2 (Pembina) CPP region, in Alberta, Canada. The *P*-values correspond to results of linear mixed effect models where pre-drought, drought, and post-drought BAI growth were the response variables, and sex was the explanatory variable (stand age was included as a covariate and area as a random effect). Significant differences at $\alpha = 0.05$.

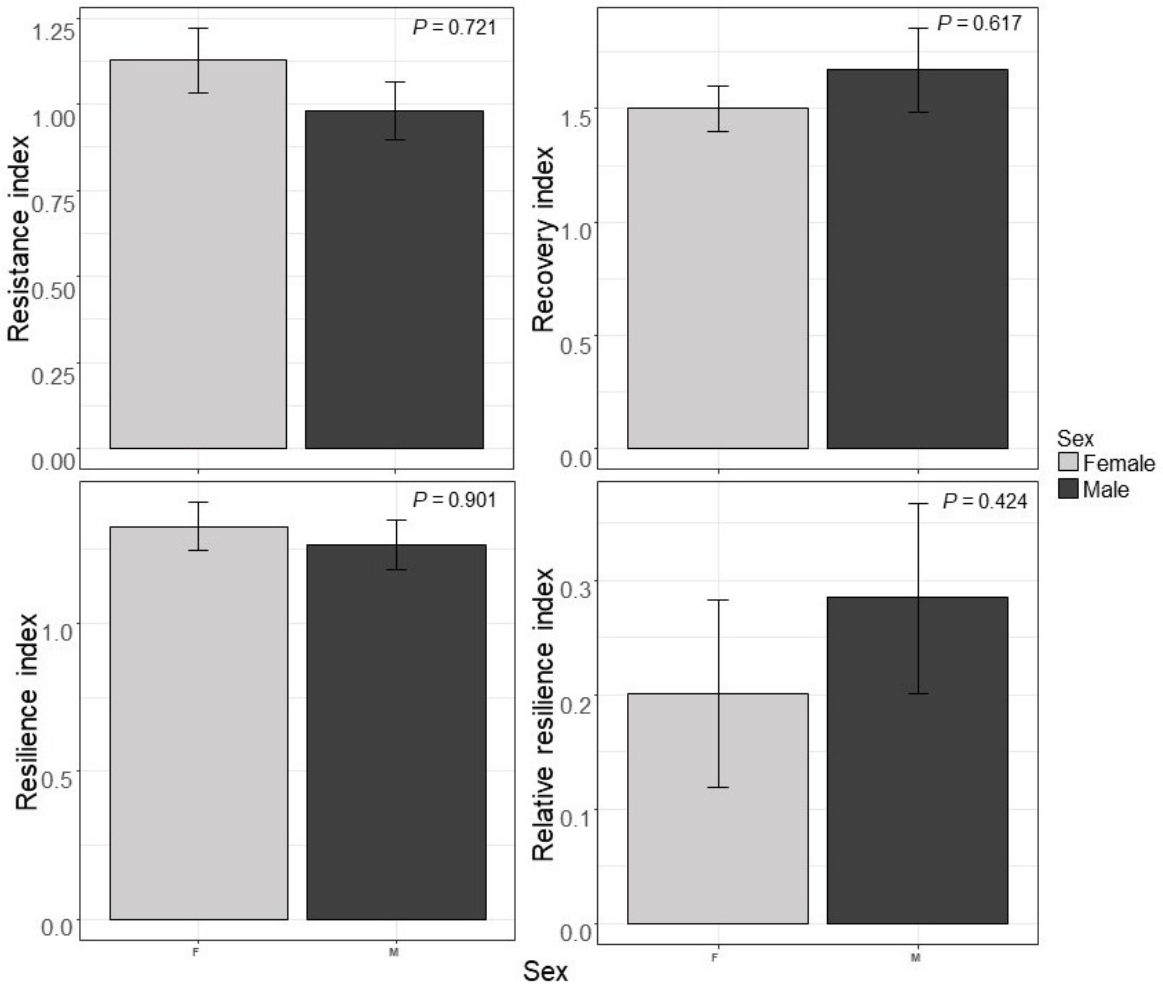

Figure 3-9. Mean ± SE of resistance, recovery, resilience, and relative resilience indexes obtained from sampled trees in study areas within the AW2 (Pembina) CPP region, in Alberta, Canada. The *P*-values correspond to results of linear mixed effect models where resistance, recovery, resilience, and relative resilience indexes were the response variables, and sex was the explanatory variable (stand age was included as a covariate and area as a random effect). Significant differences at $\alpha = 0.05$.

Chapter 4. Genetic variability of mature aspen populations in a tree improvement region in Alberta

4.1. Introduction

The genetic diversity of aspen (*Populus tremuloides* Michx.) stands is more complex than what was believed in the past century and can be influenced by a variety of factors (Jelínková et al. 2009; Latutrie et al. 2019). Initially, an aspen stand can be formed by original seedlings (or ortets), which are established from seed after disturbance (Landhäusser et al. 2019). With time, ortets spread their root systems, subsequently generating ramets (stems with the same genetic material as the original seedling), and those ramets form the original aspen clones in an area (Stout 1929). In sequence, disturbance events might clear patches in the original clones, opening space for new aspen seedlings to become established (Jelinski and Cheliak 1992; Mock et al. 2008). New seedlings (new genotypes) established after disturbance may be outcompeted by root suckers from the pre-established clones, yet some new seedlings may survive to maturity (De Woody et al. 2009). Moreover, these pre-established clones potentially accumulate somatic mutations with time (Mock et al. 2008), and therefore may also appear to be different genotypes (Yeh et al. 1995; Wyman et al. 2003; De Woody et al. 2009). Lastly, root systems of pre-established clones can form connections through natural root grafting even when the aboveground stems have died, which may also have an influence on the genetic diversity of aspen stands, by the emergence of 'new' root suckers from genotypes thought to be extirpated from the stand (Jelínková et al. 2009).

Before the use of molecular genetic tools for aspen clone identification, putative aspen clones were delineated and described based on morphological and phenotypical characteristics, such as time of flowering, flower sex, leaf flushing and senescence, bark texture and colour, stem form and branching habit, and susceptibility to injury (Baker 1921; Barnes 1966; Kemperman and Barnes 1976; Mitton and Grant 1980; Jones and DeByle 1985). However, this method could induce an underestimation of clonal diversity if genetically different clones that have similar phenotypes (Levins 1968) grow in the same area (Wyman et al. 2003).

Furthermore, the same clone could express different phenotypes (plasticity) for characteristics that express themselves differently under varying environmental conditions (Mitton and Grant 1980; Jelínková et al. 2014). Aspen clones in western North America were found to be generally smaller than 0.4 ha (De Woody et al. 2009), as opposed to the several hectares found on 'Pando'; an aspen clone considered to be the largest living organism (De Woody et al. 2008). In western Canada, more specifically in Alberta, large aspen stands tend to be comprised of several smaller clones, and therefore retain relatively high levels of genetic diversity (Yeh et al. 1995; Wyman et al. 2003).

Evidence shows that the distribution of aspen clones and patterns of clone presence on the landscape may be directly influenced by available soil moisture, as seedlings need accessible moisture to become established (Landhäusser et al. 2019, and references therein). Additionally, environmental fluctuations and stress can increase the presence of polyploids as those conditions can stimulate the formation of unreduced gametes (Van de Peer et al. 2017). Aspen triploids can arise from the union of an unreduced male gamete with a reduced female gamete, and tetraploids are formed from the union of an unreduced triploid male gamete and a reduced female gamete (Einspahr and Winton 1977; Mock et al. 2012; Van de Peer et al. 2017). Triploids cannot produce gametes with even-numbered chromosome sets, and therefore their gametes are believed to be infertile or have reduced fertility (Einspahr and Winton 1977; Mock et al. 2008). Previous studies have indicated the possibility of a high proportion of polyploids in natural aspen stands, particularly in larger clones, with a higher frequency of triploids in drought-prone regions (Mock et al. 2008; Mock et al. 2012). Furthermore, triploid aspen clones were found to have greater general vegetative growth (ramet growth and clonal expansion rates) and higher wood density than diploids (Einspahr and Winton 1977; Mock et al. 2008; Mock et al. 2012).

In Alberta, two aspen improvement regions (AW1 and AW2) were delineated for selection and breeding based on genetic variation (Li 1995; Gylander et al. 2012). Still, the genetic variation of those aspen populations was determined from phenotypic assessments of putative aspen clones in the field (Li 1995), as the use of genetic techniques for aspen clonal fingerprinting was still not available. The objectives of this chapter are to: i) describe aspen clonal structure (genotype richness, clonal diversity, clonal evenness, sex, and ploidy) in the AW2 active aspen forest management region in Alberta using phenotypic assessments and microsatellite markers; ii) determine if historical patterns of water availability (precipitation, PET, and CMI) influence the distribution of aspen ploidy; and iii) explore if aspen diploids and polyploids (triploids and tetraploids) differ in growth and wood density, and if they are affected

differently by a past drought event (using basal area increment and resilience components). I hypothesized that: i) mature aspen stands within each area would include patches of small clones interspersed within original larger clones; and microsatellite markers are expected to indicate higher clonal diversity for a site than was previously found using only phenotypic characteristics for putative clone delineation, and sex and ploidy will not vary within a clone; ii) if polyploidy is stimulated by drought, then areas of lower moisture availability will have higher presence of polyploids; and iii) triploid aspen will have greater diameter growth and wood density, and diploids and polyploids will respond differently to a past drought period, with polyploids showing higher indices of recovery, resilience, and relative resilience post-drought.

4.2. Methods

Study area

See Methods in Chapter 3 for the description of the study area.

Plot selection

Within each of the four study areas (Eta Lake, Dominion, Cathedral, and Tower), three mature aspen populations were selected and delineated as putative aspen clones (named study sites 1-12 in Chapter 3), based on phenotypic assessments (bark colour and texture, and branching pattern; Figure 4-1; Table 4-1). Four trees (or outside trees), one on each cardinal direction, were selected and determined to be outside the clone of interest based on phenotypic characteristics. Plot size equalled the putative clone size, with plot limits being delineated by the outside trees, therefore plot size was not uniform for all stands. The FGRMS (2016) defines that seed lot collections of aspen must be separated by a minimum of 500 m from the edge of aspen patches or clones. Mitton and Grant (1980), for a study in Boulder, Colorado, considered aspen clones to be different where tree stands were at least 400 m apart, without aspen stems along the way, or separated by ecological barriers. In this study, putative clones were at least 500 m apart from each other, following policy statements described on the FGRMS (2016). To map out the extent of a clone, in case the clone was larger than the limits pre-established from phenotypic assessments, transects were placed between putative clones in Eta Lake, Dominion and Tower. DNA was extracted from trees along those transects. Transect locations were as follows: transect (TR) 1 (600 m) connected C1 and C2; TR2 (630 m) connected C2 and C3; TR3 (770 m) connected C4 and C5; TR4 (1000 m) connected C5 and C6; TR5 (660 m) connected C10 and C11; TR6 (650 m) connected C11 and C12.

Sampling

See Chapter 3 for details.

DNA analysis: extraction

See Chapter 3 for details.

Sex screening

See Chapter 2 for details.

DNA analysis: microsatellite and genotyping

Microsatellites are sequences of DNA bases that are repeated several times throughout the genome; they serve as good genetic markers due to their instability (Vieira et al. 2016). Microsatellites present high mutation rates per cell generation, this way, different genotypes are identified based on variation in repeat number of base pairs (Vieira et al. 2016).

DNA was amplified using a Qiagen Type-it Microsatellite PCR reaction (Qiagen Cat#206241) and seven primers (Latutrie et al. 2015; Table 4-2). Dayanandan et al. (1998), identified the loci PTR1, PTR2, PTR3, and PTR4, which can uniquely identify 89% of aspen individuals. PTR1, PTR2, PTR3, and PTR4 are under one nucleotide locus, and Rahman et al. (2000) identified PTR5, PTR6, PTR7, PTR8, and PTR14, to be polymorphic. The four microsatellites under single nuclear locus control (PTR1, PTR2, PTR3, and PTR4), used in addition to one polymorphic microsatellite (PTR8), could identify all unique genotypes from Dayanandan et al. (1998) and Rahman et al. (2000). The previously mentioned microsatellite DNA markers can be used for clonal identification of *P. tremuloides* (Dayanandan et al. 1998; Rahman et al. 2000; Latutrie et al. 2015; Latutrie et al. 2019). For each reaction 12.5 µL 2X type-it multiplex PCR master mix, 2.5 µL of each 2 µM primer, 5 µL DNA template, and 2.5 µL water were made. Each PCR primer set was processed independently using the following program: 95°C, 5 min; 95°C, (30 sec; 60°C, 90 sec; 72°C, 30 sec) x 28; 60°C, 30 min. PCR products were wrapped in foil and stored at -20°C. 5 µL of PTR1, PTR2, PTR3, and PTR4 were combined into one plate; and 7 µL of PTR6, PTR14, and WPMS16 were combined in a second plate.

The PCR fragments were sized at the University of British Columbia (UBC) using Sanger sequencing microsatellite analysis, and the sizing files were analyzed at InnoTech Alberta to determine fragment sizes. Alleles were called using the Thermofisher Connect microsatellite

analysis suite. Different alleles of a microsatellite marker are recognized from the molecular mass of the amplified fragment, the resulting peaks correspond to each amplified allele (Vieira et al. 2016). A Lynch distance matrix of those results was created using the polysat 1.7-4 (Clark and Drauch Schreier 2017) R-package (R Core Team 2023), which allowed for potential polyploid distance calculations. Lynch (1990) described a measure of similarity between DNA fingerprints, where 2 times the similar bands between two genotypes are divided by the total number of bands of both genotypes. The Lynch distance returns a dissimilarity where: dissimilarity = 1 – similarity (Clark and Jasieniuk 2011). This distance matrix was then imported into MEGA11 (Tamura, Stecher, and Kumar 2021) to create the neighbour-joining phylogenetic tree for the individuals.

The primer set PTR4 did not amplify well, and most of the peak calls for it were of poor quality. This is likely due to the substitution of the PET fluorophore for Cyanine3. PET is a proprietary dye used by ABI systems; when the primers were ordered ABI had just been taken over by Thermofisher and custom PET primers were not available at that time; so, a substitute fluorophore was chosen and ordered through Sigma instead. Overall, the allele calls from the PTR4 primer set are suspect, so a second set of distance matrixes was created, and a tree was produced based on that. Apart from the PTR4 reactions, only 15 samples showed failed reactions over all the PCRs, most of the failures were a failure to read a single microsatellite (PCR set), so the remaining microsatellites could be used to genotype the sample.

Phylogeny

The analysis of the aspen genotyping data indicated several potential triploid individuals as well as one primer set (PTR4) which did not work. A phylogenetic tree was made considering polyploidy, using the distance matrix of the R polysat 1.7-4 package (Clark and Drauch Schreier 2017), and the Lynch distance matrix was imported into MEGA11 (Tamura, Stecher, and Kumar 2021) to create a neighbour-joining tree. This did not consider any information about the known population or sampling structure, and consequently, the tree did not show any clear clusters. Since observations of the previous neighbour joining trees showed no clear clusters that matched the known population structures, allele calls were redone based on a diploid only assumption to access the tools for more in-depth creation of phylogenetic trees that take known population groupings into account. The diploid allele calls were converted from fragment length to repeat number using the R polysat 1.7-4 package (Clark and Drauch Schreier 2017) and exported to PoptreeW (Takezaki et al. 2014) to create trees that accounted for the sampling population structures.

PoptreeW creates phylogenetic trees from allele frequency data with the use of the neighbour-joining (NJ) method and the unweighted pair-group method with arithmetic mean (UPGMA; Takezaki et al. 2014). A UPGMA tree with a sample size bias corrected F_{ST} distance (Latter 1972) and bootstrapping of 1,000,000 was used to create the overall population tree. UPGMA, with a $(\delta\mu)^2$ distance (aka Dmyu; Goldstein et al. 1995) and bootstrapping of 100,000 was used to create each of the trees showing individual relationships.

The UPGMA tree with sample size bias corrected F_{ST} distance showed the best correspondence to the known populations. F_{ST} is a distance measure that measures genetic differentiation between populations and is a number between 0 and 1, with 0 indicating genetically identical populations. Because the different populations are genetically similar, phylogenetic trees were made for each group to show the relationships at the individual level. The $(\delta\mu)^2$ distance was used to assign individual relationships. This distance was designed specifically for microsatellites, it accounts for mutation patterns and does not rely on multiple individuals being assigned to a population to initiate the tree construction. The specialized microsatellite distance calculation does not perform as well as other distance calculations in building the trees for populations but will indicate the relationship between individuals. Bootstrapping of UPGMA trees pairs the members with the smallest distance between them into a cluster and repeats that process until a rooted tree is created. Bootstrapping is a technique in which many trees are created using resampled loci as starting points, and the percent branch (the grouping of the taxa separated by the branch) occurrences are calculated and noted with each branch. Figure 4-2 includes a phylogenetic tree obtained with this chapter's data and a description of how to interpret the tree. Appendix 4 includes phylogenetic trees obtained for each putative clone with and without outside trees and connecting transects.

When describing clonal structure, genotypes belonging to the same clone might be described as unique genotypes if the organism developed somatic mutations, this would lead to the overestimation of clones in a population (Arnaud-Haond et al. 2007). This overestimation can be avoided by defining a threshold of genetic distance in which very low genetic distances (below the threshold) cannot be considered as unique genotypes (Arnaud-Haond et al. 2007). In aspen, a ramet might have zero to one mutation, while clones can have more than one somatic mutation (Ally et al. 2008). In the first round of analysis, individuals were considered as belonging to the same clone if the total genetic distances between them were equal to zero. A second round of analysis accounted for possible somatic mutations, considering the threshold of two $(\delta\mu)^2$ distances, also equivalent to two genetic mutations, as the cut-off for considering genotypes as belonging to the same clone. A threshold of two mutations means that ramets in a

clone might differ by up to two alleles at up to two loci, but still share the same allele frequency at all other loci (Ally et al. 2008).

DNA analysis: microsatellite and polyploidy

From the microsatellite analyses, several samples seemed to have more than two microsatellite peaks, indicating a non-diploid organism (Clark and Jasieniuk 2011). Individuals were designated as triploids or tetraploids if they had three or four alleles at any locus and peak height above 100 rfu (relative fluorescence units), which was not designated a stutter peak via the applied biosystems microsatellite analysis module, available through Fisher Scientific. Peaks were also examined visually.

Samples were reanalyzed as having a maximum of four alleles, which indicated polyploidy. This reanalysis produced better results than the initial diploid analysis, and all the data provided were set to show three or four alleles per loci. Distance matrixes used for the creation of the neighbour-joining tree were adjusted for the various ploidies found in each sample.

Triploidy has been reported in aspen (Mock et al. 2012; Goessen et al. 2022), so this result is not unexpected for the population. Tetraploidy can occur in trees that were crossed by laboratory methods (Winton and Einspahr 1970), and it has been seen in natural populations (Jones 1985). Tetraploidy occurs during self-fertilization or indicates duplication of the genetic region that the microsatellite is in (Van de Peer et al. 2017). Polyploidy in aspen may be due to drought stress (Greer 2018). Since not all the primer sets show the same number of alleles and there is an inconsistency between the microsatellites for the number of alleles detected this suggests gene duplication or polysomy occurring in specific chromosomes rather than genome-wide duplication (polyploidy) or sample contamination.

Cytology

Cytology was performed to confirm the potential polyploidy of the studied trees. In May 2021 root segments of 1 to 7 cm in diameter were collected in the field from 12 trees: four putative diploids, triploids, and tetraploids (based on microsatellite genotypes). Two to five root segments of 30 to 100 cm were collected from each tree in two different study sites (C1 and C3). Collected roots were placed in labelled large bags, kept in coolers containing ice packs and refrigerated until propagation.

For propagation of root suckers in the greenhouse, in June 2021 field-collected root segments (two to three segments per tree, 30 to 50 cm in length, of about two to three

centimetres in diameter) were washed with a soft brush, laid out on flat trays, and covered with an artificial soil mixture (Sunshine Mix #4; SunGro Horticulture, Vancouver, BC, Canada; Ingredients: Canadian Sphagnum Peat Moss, RESiLIENCE®, Perlite, Dolomite Lime, Wetting Agent). Root suckers were grown in a greenhouse at the University of Alberta, Edmonton, AB, Canada under an approximate 18 h photoperiod (6 am - 12 am), in which natural light was supplemented with artificial lights. The artificial lights provided a photosynthetic photon flux density ranging between 200 and 300 $\mu mol.m^{-2}s^{-1}$ at pot level. Humidity and temperature were not monitored, but suckers were sprayed with water mist and kept covered to maintain the relative humidity high. Root segments were watered daily. Two to three weeks after potting, shoots started emerging from the root segments (root sprouts). When suckers reached 3 to 5 cm in height (approximately one week after sprouting), shoots were cut, dipped in rooting hormone (Stim-Root #3, 0.8% IBA rooting powder, Master Plant-Prod Inc.), and transferred to seed starters containing artificial soil mixture (Sunshine Mix #4). Trays containing the potted shoots were covered to shade reduce temperature and avoid water loss and sprayed with water mist to maintain relative humidity high. After approximately two weeks, shoots were rooted, and tray covers were removed.

Following the protocol provided by Dr. M. Nurul Islam-Faridi (Mock et al. 2012; personal communication, 2021) for the preparation of chromosome spreads, rooted shoots (or seedlings) were watered the night before. Actively growing root tips (about 1 cm long) were removed with a scalpel (disinfected with 70% alcohol), washed with distilled water, and pretreated with 1 ml of 0.8% Bromonaphtalene (800 ul/99.2 ml DDH_2O) for 2h in the dark at room temperature. Subsequently, roots were washed with distilled water and fixed in 4:1 (4 ml ethanol to 1 ml glacial acetic acid) solution (10 min with a change of solution halfway). After fixing, roots were added to 1 ml of 0.2 NHCl hydrolyzation (16.36 ml concentrated HCl in 1000 ml DDH_2O) and placed in the oven at 60°C for 5 min, then they were rinsed, and 0.01M cold (4°C) citrate buffer (1.47 g trisodium citrate-dihydrate, 1.05 g citric acid -monohydrate, and 400 ml DDH_2O, pH 4.5 then adjust volume to 500 ml) was added for 10 minutes with a fresh change halfway. Finally, the meristematic portion of the root tips was cut off to be processed enzymatically. Root tips were placed in an enzyme digestion solution (5% Onozuka R-10, 1% Pectolyase Y-23, 10 ml of 0.01M citrate buffer), then placed on a float in a warm bath at 37°C for 20 to 40 minutes, depending on root thickness. Next, root tips were rinsed with 0.01M citrate buffer first, then with distilled water and kept in distilled water for 10 minutes, before slide preparation. One single root tip was used per slide, tissue was spread thin over slide surface, any chunks or debris were removed. The slide preparation was then stained with 0.2% Azure Blue (Sigma; 200 ul/99.8 ml

DDH_2O) for 40 to 60 minutes and made permanent with a drop of Permount (Fisher Scientific™, S70104) and a coverslip.

Chromosome spreads were viewed under 63X (ZEISS AXIO A1 Compound Light Microscope with SeBaCam 5.1MP Camera) and 100X (ZEISS Axio Imager M2, Axiocam 105 Colour Camera and Axiocam 506 Monochrome Camera). Digital images were recorded and processed using ZEN 2 (blue edition; Carl Zeiss Microscopy GmbH). Multiple spreads from each of the 12 genotypes were prepared and examined.

In *Populus* spp. (Xin et al. 2020; Zhong et al. 2022) and more specifically *P. tremuloides*, Chromosome 1 is the largest of the genome (Mock et al. 2012). In this study, Chromosome 1 was used as a reference to determine if an individual was diploid, triploid, or tetraploid. Ploidy was assigned based on the number of Chromosome 1's found in several cells of an individual undergoing mitosis and then compared to the ploidy obtained from microsatellite analyses. Spreads were interpreted by an observer blind to the microsatellite genotypes. All ploidies matched the ploidies assigned based on cytology (Table 4-3). Still, caution must be taken as per the interpretation of the tetraploid samples. Aspen chromosomes are very small and difficult to see under the microscope and, in some situations, one same cell could look like a diploid in anaphase, or a tetraploid in prophase, for example. Cells on metaphase were easier to interpret, but difficult to find. Figure 4-3 includes a selection of images obtained for assigned diploid, triploid, and tetraploid individuals.

Spatial distribution of clones and clonal structure

The position of each sampled tree within a population and transect was extracted using GPS coordinates on the ArcMap (ESRI, Redlands, CA) software. After obtaining the results from clonal fingerprinting, sex screening, and ploidy, each tree was assigned its specific clonal, sex, and ploidy information, which was reflected on the maps of spatial distribution of trees within populations (Figure 4-4). All the figures including spatial distribution and clonal structure of studied aspen populations within the AW2 region, can be found in Appendix 5.

To investigate if the same genotypes or clones extended beyond the spatial area of the study populations, transects were placed connecting the populations. The clonal fingerprinting of trees on those transects was assigned to each tree and reflected on maps of spatial distribution of transects within each area (Eta Lake, Dominion, and Tower).

Climate data

See Chapter 3 for details. To determine if historical patterns of water availability (precipitation, PET, and CMI) influenced the distribution of trees with different ploidy the climate normal period of 1960-1990 was used. And the climate normal period of 1991-2020 was used to explore if aspen diploids and polyploids (triploids and tetraploids) are affected differently by a past drought event (using basal area increment and resilience components).

Increment core sampling and processing

See Chapter 3 for details.

Wood specific gravity

See Chapter 3 for details.

Drilling resistance

See Chapter 3 for details.

Basal area increment and resilience components

See Chapter 3 for details.

Selection of drought period

See Chapter 3 for details.

Data analyses

In order to analyze potential differences between areas, three study sites were nested within each area, with areas considered as blocks. Data were analyzed in R 4.3.0 (R Core Team 2023). A significance level of $\alpha < 0.05$ was used. Model assumptions of homogeneity of variances and normality were verified, and data transformations were applied when needed. The variables resistance, recovery, resilience, and relative resilience were log-transformed for data analyses.

Clonal structure was described for the aspen populations with the use of two thresholds of genetic distance: zero and two $(\delta\mu)^2$ distances.

To explore patterns associated with ploidy, all analyses were performed under two assumptions. The first assumption was that individuals were diploids or triploids, in this case, tetraploid samples were assumed to be diploid trees that had somatic mutations; this approach accounts for the possibility that what appears as polyploidy are mutations in the somatic cells of

diploid organisms, caused by cell fusion or abortion of cell division during cytokinesis (Van de Peer et al. 2017). The second assumption was that observed tetraploid trees were originated by unreduced gametes or whole genome duplication, as tetraploids of aspen have been reported to occur naturally or to be produced for greater growth (Winton and Einspahr 1970; Einspahr and Winton 1977) and whole genome duplication of diploids could be induced under drought (Van de Peer et al. 2017).

Patterns of clonal structure and diversity

Clonal diversity of the study aspen populations was described using the following components of diversity standardized for populations of clonal species as described by Arnaud-Haond et al. (2007):

Dorken and Eckert's Richness (*R*, Dorken and Eckert 2001, as cited in Arnaud-Haond et al. 2007), hereafter genotype richness, is the proportion of distinct genotypes present in a population relative to the total number of sampled trees:

$$R = \frac{(\mathrm{G} - 1)}{(\mathrm{N} - 1)}$$

Eq. (1)

Where G is the total number of genotypes and N is the sample size. Genotype richness ranges from 0 (for a population comprised of one single clone) to 1 (for a population including only unique genotypes; Arnaud-Haond et al. 2007).

Shannon-Wiener's diversity (*H*, Wiener 1948, Shannon and Weaver 1949, as cited in Arnaud-Haond et al. 2007), hereafter clonal diversity, which is characterized by relative abundance of individuals of a same clone in the population:

$$H = -\sum_{i=1}^{Gpop} \frac{ni}{N} log \frac{ni}{N}$$

Eq. (2)

Where G_{pop} is the total number of multi-ramet clones found in the population, n_i is the number of sampled trees within a multi-ramet clone, and N is the sample size. The diversity index increases where the number of multi-ramet clones and the evenness in the assignment of individuals to a clone is higher (Arnaud-Haond et al. 2007). This index can be interpreted as the estimated probability that two randomly selected trees in a population would belong to the same clone (Arnaud-Haond et al. 2007).

Pielou's evenness (*J,* Pielou 1975, as cited in Arnaud-Haond et al. 2007), hereafter clonal evenness, describes the uniformity of distribution of ramets among different clones.

$$J = \frac{H}{Hmax}$$

Eq. (3)

Where H is Shannon-Wiener's diversity and H_{max} = logG. This index varies from 0 to 1, with 1 representing equal abundance of individuals within each clone.

Latutrie et al. (2019) defined three clone size classes on even-aged mature (older than 50 years) aspen-dominated stands based on genotype richness (*R*): U, unique clones, MS, mostly small clones, and FL, few large clones. This study includes a modificaiton of the method applied by Latutrie et al. (2019), with the following parameters to describe the presence of an aspen clone: U, only unique clones where *R* = 1; MS, mostly small clones, where *R* ranges from 0.40 to 0.99; and FL, few large clones where *R* is below 0.39.

Descriptive statistics were used to calculate the mean proportional occurrence of single-ramet genotypes and multi-ramet clones (clone presence in number of ramets per clone).

The R package corrplot (Wei and Simko 2021) was used to investigate correlations between multi-ramet clones, single-ramet genotypes, unique genotypes, genotype richness, clonal diversity, clonal evenness, differences in DNA and sex and ploidy, the proportion of males and females, proportion of diploids, triploids, and tetraploids, and stand age.

Distribution of aspen ploidy and patterns of water availability

To test if there is an association between the distribution of ploidies among areas, a chi-squared test of independence was applied. A Bonferroni-adjusted pairwise nominal independence test (Mangiafico 2016) was applied as a post hoc test to explore the differences in ploidy distributions between areas.

Differences in climate between the four areas for the normal period of 1960-1990 were tested previously in Chapter 3. To test if ploidy distribution was influenced by climate moisture availability, two approaches were applied. When analyzing ploidy as a two-level response variable (diploid and triploid), a generalized linear mixed-effect model fit by maximum likelihood (Laplace approximation) was used to test if ploidy distribution was influenced by CMI, where ploidy (binary, coded as 0 for diploid and 1 for triploid) was the response variable, CMI (mm) was the explanatory variable, and area (Eta Lake, Dominion, Tower, and Cathedral) was included as a random effect. The glmer() function on the lme4 package (Bates et al. 2015) was used to fit a logistic regression model, with the 'family' argument included as "binomial". When analyzing ploidy as a three-level response variable (diploid, triploid, and tetraploid), a mixed-effects multinomial logistic regression model was used, where ploidy was included as dummy

variables using the 'within' and 'cbind' functions in R to specify the outcome as a matrix of counts for each level, CMI (mm) was the explanatory variable, and area (Eta Lake, Dominion, Tower, and Cathedral) was included as a random effect. The glmer() function on the lme4 package (Bates et al. 2015) was used to fit a multinomial logistic regression model, with the 'family' argument included as "binomial".

Growth, wood density, and drought responses (basal area increment and resilience components)

Analyses of variances (ANOVA) with pooled variance were used to explore if diameter growth and wood resistance (wood specific gravity) differed on trees of different ploidies. DBH (cm) and wood specific gravity (g/mL) were the response variables and ploidy was the explanatory variable.

Linear mixed-effect models were applied to test if there were differences in resistance, recovery, resilience, and relative resilience indexes in trees from different ploidies. The pre-drought (1997 to 2001), drought (2002 and 2003) and post-drought (2004 to 2007) periods used to calculate the resilience components are described in Chapter 3. The lmer function on the lme4 package (Bates et al. 2015) was used to fit the linear models. Resistance, recovery, resilience, and relative resilience were included as response variables, ploidy was included as an explanatory variable, stand age was included as a covariate, and area was included as a random effect. Tukey-adjusted contrasts were performed with the 'glht' function on the multcomp package (Hothorn et al. 2008) to obtain two-way comparisons between ploidies.

4.3. Results

Patterns of clonal structure and diversity

The results of clonal structure including two cut-offs for determining when individuals belong to the same clone (zero and two mutations) are presented in Table 4-4. The sampled aspen populations were first delineated as putative aspen clones, based on phenotype, yet all populations contained a diversity of genotypes. A total of 230 trees were genotyped and had genetic distances successfully calculated, from those 194, unique genotypes were found when considering only identical phylogenetic distances as clones, that is 84% unique genotypes and 16% multi-ramet clones (MRCs), while 116 unique genotypes were found when considering

trees with DNA that differed by two mutations as belonging to the same clone, that is 50% of the samples being unique genotypes and 50% MRCs.

Using the threshold of zero mutations or identical $(\delta\mu)^2$ distances to determine clones, almost all populations were grouped as having mostly small clones, while one population (C11) had only unique clones. Clonal diversity (*H*), or diversity of MRCs, was low, in which the chances of two randomly selected trees in a population belonging to the same clone were as low as 0% for C11, with the highest probability of 39% at C2. Clonal evenness (*J*) was also low, ranging from 0 to 0.04, for all sampled populations. *H* and *J* were highly positively correlated (r^2 = 0.96, $P < 0.001$; Figure 4-5). The average percentage of single-ramet genotypes (SRG) was 75%, with lowest percentage at C2, 47%, and highest at C11, 100%. Presence of SRGs was negatively correlated with *J* (r^2 = -0.89, $P < 0.001$) and *H* (r^2 = -0.81, $P < 0.001$), and positively correlated with genotype richness (*R*, r^2 = 0.76, $P = 0.004$) and stand age (r^2 = 0.58, $P = 0.04$; Figure 4-6). The number of ramets per clone on MRCs ranged from 0 to 7, with an average clone presence of 2.33 ramets per clone. From the 57 ramets included in MRCs, 48 were screened with the same sex, resulting in 84% match of DNA identification and sex, while 9 ramets were screened as a different sex than the majority of ramets within an assigned clone, in total 16% of ramets did not match DNA and sex. Ploidy showed similar results, with 46 out of 57 ramets identified as the same DNA and same ploidy (81%), and 11 out of 57 showing different ploidy from the clone (19%). The clonal fingerprinting of trees along transects connecting populations in Eta Lake, Dominion and Tower did not identify genotypes exceeding the area of study populations or the same genotypes in different populations within an area (Figure 4-7).

When considering two mutations, or $(\delta\mu)^2$ distances, as a threshold to determine when individuals belong to the same clone, eight populations were characterized as containing mostly small clones, four populations contained few large clones, and zero of the study populations contained only unique clones. *H* was high, with chances of randomly selecting two trees from a same clone in a population being lowest at C3, 37%, and highest at C4, 71%. *J* remained low, ranging from 0.03 to 0.1. *H* and *J* were not significantly correlated, yet *J* was higher on a few large clones (Figure 4-8), this relationship is also noted by the significant correlation between *J* and *R* (r^2 = -0.85, $P < 0.001$; Figure 4-9), which is the parameter used to calculate clone presence classes. Average percentage of SRGs was 26%, with the lowest at 0% in C5, where all trees sampled belonged to a clone. The highest average of SRGs occurred at C11, 43%. Presence of SRGs was negatively correlated *J* (r^2 = -0.97, $P < 0.001$) and positively correlated with *R* (r^2 = 0.87, $P < 0.001$). Clone presence varied from 2 to 14 ramets, with an average of 3.07 ramets per clone. From the 171 ramets included in MRCs, 130 were assigned to the same

sex, resulting in 76% match of DNA identification and sex, while 41 ramets were screened as a different sex than the majority of the ramets within an assigned clone, in total, 24% of the ramets did not match DNA and sex (not for a single MRC, but all MRCs combined). Ploidy again showed similar results, with 126 out of 171 ramets identified as the same DNA and same ploidy (74%), and 45 out of 171 were assigned different ploidy than the clone (26%). Clonal fingerprinting of trees along transects connecting populations in Eta Lake, Dominion, and Tower showed that most of the sampled trees along transects belong to a MRC. In Eta Lake 15 out of 22 (68%) transect trees belonged to MRCs, from those, one clone extended beyond the C1 population, five clones extended beyond the C2 population, and two clones extended beyond the C3 population. Populations C1 and C2 (600 m apart) had both one ramet each of the same clone, while populations C2 and C3 (630 m apart) shared three ramets of two different clones and one ramet of a third clone. In Dominion 28 out of 34 (82%) transect trees belonged to MRCs from those, one clone extended beyond C4 and was identified in both transects and in C6, this clone was present throughout 1700 m. C5 and C6 (1000 m apart) also had one ramet each of the same clone. C5 had three MRCs that extended beyond the established limits of the population, and C6 had seven MRCs that were also identified beyond the limits of the population. And lastly, Tower had 27 trees sampled on transects, from those 21 were identified as belonging to MRCs (77%). In C10 four MRCs extended beyond the limits of the population, C11 had seven, and C12 had four. C10 and C11 (660 m apart) had seven ramets in common for one MRC, four for a second, and two for a third, and C11 and C12 (650 m apart) had three ramets in common for two different MRCs (Figure 4-10).

The number of tetraploids was correlated with stand age ($r^2 = 0.62$, $P = 0.03$), but the proportion of tetraploids in relation to sample size was not significantly correlated with stand age, yet the proportion of diploids in relation to sample size was significantly negatively correlated with stand age ($r^2 = -0.64$, $P = 0.02$; Figures 4-11 a, b, and c).

Distribution of aspen ploidy and patterns of water availability

Assumption of diploids and triploids

Under the assumption that samples were only diploid (diploid + tetraploid) or triploid, the proportion of ploidy in the study aspen populations showed 63% diploid and 37% triploid aspen trees. A chi-square test of independence showed that the total proportions of diploids and triploids were different ($\chi^2 = 578.07$, df = 1, $P < 0.001$); Figure 4-12a). When exploring the proportion of ploidies by study area, all areas showed higher proportions of diploids than

triploids. A second chi-square test of independence showed that the association between ploidy and area was significant ($\chi^2 = 270.67$, df = 3, $P < 0.001$; Figure 4-12b).

When exploring ploidy proportions as a function of CMI the generalized linear mixed-effect model results indicate that CMI was not statistically significant in explaining ploidy occurrence ($\beta = -0.0004$, SE = 0.02, $P = 0.985$; Figure 4-13).

Assumption of diploids, triploids, and tetraploids

When interpreting tetraploids as a separate level of ploidy, the study aspen populations were composed of 10% diploid, 52% tetraploid, and 37% triploid aspen trees. A chi-square test of independence showed that the total proportions of diploids, triploids, and tetraploids were different ($\chi^2 = 2343.3$, df = 2, $P < 0.001$); Figure 4-14a). When exploring the proportion of ploidies by study area, Cathedral, Dominion, and Eta Lake had higher proportions of tetraploids, followed by triploids, while Tower had higher proportions of triploids, followed by tetraploids. A second chi-square test of independence showed that the association between ploidy and area was significant ($\chi^2 = 531.8$, df = 6, $P < 0.001$; Figure 4-14b).

When exploring ploidy proportions as a function of climate moisture index the generalized linear mixed-effect model results indicate that CMI was not statistically significant in explaining ploidy occurrence ($\beta = -0.002$, SE = 0.02, $P = 0.947$; Figure 4-15). Still, while not significant, the areas with the lowest CMI had a pattern of higher proportions of polyploids (tetraploids and triploids) than diploids.

Growth, wood density, and drought responses (resilience components)

Assumption of diploids and triploids

When comparing diploid (diploid + tetraploid) with triploid trees, diploids showed greater diameter at breast height (DBH) than triploids ($F_{1, 273} = 4.23$, $P = 0.041$) where diploids had an average DBH of 31.7 $\pm$ 0.73 cm and triploids had 29.3 $\pm$ 0.90 cm; Figure 4-16a). Wood specific gravity was not different between diploid and triploid aspen trees ($F_{1, 117} = 0.006$, $P = 0.940$; Figure 4-16b).

Diploids had significantly higher resistance index to drought than triploids ($\beta_{Resistance} = -0.263$, $SE_{Resistance} = 0.13$, $P_{Resistance} = 0.048$), while triploids had significantly higher recovery index after drought ($\beta_{Recovery} = 0.209$, $SE_{Recovery} = 0.10$, $P_{Recovery} = 0.051$). The resilience ($\beta_{Resilience} = -0.055$, $SE_{Resilience} = 0.11$, $P_{Resilience} = 0.632$) and relative resilience ($\beta_{RelResilience} = -0.112$, $SE_{RelResilience} = 0.25$, $P_{RelResilience} = 0.662$) of diploids and triploids did not differ (Figure 4-17).

Assumption of diploids, triploids, and tetraploids

When comparing all three ploidies (diploids, triploids, and tetraploids), tetraploids showed the greater DBH (32.4 $\pm$ 0.79 cm), followed by triploids (29.3 $\pm$ 0.90 cm), and lastly diploids (28.0 $\pm$ 1.79 cm), indicating greater diameter growth of polyploids ($F_{2, 272} = 4.23$, $P = 0.008$; Figure 4-18a). Wood specific gravity was not different between aspen trees of different ploidies ($F_{2, 116} = 0.113$, $P = 0.893$; Figure 4-18b).

Resistance, recovery, resilience, and relative resilience indexes did not differ between diploids, triploids, and tetraploids (Table 4-5; Figure 4-19).

4.4. Discussion

Patterns of clonal structure and diversity

The delineation of aspen clones within the AW2 CPP tree improvement region based on phenotypic assessments has showed inconsistent results with the identification of clones based on microsatellite markers. Microsatellites are tandem repeats or repetitive genomic regions that serve as a tool to determine genetic diversity of clonal species, due to their known instability and capacity to mutate at high rates (Vieira et al. 2016). Microsatellites are, in most cases, non-coding sequences of DNA, from which mutations evolve neutrally (i.e., changes in base pairs do not change protein-coding genes; Ally et al. 2008). Microsatellites are capable of capturing mutations that occur through cell division and over time (Ally et al. 2008). The discrepancy in clonal identification between phenotypic assessments and microsatellite markers is of importance if considering the history and delineation of aspen breeding regions in Alberta. When Li (1995) developed the first breeding strategy for the improvement of aspen in Alberta, all information on genetic variation among natural populations of aspen was based on phenotypic assessments, as microsatellites for aspen clonal fingerprinting were yet to be discovered. During the development of aspen tree breeding strategies, aspen natural populations in Alberta were considered as having low genetic differentiation and breeding regions were selected based on patterns of geographic variation (Cheliak and Dancik 1982; Li 1995).

The results from this study showed that the diversity of genotypes in aspen populations within the AW2 CPP tree improvement region identified from microsatellite markers is much greater than what would be predicted based only on physical characteristics of trees. Other

studies also found high diversity of genotypes within aspen populations that were predicted to be mono-clonal (Ally et al. 2008; De Woody et al. 2008; Mock et al. 2008). Jelínková et al. (2014), while exploring if the identification of aspen stands defined as putative clones based on phenotype and phenological characteristics would be confirmed by microsatellite markers, found that putative clonal groups were not confirmed by molecular markers. In addition, multi-ramet clones identified by microsatellite markers had different times of leaf-flushing, which was unexpected based on the previously commonly accepted assumption that ramets within a same aspen clone would have similar phenology (Jelínková et al. 2014). In the present study, both thresholds for clonal identification, zero and two mutations, showed a high diversity of unique genotypes in the study populations, which were initially delineated as putative aspen clones based on phenotype. The use of a zero-mutation threshold can lead to an overestimation of unique genotypes in the populations if multi-ramet clones developed somatic mutations over time (Arnaud-Haond et al. 2007). With that in mind, clonal structure and diversity were described in this study assuming zero and two mutations. Still, both methods found high levels of genetic diversity, with 84% unique genotypes found when not considering mutations, and 50% unique genotypes found when considering mutations. Since the occurrence of somatic mutations is common to clonal species, including aspen (Ally et al. 2008; Mock et al. 2008; De Woody et al. 2009; Ally et al. 2010; Barrett 2015; Latutrie et al. 2019), this discussion will focus on the results obtained when considering two mutations as a threshold to determine that ramets belong to the same clone.

Aspen clones are likely to spread their root systems and grow in varied shapes and lengths determined by resource availability, resulting in shapes that are generally complex, random, or elongated (Namroud et al. 2005; Ally et al. 2008), thus measuring clone size in aspen is a challenging task. Aspen clone size has been described based on number of ramets per clone, as in Latutrie et al. (2019), and on size of stand in which an aspen clone is located (Kemperman and Barnes 1976). In the present study, clone presence, instead of size, was described following the method used by Latutrie et al. (2019), based on parameters of clonal diversity. In Latutrie et al. (2019), a focus aspen population could be comprised of only unique genotypes, mostly small clones, or a few large clones; and multi-ramet clones were described by the number of ramets sampled and identified per clone. In the present study, 67% of the populations contained mostly small clones and 33% of the populations contained a few large clones.

Our results showed high levels of genotype richness and diversity of clones within the studied populations. Genotype richness is the proportion of distinct genotypes present in a

population relative to the total number of sampled trees and clonal diversity is characterized by relative abundance of individuals of a same clone in the population. This means that almost all study populations included ramets of unique genotypes, except for C5 where only MRCs were identified. All study populations included more than one MRC (a minimum of three different MRCs were identified in two populations, and a maximum of seven in one). Latutrie et al. (2019) found, for a region Southwest of Edmonton, a mean clone size of 2.86 ramets per clone, with a maximum of 9 ramets per clone. In my study, clone presence was on average 3.07 ramets per clone, with a maximum of 14 ramets in the same clone. The higher genetic diversity of aspen stands in northern latitudes of North America, throughout Canada and Alaska, has been attributed to a post-glacial expansion that might still be occurring, delivering high levels of gene flow to those areas (Callahan et al. 2013). Namroud et al. (2005) found a high genotypic diversity of aspen on their study sites in northwestern Quebec, where more than 75% of clones were comprised of one or a few ramets. The authors did not find evidence of seedling establishment in the areas, so they attributed the genetic diversity observed to root suckering from a potential original genetic pool that was established in the past and is still alive via the root system of the stands (Namroud et al. 2005).

The diversity of single ramet genotypes found at most of the study aspen populations in the AW2 (Pembina) region suggests that sexual recruitment of aspen might be occurring in these areas or has occurred in the past. The establishment of aspen seedlings can occur under a narrow set of environmental conditions, consequently, aspen seedling establishment is considered a rare event in nature, still, aspen seeds potentially have a higher chance of establishment in moist environments and low chances of germinating in arid environments (Einspahr and Winton 1977; Mitton and Grant 1980; Sakai and Burris 1985; Landhäusser et al. 2019). Aspen seedlings are small and susceptible to environmental conditions, in effect, it takes time for seedlings to develop a root system that can effectively penetrate the soil (Barnes 1966). The small radicle and root hairs collect water and nutrients from resources available in the area of germination, such as leaves, bare soil, and decaying wood (Barnes 1966). Under those circumstances, specific conditions of ground cover, competition, and water availability play an important role in seedling establishment and survival of aspen seedlings (Barnes 1966; Jelinski and Cheliak 1992; Mock et al. 2008). Aspen is an early successional species (Einspahr and Winton 1977) and colonizes areas without much competing vegetation and with high light availability (Landhäusser et al. 2019). Therefore, seedlings are more likely to survive and become established in post-disturbance sites, such as post-fire, or cleared areas (Mitton and Grant 1980; Landhäusser et al. 2019). Considering aspen's specific conditions for germination

and establishment, the ramets presenting unique genotypes could have been established after disturbance, such as fire or diseases (Namroud et al. 2005; Ally et al. 2008).

Alternatively, the diversity of new genotypes could be part of a genotype bank stored in the root systems of those aspen stands (Jelínková et al. 2009). Aspen can reproduce vegetatively through root suckers, and aspen genotypes from which ramets have died back can still be alive through the root system of the aspen stand (Barnes 1966; Grant and Mitton 1979; Jelínková et al. 2009). Those roots are capable of re-sprouting and bringing ramets of the extinct aspen clones back to the surface (Namroud et al. 2005; Jelínková et al. 2009). Arnaud-Haond et al. (2007) justified that for clonal plant species, the same species can occur in mono-clonal and genetically diverse stands showing plasticity in the allocation of energy to vegetative versus sexual reproduction. This might occur independently of environmental characteristics, as in Latutrie et al. (2019) aspen clonal structure did not vary under different environmental conditions and disturbance regimes (Latutrie et al. 2019). In aspen stands, the vegetative and sexual modes of reproduction combined increase the genetic variability of stands, in a way that seems to be independent of environmental conditions (Latutrie et al. 2019).

The clonal mode of reproduction in aspen might facilitate the development and accumulation of somatic mutations (Barrett 2015). In clonal species somatic mutations can be passed to future ramet generations (Ally et al. 2010; Barrett 2015). As aspen clones grow, spread ramets, ramets die back and grow again, somatic mutations accumulate over time, if those mutations do not negatively affect clonal growth (Ally et al. 2008; Barrett 2015). If this happens, a single original aspen clone might be comprised of a mix of mutant and non-mutant cell lineages (Ally et al. 2008). Ally et al. (2008) found several aspen clones with somatic mutations shared amongst ramets, some of the ramets that shared somatic mutations were physically distant (20 to 60 m apart). Somatic mutations can occur in the shoots or rootstock and might be transferred from shoot meristems to gametes and subsequently to sexual offspring (Ally et al. 2008; Ally et al. 2010; Barrett 2015). Older aspen clones are more susceptible to somatic aneuploidy (Mock et al. 2008), which could cause the addition or subtraction of chromosomal material (Stohl and Platt 2018).

Our results showed that 26% of ramets in multi-ramet clones showed different ploidy from the original clone. This difference in ploidy could be attributed to possible somatic aneuploidy, especially since the study populations contain mature aspen trees, which could have developed somatic mutations over time. Older tissue has been found to accumulate a higher frequency of point mutations than young tissue, because of longer exposure to stress (Pla et al. 2000, as cited in Ally et al. 2008). The studied aspen populations might also be

formed by potentially old aspen clones, that could have developed and accumulated mutations after cycles of ramet growth, development, and death (Ally et al. 2008). High numbers of tetraploids and a lower proportion of diploids were found in older stands, this might indicate that tetraploid samples could belong to original diploid genotypes which could have undergone genome duplication or somatic aneuploidy over time (Van de Peer et al. 2017). Mock et al. (2008) found similar results of multi-ramet clones in which most ramets were diploids and some ramets had an additional third allele. The authors also attributed this occurrence to possible somatic aneuploidy or somatic mutations (Mock et al. 2008). It must be noted that a diploid-only assumption was used when building the phylogenetic trees for individual relationships. This approach could have led to the misidentification of different genotypes as the same MRC if by chance ramets of different ploidies were genetically identical at the first two alleles, but different on the extra alleles.

Like ploidy, 24% of ramets within MRCs did not match the sex of the clone. Lester (1963) described variation in sex expression in aspen where bisexual trees and hermaphroditic flowers in unisexual trees were found. This variability in sex expression was then attributed to the interaction of genotype and environment (Lester 1963). Yet, the exact mechanism of sex determination in *Populus* spp. is still not completely understood (Kersten et al. 2017). A sex-linked marker was described for aspen, the gene TOZ19 is in a central region of chromosome 19 (Pakull et al. 2015). TOZ19 is not present, or it is fragmentally present in females of aspen, while the genes neighbouring TOZ19 are present in both males and females (Pakull et al. 2015). Additionally, a paralogous gene (homologous gene to TOZ19 that has diverged due to *Populus* genome duplication), TOZ13, is in chromosome 13 and amplifies in all male and female aspen individuals (Pakull et al. 2015). Kersten et al. (2017) suggests that other than protein-coding genes, factors such as non-coding regulatory small RNAs or epigenetic regulation may be involved in the sex determination of dioecious species. Environmental factors might also influence the sex expression and determination in trees. Hormonal changes, and abiotic factors such as drought, light intensity, and nutrient levels have been shown to change sex expression in trees (Kersten et al. 2017). The genetic and complex nature of sex determination in *P. tremuloides* raises the question if mutations, polysomy, or whole genome duplication could lead to sex changes on the ramets of a clone. This might be possible since whole genome duplication is followed by epigenetic modifications that can modify homologous gene expression patterns (Van de Peer et al. 2021).

In my study, ramets of the same aspen clone were found throughout 1700 m. This finding is surprising considering that the description of aspen clones in Alberta and Canada has

pointed to small clone sizes (Woody et al. 2009; Latutrie et al. 2019), also traditional studies defined 400 m as enough space to determine that aspen clones are independent (Mitton and Grant 1980). In Alberta, collections of seed or vegetative materials from stands of native species must be separated by at least 500 m, to avoid collecting material from the same clone (FGRMS 2016). In the current study, ramets of the same aspen clones were found at distances ranging from 600 to 1700 m. Still, the possibility that two different genets could present identical allele calls by chance must be considered (De Woody et al. 2009), especially when the analysis extrapolates the limits of the known populations. Like the results obtained here, De Woody et al. (2009) found clones (i.e., genets) that occurred in more than one stand, indicating that adjacent stands could have been connected at some point in the landscape history. Mock et al. (2008) found ramets of a single aspen clone to be spaced over 500 m apart. The degree of distribution of ramets of an aspen clone suggests the extension of aspen root systems (Ally et al. 2008) and potential root connections between old clones and new genotypes (Jelínková et al. 2009). The area occupied by a single aspen clone depends on biotic and abiotic factors within its environment, events such as fire and diseases can cause the decline of a large, and potentially original clone, creating space for new genotypes to become established (Namroud et al. 2005; Ally et al. 2008).

Distribution of aspen ploidy and patterns of water availability

Diploids, triploids, and tetraploids of aspen have been reported in the literature (Einspahr and Winton 1977). From those, triploids are supposed to present good form, fast growth, and improved fibre properties (Einspahr and Winton 1977). The haploid aspen chromosome number is n=19, the diploid chromosome number is 2n=38, the triploid number is 3n=57, and the tetraploid number is 4n=76 (Erlanson and Hermann 1927, as cited in Einspahr and Winton 1977). From the trees sampled in the present study, 10% were identified as diploid, 37% triploid, and 52% tetraploid. These results do not align with previous studies that found higher proportions of diploids than triploids on natural aspen stands (Greer et al. 2018; Goessen et al. 2022). Mock et al. (2012) found high proportions of triploids, yet diploids were also abundantly found in the study areas. From the studies available up until now and to this author's knowledge, tetraploids have not been described as abundantly occurring on native aspen stands. Taking the literature into account and the results obtained from this study that indicate that diploid trees were more frequently found at younger stands and tetraploids were abundant at older stands, it seems reasonable to infer that most tetraploid samples could have been originally diploid aspen clones. Those diploid clones could have developed somatic mutations

that led to possible somatic aneuploidy, polysomy occurring in specific chromosomes or endoreduplication (somatic mutations that lead to duplication of chromosomes in the tissues; Van de Peer et al. 2021). Somatic mutations can be present at the shoot or stem cells (Ally et al. 2010; Barrett 2015), this type of mutation would be captured by the sampling method applied in the present study, as the DNA samples were collected from phloem tissue. Polyploidy (whole genome duplication) is also possible since the cells examined for cytology indicated triploid and tetraploid numbers of chromosomes when examining chromosome 1 under different stages of cell division (e.g., prophase and metaphase). Polyploidy can also arise in the somatic cells of diploid organisms by cell fusion or abortion of cell division during cytokinesis, this process, called programmed polyploidy, is known to happen in fibers of plants (Van de Peer et al. 2017). The genome duplication in somatic cells might be induced by drought, and it can increase whole-organism stress resilience in plants (Van de Peer et al. 2017). Therefore, even if tetraploid cells were part of originally diploid organisms, the growth and wood characteristics of such mutant tetraploid cells might differ from the original diploid cells. This way, the results on ploidy were considered as interpreting diploids, triploids, and tetraploids separately, and interpreting diploids and tetraploids together as original diploid organisms.

This study sampled mature aspen stands with average age ranging from 52 to 113 years old. When considering the occurrence of true tetraploids, diploid aspen ramets only represented 10% of the sampled population, while if tetraploids are considered as mutant diploids, that would increase the presence of diploids to 63%, versus 37% of triploids. When looking at the distribution of ploidy within aspen clones, the largest MRCs were mostly comprised of triploid ramets, the second most frequent cytotype on large clones was tetraploid. Mock et al. (2012) suggested a relationship between clonality and triploidy, as larger clones in the western United States are comprised of mostly triploid ramets. Triploid aspen clones had more ramets and greater expansion rates than diploids (Mock et al. 2008; DeRose et al. 2015). Greer et al. (2018) on the other hand described higher ramet densities for diploid clones. Theories have been raised to explain the high proportion of aspen triploids in natural settings, such as: aspen triploid clones could be older clones that have remained on the landscape and undergone somatic aneuploidy (Mock et al. 2008), and; triploid clones might expand rapidly due to the possible advantage that triploids have over diploid clones on general vegetative growth (ramet growth and clonal expansion rates) and persistence (Mock et al. 2008; Mock et al. 2012). On the other hand, some authors suggest that triploid aspen clones are potentially sterile, which would negatively affect triploids under conditions of extreme climate events if they cannot re-colonize areas through wind dispersal (Greer et al. 2018). Similarly, Latutrie et al. (2019) found that the

presence of triploids decreased with an increase in the age of aspen stands, they attributed this pattern to the higher frequency of sexual reproduction events, which would result in a reduction of triploids and an increase in diploids, since triploids are allegedly unlikely to reproduce sexually. On the other hand, triploids might not be sterile, as aspen triploids were found to produce viable seeds and germinate without much disadvantage over diploids (Goessen et al. 2022).

Studies indicate that triploids of aspen occur more frequently in warmer and drier climates (Greer et al. 2018; Goessen et al. 2022). This has been associated with aspen's narrow conditions of seed establishment and the potential infertility of triploid clones. Since germination and growth of aspen seedlings are dependent on available moisture (Barnes 1966; Jelinski and Cheliak 1992; Mock et al. 2012), clonal growth would be favoured under dry conditions (Mock et al. 2012). Additionally, triploids have been shown to expand faster than diploids and to occur more frequently in areas where aspen does not establish from seed (Mock et al. 2012; DeRose et al. 2015). In the present study, the occurrence of different ploidies was not significantly influenced by climate when considering the climate moisture index. Still, while not significant, the areas with the lowest CMI had the highest proportions of polyploids (tetraploids and triploids) over diploids. While this might not be strong evidence of polyploidy occurring in drier areas, it aligns with the hypotheses that triploids occur in drier areas (Greer et al. 2018; Goessen et al. 2022) and tetraploids might arise from the genome duplication in somatic cells of diploid organisms under drought (Van de Peer et al. 2017). In Goessen et al. (2022) aspen triploid occurrence was moderately correlated with dry and warm environments, yet the authors found a prevalence of male triploid aspen in dry environments, their theory is that sex might influence aspen distribution under drought conditions instead of ploidy (Goessen et al. 2022).

Drought responses (basal area increment and resilience components)

While exploring if aspen diploids and polyploids (triploids and tetraploids) differ in growth and wood density, polyploids had greater diameter growth than diploids, but when diploids and tetraploids were considered together, diploids had greater growth than triploids. Studies show that aspen triploids have higher growth rates than diploids (Greer et al. 2018; DeRose et al. 2015; DeRose et al. 2022). Triploids might grow twice as fast as diploids by age 10 and likely have longer wood fibers (Einspahr and Winton 1977). In Greer et al. (2018) aspen triploids had greater intrinsic water-use efficiency, assimilation, and stomatal conductance. Having higher water-use efficiency and assimilation rates would give triploids an advantage in resource uptake

and use, reflecting in greater growth and expansion rates. In our study, polyploids showed greater growth, indicating a possible advantage in growth on polyploid aspen. Wood density did not differ between cytotypes, contrasting to available literature that suggests that polyploids of aspen have higher wood density (Einspahr and Winton 1977).

Based on the commonly accepted concept that triploids, and potentially polyploids, of aspen present better performance under drought conditions (Mock et al. 2012). In this study, we hypothesized that polyploids would have higher indices of recovery, resilience, and relative resilience post-drought. In DeRose et al. (2015) triploid aspen grew faster than diploid in the early stages of stand development, while older ramets did not show a difference in growth between cytotypes. The authors suggest that in ramets older than 50 years growth differences between diploids and triploids might be obscured (DeRose et al. 2015; DeRose et al. 2022). Yet, in our study, radial growth differed between diploids, triploids, and tetraploids during and after a drought event. The results showed that diploids had greater resistance to drought, while triploids had greater recovery after drought. When considering tetraploids separately, significant differences were not found, yet a similar pattern was observed, with the exception that tetraploids had average greater resistance and recovery than diploids (yet not significant).

The resistance index compares performance during and before the drought event while the recovery index considers the post-disturbance performance as a function of the changes caused by the drought event (Lloret et al. 2011). Resilience components are calculated from annual tree increment growth or basal area increment. In DeRose et al. (2015) diploids and triploids had similar tree ring growth responses to climate for precipitation, temperature, and drought over the period of 1895 to 2007 (DeRose et al. 2015). Here, tree ring growth responses to drought varied on diploids and polyploids. Forest trees might exhibit a trade-off between resistance and recovery after drought events if performance during and after drought depends on stored reserves (Galiano et al. 2011, as cited in Lloret et al. 2011). The higher resistance in diploids and tetraploids could indicate that triploids have greater consumption of reserves during drought stress (Galiano et al. 2011, as cited in Lloret et al. 2011). This would align with the findings that triploids have higher assimilation rates and stomatal conductance than diploids (Greer et al. 2018). Greer et al. (2018) suggest that triploids might have greater transpiration rates and consequently need greater water supply than diploids, therefore being more prone to drought stress. Yet, the same characteristics of triploids would explain why they had greater recovery after drought. If the hydraulic system of the triploid trees did not suffer substantive cavitation damage during the drought event, which is generally expected to happen on efficient

hydraulic systems (Franklin et al. 2023), then triploids would recover faster due to higher photosynthesis rates.

The characteristics shown by tetraploids (e.g., greater DBH, resistance and recovery to drought when compared to diploids), show an advantage of tetraploids over diploids and potentially triploids. While triploids likely have advantages in photosynthesis and growth, their generally higher hydraulic efficiency could be detrimental under drought. Tetraploids, in contrast, seem to have an advantage over diploids in growth but also an advantage over triploids in resistance to drought events. These tetraploid trees could be a result of whole genome duplication by the union of unreduced gametes (polyploidy) or somatic mutations that lead to duplication of chromosomes in the tissues (endoreduplication; Van de Peer et al. 2021). Endoreduplication is known to occur in plant fibres, shoots, or stem cells, and to promote plastic responses to stress at the organism level, which can even be inherited genetically (Ally et al. 2010; Barrett 2015; Van de Peer et al. 2021). The potential inheritance of polyploidy makes this characteristic a possible adaptive advantage (Van de Peer et al. 2021). In general (not just for aspen), polyploids have a higher tolerance to abiotic stress (Van de Peer et al. 2021). The occurrence of polyploids is directly related to periods of global change (over time, in history) and to areas of dry and cold environments (over the landscape; Van de Peer et al. 2021). In the final analysis, the existence of polyploids (triploids and tetraploids) of aspen improves the genetic variability of the species and its adaptive potential. This is a great finding considering the changes predicted to happen in climate still in this century (IPCC 2022). Notably, natural aspen stands within the AW2 (Pembina) tree improvement region carry great genetic variability expressed in a diversity of genotypes, sex expression, and ploidy levels. Understanding the characteristics and performances of each of these genotype variables can be a valuable tool for the development of aspen improvement programs and future management of aspen stands.

4.5. Conclusion

Tree improvement regions are delineated based on available information on the genetic and ecological characteristics of the region, keeping in mind the selection and heritability of desired traits and the maintenance of genetic diversity within populations (FGRMS 2016). In Alberta, aspen clones propagated from the AW1 and AW2 breeding regions, that were delineated based on geographic variation, showed variable performance that would confirm genetic variation of aspen populations between those areas (Li 1995; Gylander et al. 2012). However, there were less techniques available in the 1990s to describe genetic diversity of

aspen populations. Cheliak and Dancik (1982) found high levels of genic diversity in seven natural aspen populations in Alberta using molecular genetics. Still, most of the genetic variation was attributed to individuals, while populations were similar amongst each other, suggesting the delineation of large-sized breeding populations (Cheliak and Dancik 1982; Li 1995)

The present study revealed new insights into the genetics of naturally occurring aspen populations in Alberta. The results showed high levels of genetic diversity in aspen natural populations within the AW2 (Pembina) tree improvement region of Alberta based on high levels of unique genotypes and the presence of multi-ramet clones of varying presences identified. The evidence of the occurrence and accumulation of somatic mutations on aspen clones amplifies the potential for genetic variability. Tissue-level mutations might change the cytotype of a clone from diploid to tetraploid, and from diploid to triploid (Mock et al. 2008; Van de Peer et al. 2017). The extra sets of chromosomes on mutant cells might alter gene expression under stress conditions, providing advantages in the performance of triploid and tetraploid organisms (Ally et al. 2010; Barrett 2015; Van de Peer et al. 2021). Moreover, there may be a connection between somatic mutations and sex change in aspen.

Considering the results obtained on the diversity of aspen genotypes within populations in Alberta, made possible by clonal fingerprinting, I recommend that new selections of plus trees include variable genotypes, such as unique genotypes of males, females, diploids, triploids, and tetraploids. This would maximize the growth performance of selected materials under varied environmental conditions. In the current study, polyploids showed better growth and performance during and post-drought than diploids. Yet, diploids are likely favoured in young stands and under regular environmental conditions due to natural adaptation to conditions (Van de Peer et al. 2021) and potentially successful sexual reproduction (Mock et al. 2012). Polyploidy is considered a mode of speciation, as it has ecological and evolutionary consequences (Van de Peer et al. 2021). Polyploid individuals show a pattern of higher resilience to extreme environments attributed to greater genetic variability brought about by the duplication in the number of genes (chromosomes), leading to increased adaptability in the short term and potential speciation in the long term (Van de Peer et al. 2017). All things considered, including individuals of different ploidies, sexes, and genotypes in tree improvement programs would broaden the levels of performance of improved trees allowing for a higher selection intensity and making sure that the genetic material available can perform under varied environmental conditions.

Tables

Table 4-1. Putative aspen clones and physical characteristics used to delineate them, including bark colour, pattern of top branches, and approximate live crown ratio.

Putative clone	Areas	Bark colour	Pattern of top branches	Approximate live crown ratio (%)
1	Eta Lake	Green/gray	Forked at top (two branches)	20
2	Eta Lake	Yellow/gray	One main stem	25
3	Eta Lake	Yellow/green	One main stem	20
4	Dominion	Green/yellow/white	Forked at top (three branches)	25
5	Dominion	Yellow/gray/green	One main stem	20
6	Dominion	Orange/green/gray	Forked at top (three branches)	25
7	Cathedral	Green/gray	Forked at top (two branches)	20
8	Cathedral	White/green/gray	Not available (na)	na
9	Cathedral	Green/orange/gray	One main stem	20
10	Tower	Gray/orange	Forked at top (three branches)	20
11	Tower	Green/gray	One main stem	20
12	Tower	Gray/green	One main stem	20

Table 4-2. Primer sequences, including respective fluorescent marker, obtained product size range (in base pairs, bp), expected number of alleles, and expected repeat for seven microsatellite loci of *Populus tremuloides*. Adapted from Latutrie et at. (2015).

Primer name	Fluorescent marker*	Sequence (5′→3′)	Size range (bp)	Number of alleles	Repeat
PTR1F	[6FAM] Blue	AGCGCGTGCGGATTGCCATT	201-272	12	(GGT)n
PTR1R		TTAGTTTCCCGTCACCTCCTGTTAT			(AGG)n
PTR2F	[HEX] Green	AAGAAGAACTCGAAGATGAAGAACT	187-235	10	(TGG)n
PTR2R		ACTGACAAAACCCCTAATCTAACAA			
PTR3F	[TAM] Black	CACTCGTGTTGTCCTTTTCTTTTCT	187-247	19	(TC)n
PTR3R		AGGATCCCTTCCCTTTAGTAT			
PTR4F	[Cyanine3] Red	AATGTCGAGGCCTTTCTAAATGTCT	101-250	11	(TC)n
PTR4R		GCTTGAGCAACAAACACACCAGATG			
PTR6F	[HEX] Green	AGAAAAGCAGATTGAGAAAAGAC	184-215	9	(AT)n
PTR6R		CTAGTATAGAGAAAGAAGAAGCAGAAA			
PTR14F	[TAM] Black	TCCGTTTTTGCATCTCAAGAATCAC	158-210	14	(TGG)n
PTR14R		ATACTCGCTTTATAACACCATTGTC			
WPMS16F	[6FAM] Blue	CTCGTACTATTTCCGATGATGACC	128-204	9	(GTC)n
WPMS16R		AGATTATTAGGTGGGCCAAGGACT			(ATCCTC)n

*The dye changes were recommended as appropriate current substitutes by our supplier (Sigma).

Table 4-3. Results of ploidy interpretation of spreads obtained from the cytology method. Including trees selected for cytology (Tree ID) based on the putative ploidy obtained from the microsatellite analyses and the observed ploidy from interpretable cells represented on the root tip spreads of trembling aspen.

Tree ID	Putative ploidy	Observed ploidy	Number of interpretable cells
C1T2	Triploid	Triploid	10
C1T4	Triploid	Triploid	10
		Tetraploid	4
C1T11	Tetraploid	Diploid or tetraploid	1
C1T14	Diploid	Diploid	10
		Diploid	5
		Triploid	1
		Diploid or triploid	1
		Diploid or tetraploid	1
C1T18	Diploid	Tetraploid	2
C3T46	Diploid	Diploid	10
		Tetraploid	14
		Diploid	0
C3T55	Tetraploid	Diploid or tetraploid	6
		Diploid	2
		Diploid or tetraploid	3
		Tetraploid	7
C3T62	Tetraploid	Tetraploid or triploid	1
		Diploid	3
C3T61	Diploid	Diploid or tetraploid	4
C3T64	Triploid	Triploid	8
		Diploid	3
C3T65	Diploid	Diploid or tetraploid	1

Table 4-4. Clonal structure of 12 aspen populations within the AW2 (Pembina) tree improvement region. Two cut-offs were applied to determine when individuals belong to a same clone: zero and two mutations. First the clonal structure results are shown considering zero mutations to determine a clone, then the clonal structure is shown for considering two mutations to determine a clone. Populations (Pop) are described by area, sample size (N), total number of genotypes (G), genotype richness (*R*), clone presence class (U, only unique clones where R = 1; MS, mostly small clones, where R ranges from 0.40 to 0.99; and FL, few large clones where R is below 0.39), clonal diversity (H), clonal evenness (J), mean and maximum clone presence in number of ramets per clone (Mean cs; Max cs), percent of single-ramet genotypes (SRG %), number of females (Sex F) and males (Sex M), number of diploids (2n), triploids (3n), and tetraploids (4n), number of trees with the same DNA and sex (DNA=Sex) and same DNA but different sex (DNA≠Sex), and trees with same DNA and ploidy (DNA=Ploidy) or same DNA but different ploidy (DNA≠Ploidy).

Zero mutations

Pop	Area	N	G	*R*	Clone presence	*H*	*J*	Mean cs	Max cs	SRG (%)	Sex F	Sex M	2n	3n	4n	DNA =Sex	DNA ≠Sex	*DNA =Ploidy*	*DNA ≠Ploidy*
				0.7		0.3	0.0												
1	Eta Lake	15	12	9	MS	5	3	2.0	2.0	60	13	10	4	5	14	6	0	6	0
				0.6		0.3	0.0												
2	Eta Lake	17	11	3	MS	9	4	3.0	4.0	47	14	5	4	11	4	7	2	8	1
				0.7		0.2	0.0												
3	Eta Lake	18	14	6	MS	5	2	3.0	4.0	67	19	5	4	8	12	5	1	6	0
				0.8		0.3	0.0												
4	Dominion	18	15	2	MS	2	2	2.0	2.0	67	18	2	1	10	9	4	2	6	0
				0.5		0.3	0.0												
5	Dominion	24	14	7	MS	0	2	6.0	7.0	50	10	20	1	11	18	10	2	8	4
				0.8		0.1	0.0												
6	Dominion	17	15	8	MS	1	1	2.0	2.0	88	16	4	3	10	7	2	0	2	0
	Cathedra			0.9		0.1	0.0												
7	l	20	19	5	MS	0	1	2.0	2.0	90	16	6	1	4	17	2	0	2	0
	Cathedra			0.9		0.1	0.0												
8	l	23	21	1	MS	8	1	2.0	2.0	83	10	15	0	6	19	4	0	0	4
	Cathedra			0.8		0.2	0.0												
9	l	22	19	6	MS	8	1	2.0	2.0	73	10	15	3	7	15	6	0	4	2

				0.9		0.1	0.0												
10	Tower	17	16	4	MS	1	1	2.0	2.0	88	15	6	0	9	12	2	0	2	0
				1.0		0.0	0.0												
11	Tower	23	23	0	U	0	0	0.0	0.0	100	23	2	6	8	11	0	0	0	0
				0.9		0.1	0.0												
12	Tower	16	15	3	MS	1	1	2.0	2.0	88	15	9	3	14	7	0	2	2	0

Two mutations

Pop	Area	N	G	*R*	Clone presence	*H*	*J*	Mean cs	Max cs	SRG (%)	Sex F	Sex M	2n	3n	4n	DNA =Sex	DNA ≠Sex	*DNA =Ploidy*	*DNA ≠Ploidy*
1	Eta Lake	15	9	0.57	MS	0.61	0.07	2.2	3.0	27	13	10	4	5	14	11	0	11	0
2	Eta Lake	17	6	0.31	FL	0.51	0.08	3.8	8.0	12	14	5	4	11	4	10	5	12	3
3	Eta Lake	18	12	0.65	MS	0.37	0.03	3.0	5.0	50	19	5	4	8	12	8	1	7	2
4	Dominion	18	7	0.35	FL	0.71	0.10	2.8	6.0	6	18	2	1	10	9	13	4	16	1
5	Dominion	24	6	0.22	FL	0.59	0.10	4.0	14.0	0	10	20	1	11	18	17	7	15	9
6	Dominion	17	11	0.63	MS	0.57	0.05	2.2	3.0	35	16	4	3	10	7	7	4	6	5
7	Cathedral	20	10	0.47	MS	0.50	0.05	3.5	6.0	30	16	6	1	4	17	9	5	12	2
8	Cathedral	23	14	0.59	MS	0.69	0.05	2.3	3.0	30	10	15	0	6	19	10	6	11	5
9	Cathedral	22	8	0.33	FL	0.52	0.07	4.5	7.0	18	10	15	3	7	15	16	2	11	7
10	Tower	17	9	0.50	MS	0.62	0.07	2.6	3.0	24	15	6	0	9	12	9	4	11	2
11	Tower	23	15	0.64	MS	0.52	0.03	2.6	4.0	43	23	2	6	8	11	12	1	7	6
12	Tower	16	9	0.53	MS	0.41	0.05	3.3	5.0	38	15	9	3	14	7	8	2	7	3

Table 4-5. Results of multiple comparisons of means, Tukey contrasts, for linear mixed effect models where the resilience components, resistance, recovery, resilience, and relative resilience indexes were the response variables, and ploidy was the explanatory variable (stand age was included as a covariate and area as a random effect). Includes the contrast outputs estimate, standard error, *Z*-value, and *P*-value. Significant differences at $\alpha = 0.05$.

Component	Ploidy	Estimate	Standard error	*Z*-value	*P*-value
Resistance	tetraploid - diploid	-0.144	0.258	-0.558	0.837
	triploid - diploid	-0.386	0.257	-1.503	0.279
	triploid - tetraploid	-0.242	0.139	-1.743	0.181
Recovery	tetraploid - diploid	-0.041	0.208	-0.199	0.978
	triploid - diploid	0.174	0.207	0.840	0.669
	triploid - tetraploid	0.215	0.112	1.920	0.126
Resilience	tetraploid - diploid	-0.188	0.222	-0.844	0.666
	triploid - diploid	-0.215	0.222	-0.969	0.586
	triploid - tetraploid	-0.027	0.120	-0.227	0.971
Relative resilience	tetraploid - diploid	-0.399	0.475	-0.841	0.670
	triploid - diploid	-0.439	0.466	-0.942	0.605
	triploid - tetraploid	-0.040	0.270	-0.147	0.988

Figures

Figure 4-1. Bark colours observed on putative aspen clones. Colours are described in order from the most evident to the least evident on a tree's stem (not only on square used as reference).

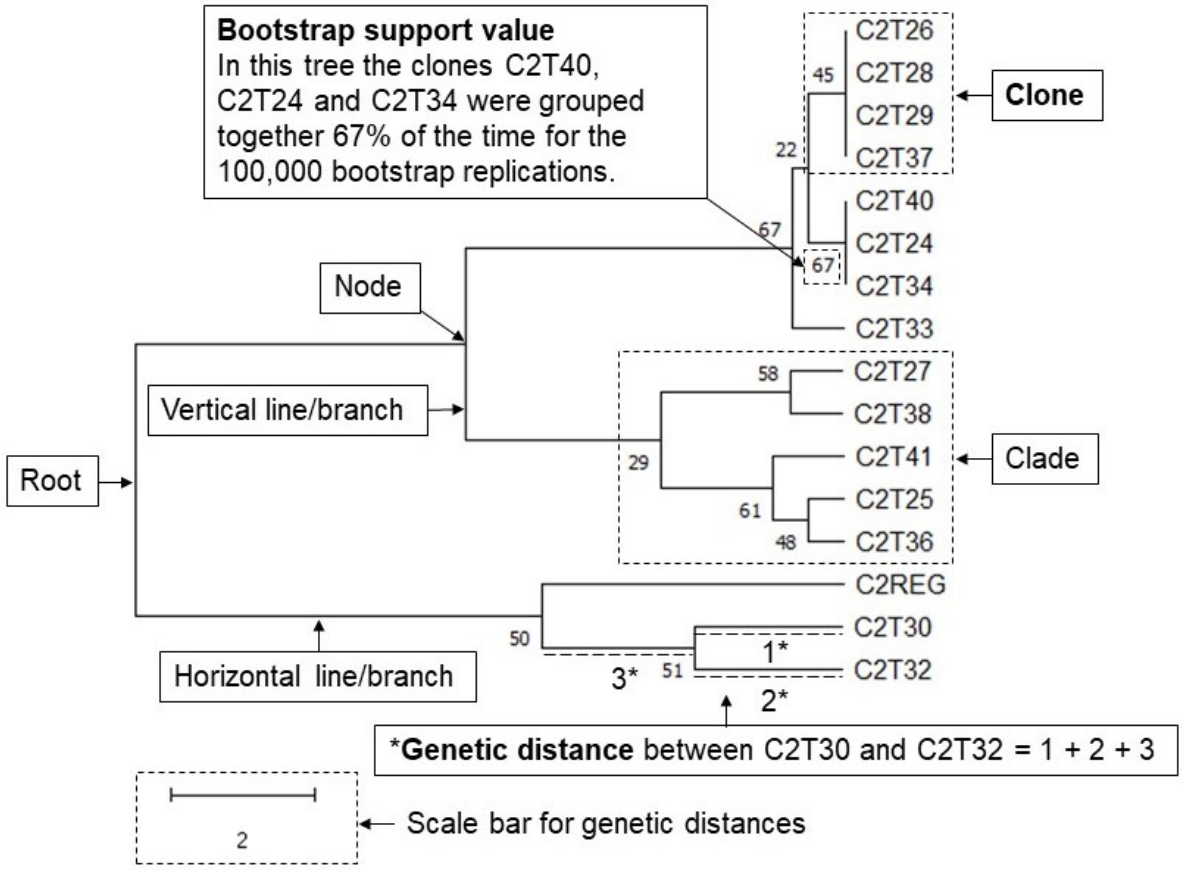

Figure 4-2. Unweighted pair-group method with arithmetic mean (UPGMA) phylogenetic tree with $(\delta\mu)^2$ microsatellite distance. Bootstrapping of 100,000 UPGMA tree pairs the members with the smallest distance between them into clusters. Bootstrap support values (%) represent the proportion of the number of times in which a specific branch was formed to the total number of bootstrap replications. The root is a common lineage from where all trees derived. Nodes represent common ancestors to the trees. Trees within a node are more closely related to each other than trees outside a node, same applies to clades. Horizontal lines or branches represent the level of sequence divergence (from the tip of the line until the node), or the difference between genotypes within that node. If there is not a horizontal line this indicates no sequence differences (or same genotype, i.e., a clone). The genetic distance or number of mutations that have accumulated between samples is a sum of horizontal branches between them. Vertical branches do not have a meaning in terms of similarity (just the horizontal lines).

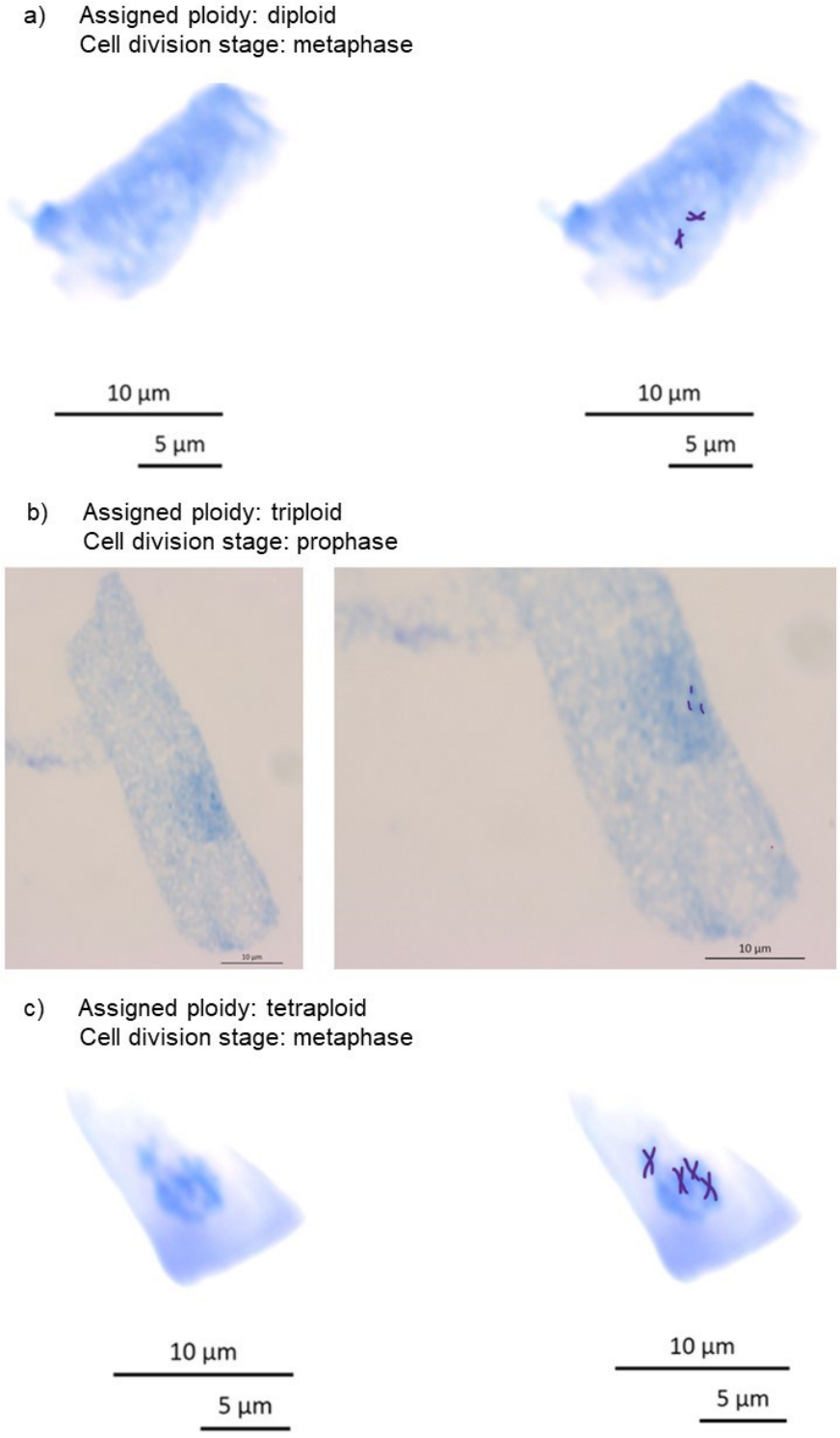

Figure 4-3. Aspen chromosome spreads including assigned ploidy and cell division stage of one diploid (a), one triploid (b), and one tetraploid (c) cells. Purple markers indicate interpretation of Chromosome 1.

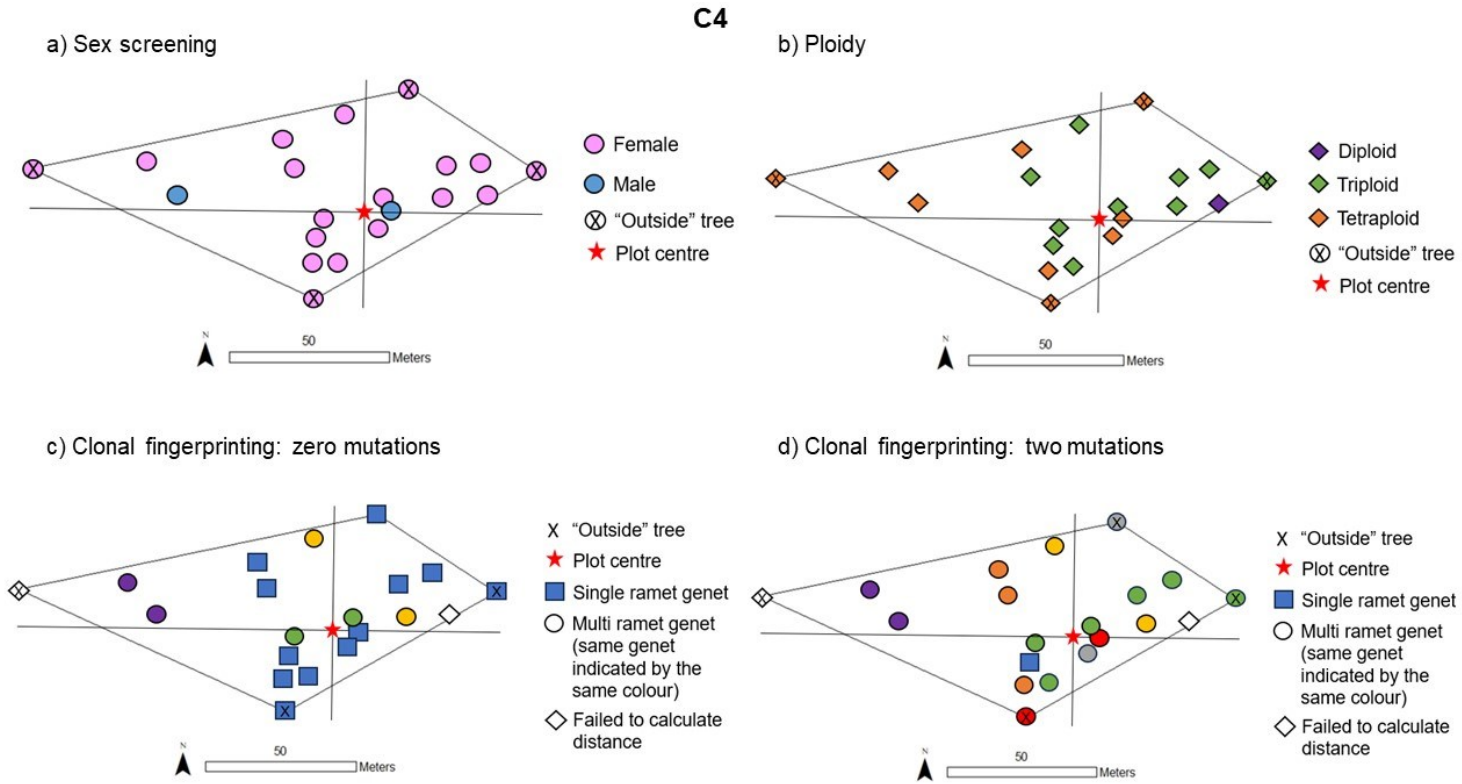

Figure 4-4. Spatial distribution and clonal structure of a study aspen population (C4) within the AW2 (Pembina) tree improvement region. Each symbol represents a sampled tree, an 'X' inside a circle represents a tree initially marked as "outside" of a putative clone, and the red star represents plot centre, including: a) sex screening, where pink circles represent females, and blue circles represent males; b) ploidy, diamond shapes of different colours represent different ploidies, where purple is diploid, green is triploid, and orange is tetraploid; and c and d) clonal fingerprinting, where blue squares represent single ramet genotypes, circles represent multi-ramet clones, with the same colour indicating the same clone, and the white diamond shapes represent trees for which a distance was not calculated. Trees were considered to belong to a same clone when the total genetic distances between them were equal to zero (c) or two (d) mutations.

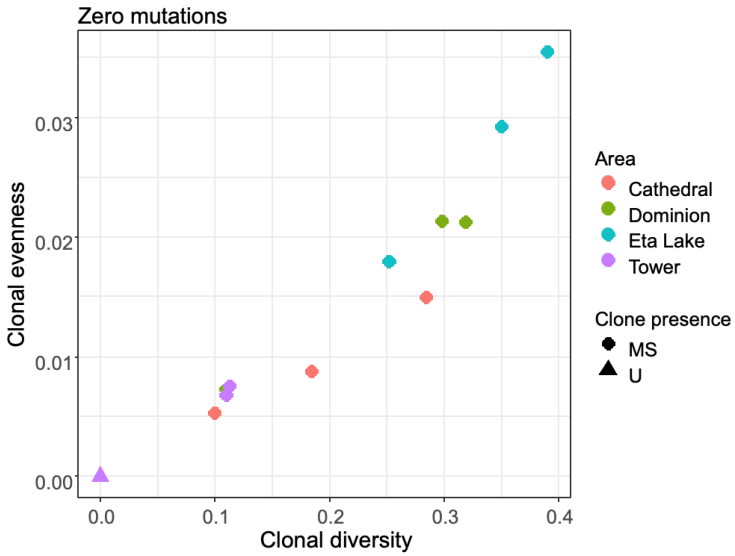

Figure 4-5. Population structure of aspen clones within the four study areas in the AW2 (Pembina) tree improvement region based on the relationship between clonal diversity and evenness. Populations were attributed a clone presence class (MS, mostly small clones, U, only unique clones) based on genotype richness. Trees were considered to belong to the same clone when the total genetic distances between them were equal to zero.

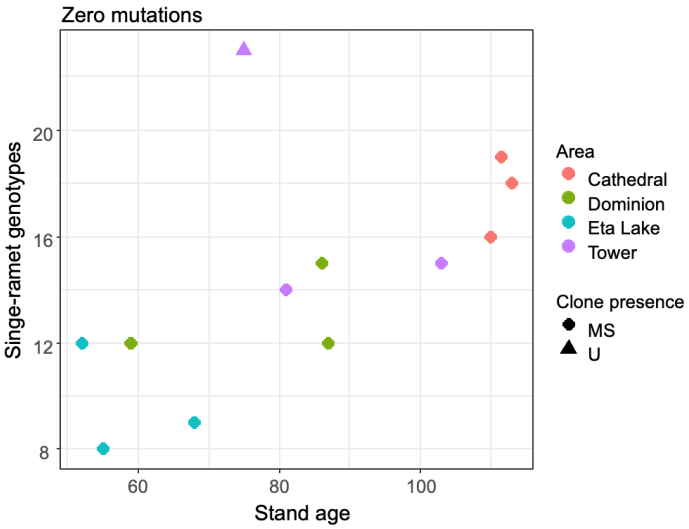

Figure 4-6. Population structure of aspen clones within the four study areas in the AW2 (Pembina) tree improvement region based on the relationship between single-ramet genotypes and stand age. Populations were attributed a clone presence class (MS, mostly small clones, and U, only unique clones) based on genotype richness. Trees were considered to belong to the same clone when the total genetic distances between them were equal to zero.

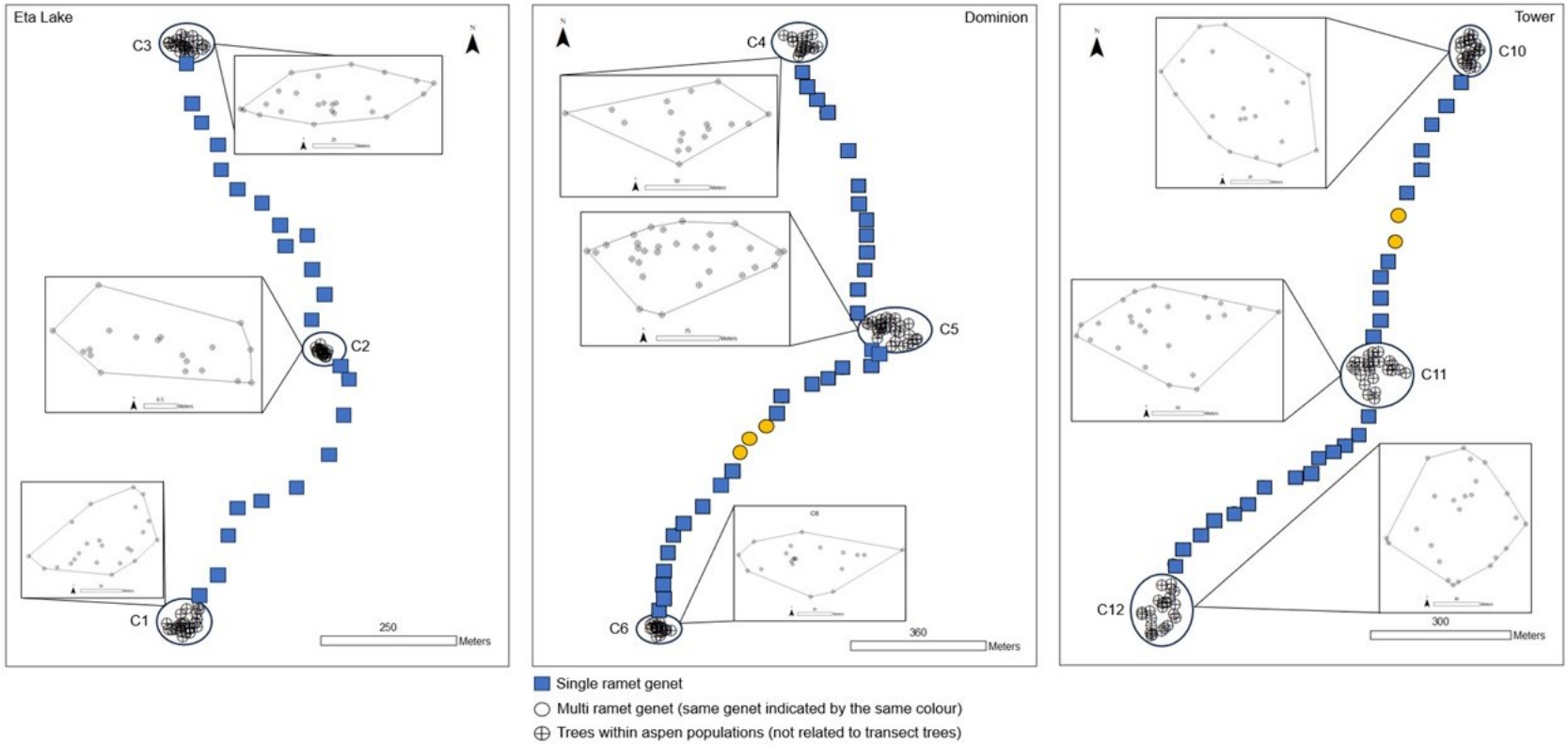

Figure 4-7. Clonal fingerprinting of trees along transects within each of the three study areas: Eta Lake, Dominion, and Tower. Transects connected aspen populations within each area. Trees were considered to belong to the same clone when the total genetic distances between them were equal to zero. Blue squares represent single ramet genotypes, circles represent multi-ramet clones, with the same colour indicating the same clone, and white circles with a cross on top represent aspen trees in a population not related to transect trees.

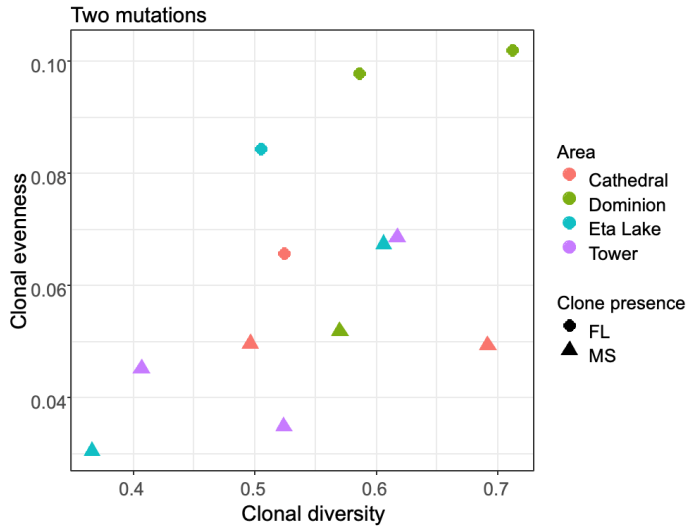

Figure 4-8. Population structure of aspen clones within the four study areas in the AW2 (Pembina) tree improvement region based on the relationship between clonal evenness and clonal diversity. Populations were attributed a clone presence class (MS, mostly small clones, and FL, a few large clones) based on genotype richness. Trees were considered to belong to the same clone when the total genetic distances between them were equal to two mutations.

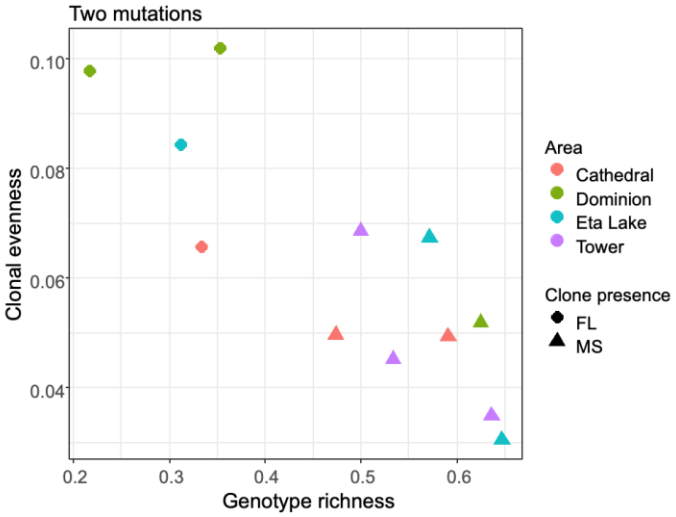

Figure 4-9. Population structure of aspen clones within the four study areas in the AW2 (Pembina) tree improvement region based on the relationship between clonal evenness and genotype richness. Populations were attributed a clone presence class (MS, mostly small clones, and FL, a few large clones) based on genotype richness. Trees were considered to belong to the same clone when the total genetic distances between them were equal to two mutations.

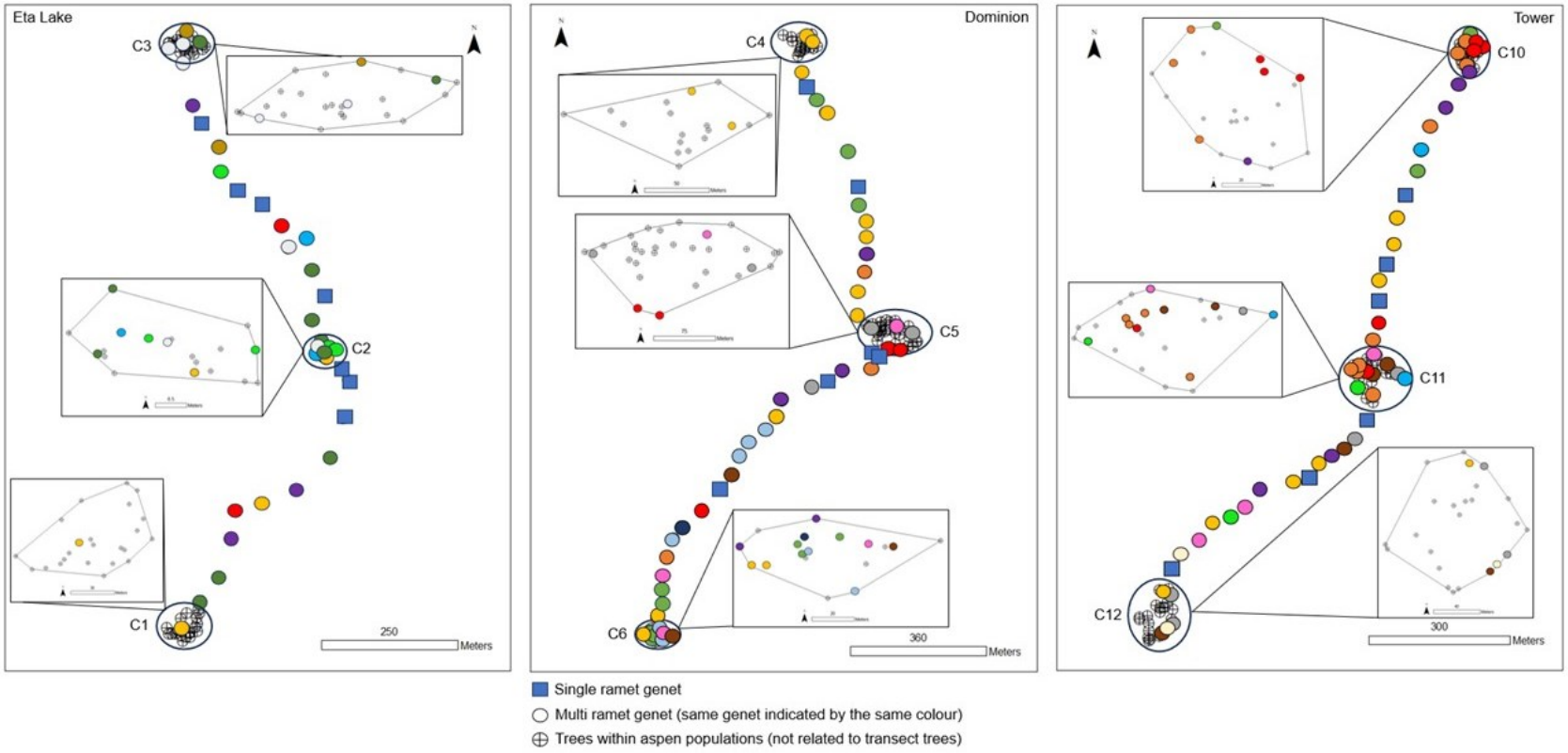

Figure 4-10. Clonal fingerprinting of trees along transects within each of the three study areas: Eta Lake, Dominion, and Tower. Transects connected aspen populations within each area. Trees were considered to belong to the same clone when the total genetic distances between them were equal to two. Blue squares represent single ramet genotypes, circles represent multi-ramet clones, with the same colour indicating the same clone within an area, white circles with a cross on top represent aspen trees in a population not related to transect trees.

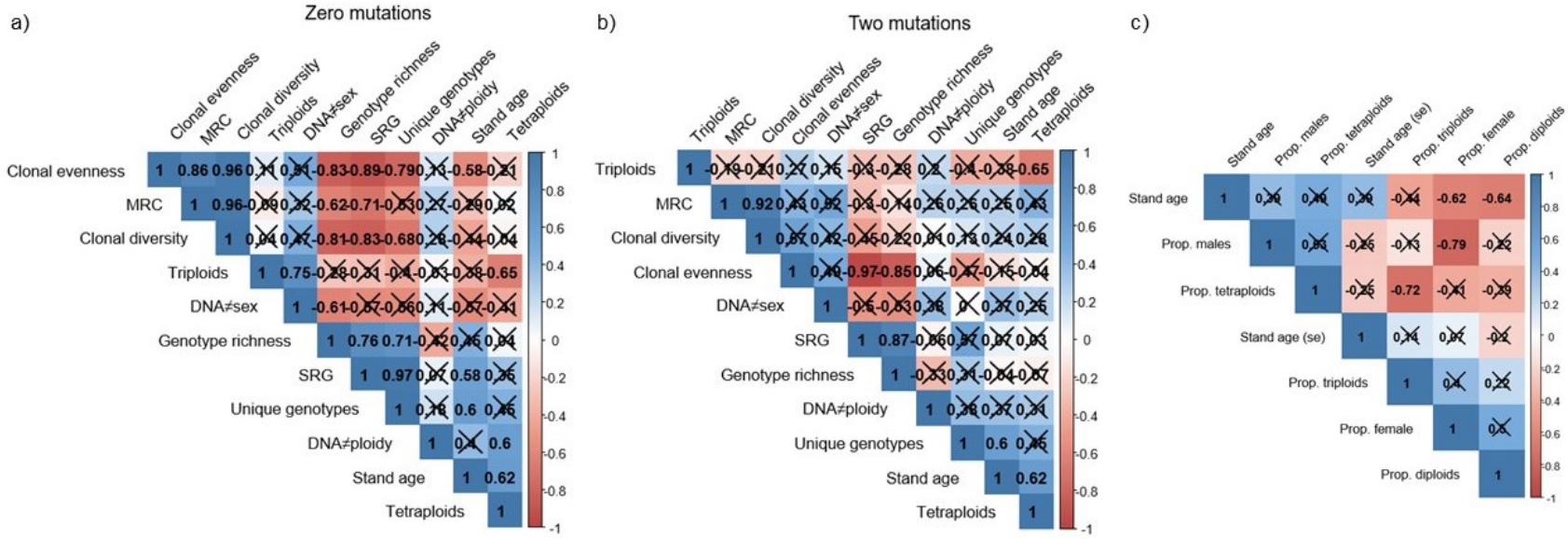

Figure 4-11. Correlation matrices for clonal structure variables including both distance thresholds to determine clones: zero mutations (a) and two mutations (b) containing the variables: multi-ramet clones (MRC), single-ramet genotypes (SRG), unique genotypes, genotype richness, clonal diversity, clonal evenness, differences in DNA and sex and ploidy, number of triploids and tetraploids, and stand age. The correlation matrix displayed at (c) is independent of clonal determination and includes the variables stand age, proportion of males and females (prop. males, prop. females), proportion of diploids, triploids, and tetraploids (prop. diploids, prop. triploids, and prop. tetraploids). Numbers represent R^2 values for correlations. Boxes containing an "X" represent non-significant correlations and boxes without an "X" on top represent significant correlations at $\alpha = 0.05$.

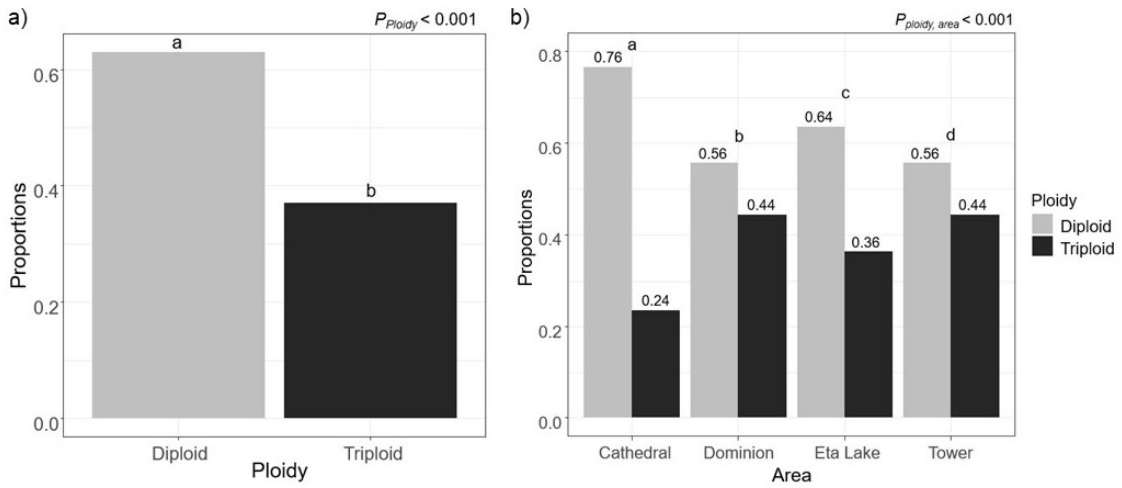

Figure 4-12. Ploidy (diploid and triploid) proportions of mature aspen populations within the AW2 (Pembina) CPP region: a) total ploidy proportions, and b) ploidy proportions by study area. The *P*-values correspond to the results of chi-squared tests on ploidy and ploidy and area. Different letters indicate significant differences at α = 0.05.

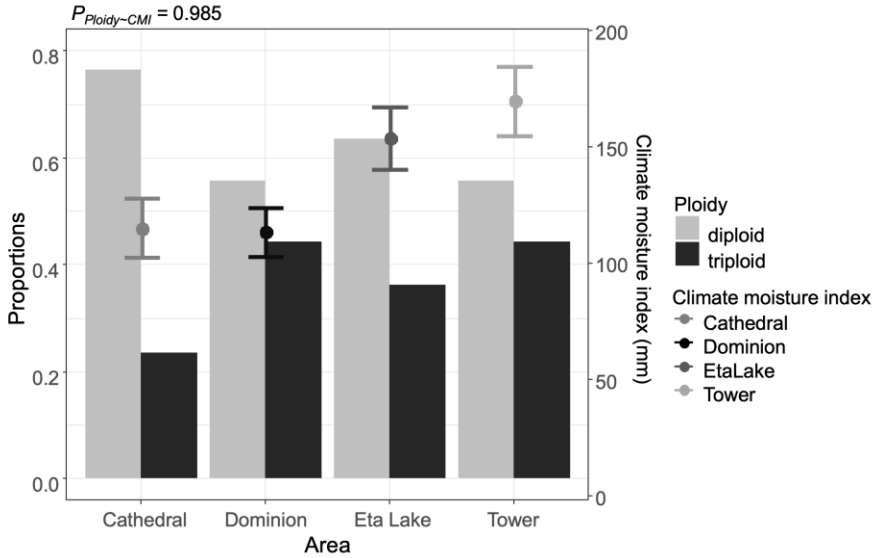

Figure 4-13. Ploidy (diploid and triploid) proportions (left Y axis) and annual climate moisture index (CMI, mm, right Y axis) of aspen populations in study areas within the AW2 (Pembina) CPP region, in Alberta, Canada. The *P*-value corresponds to the result of a generalized linear mixed-effect model where ploidy was the response variable, CMI was the explanatory variable, and area was included as a random effect. Climate normal period of 1960-1990. Significant differences at α = 0.05.

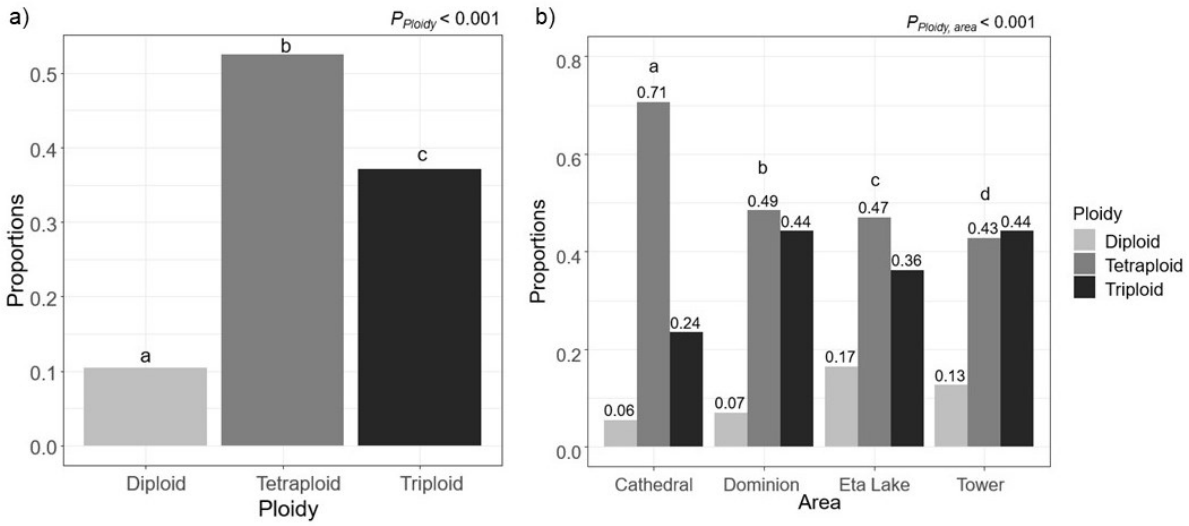

Figure 4-14. Ploidy (diploid, triploid, and tetraploid) proportions of mature aspen populations within the AW2 (Pembina) CPP region: a) total ploidy proportions, and b) ploidy proportions by study area. The *P*-values correspond to the results of chi-squared tests on ploidy and ploidy and area. Different letters indicate significant differences at $\alpha = 0.05$.

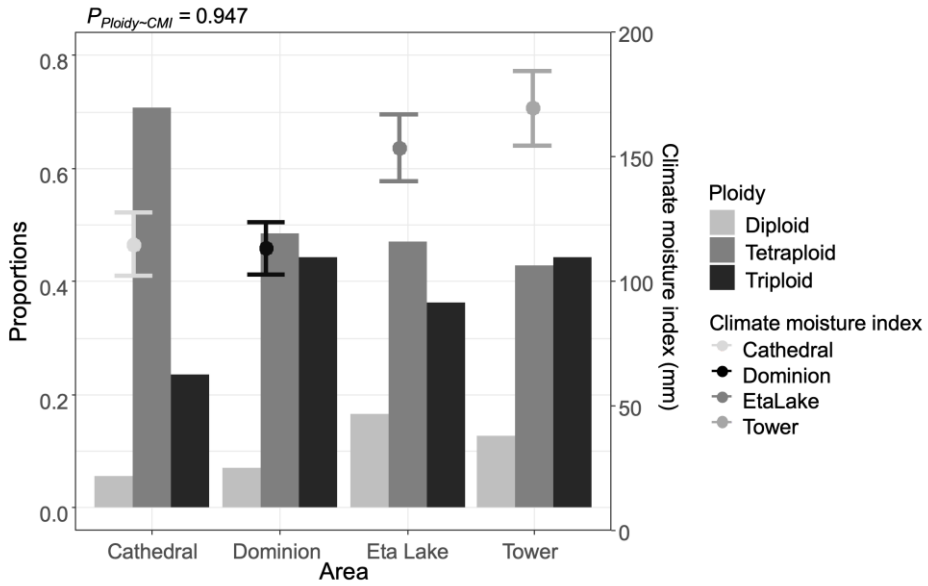

Figure 4-15. Ploidy (diploid, triploid, and tetraploid) proportions (left Y axis) and annual climate moisture index (CMI, mm, right Y axis) of aspen populations in study areas within the AW2 (Pembina) CPP region, in Alberta, Canada. The *P*-value corresponds to the result of a generalized linear mixed-effect model where ploidy was the response variable, CMI was the explanatory variable, and area was included as a random effect. Climate normal period of 1960-1990. Significant differences at $\alpha = 0.05$.

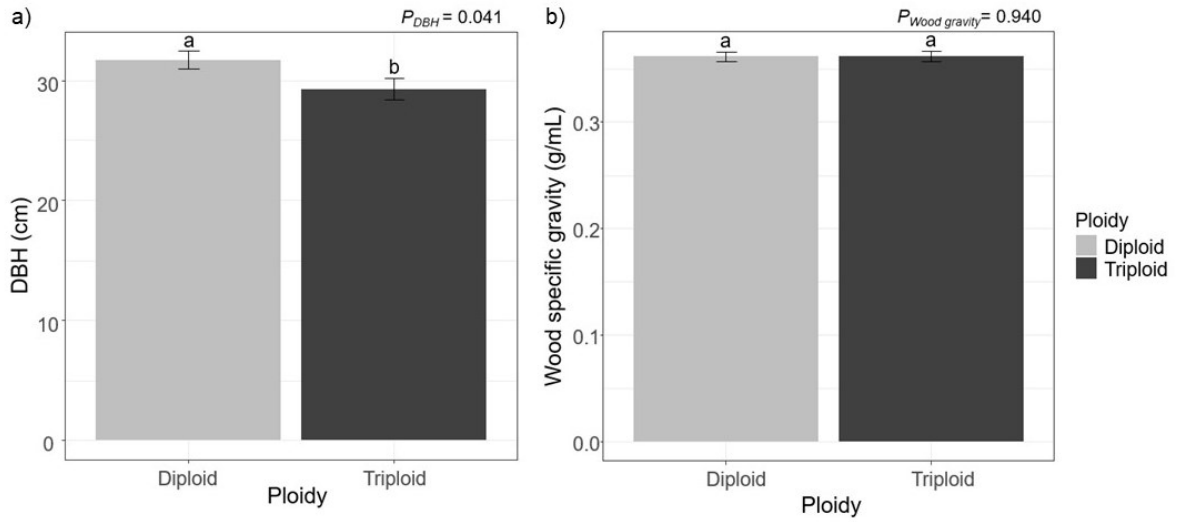

Figure 4-16. Mean ± SE of a) diameter at breast height (DBH, cm) and b) wood specific gravity (g/mL) of diploid (diploid + tetraploid) and triploid aspen trees within the AW2 (Pembina) CPP region, in Alberta, Canada. The *P*-values correspond to the results of ANOVAs with pooled variances where DBH or wood specific gravity were the response variables, and ploidy was the explanatory variable. Different letters indicate significant differences at α = 0.05.

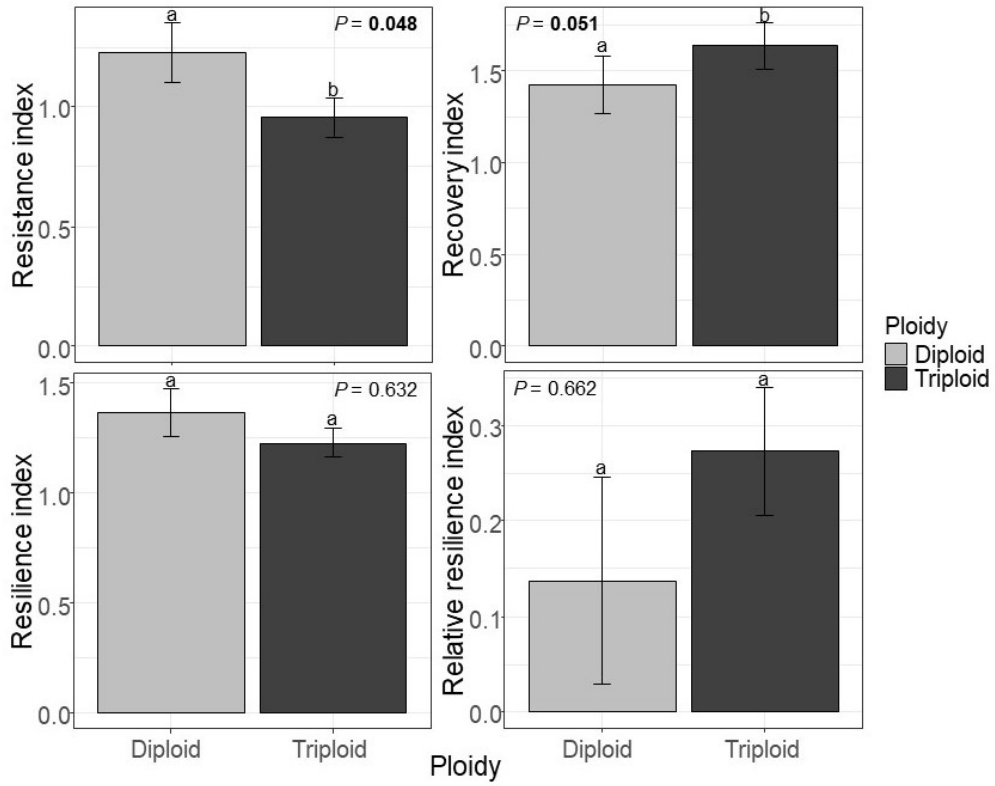

Figure 4-17. Mean ± SE of resistance, recovery, resilience, and relative resilience indexes obtained from diploid (diploid + tetraploid) and triploid aspen trees in study areas within the AW2 (Pembina) CPP region, in Alberta, Canada. The *P*-values correspond to the results of linear mixed-effect models where resistance, recovery, resilience, and relative resilience indexes were the response variables, and ploidy was the explanatory variable (stand age was included as a covariate and area as a random effect). Significant differences at α = 0.05.

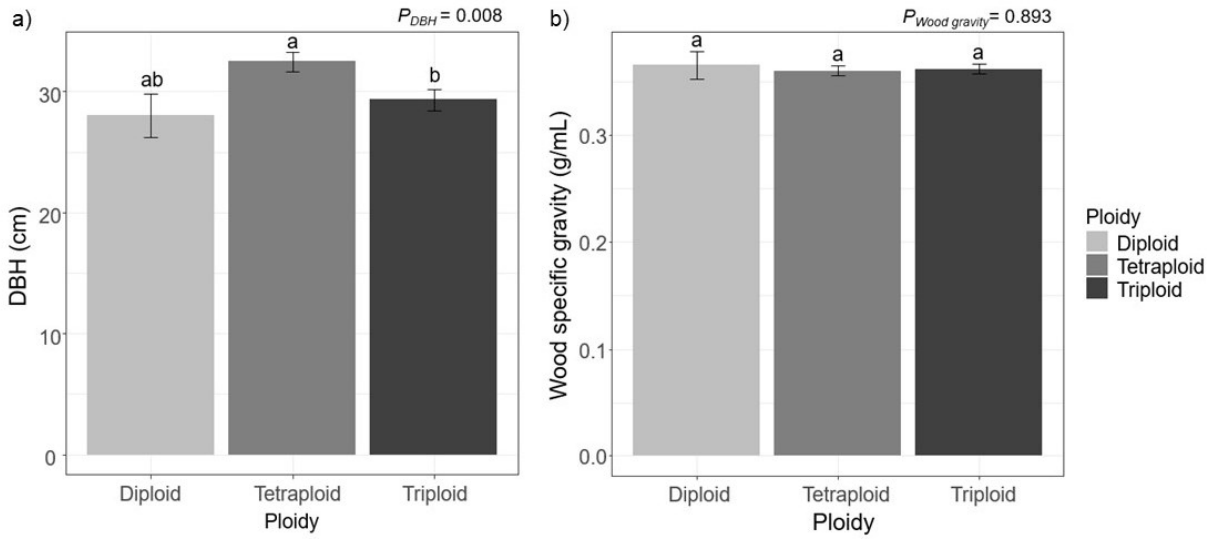

Figure 4-18. Mean + SE of a) diameter at breast height (DBH, cm) and b) wood specific gravity (g/mL) of diploid, triploid, and tetraploid aspen trees within the AW2 (Pembina) CPP region, in Alberta, Canada. The *P*-values correspond to the results of ANOVAs with pooled variances where DBH or wood specific gravity were the response variables, and ploidy was the explanatory variable. Different letters indicate significant differences at α = 0.05.

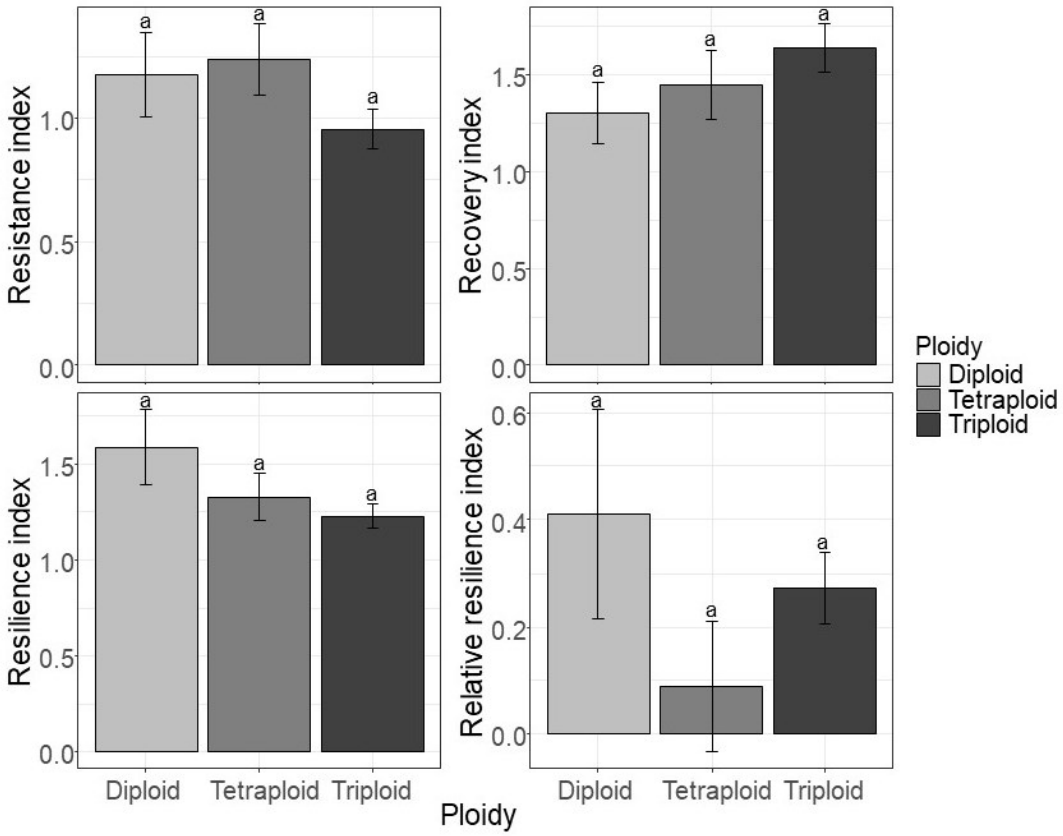

Figure 4-19. Mean ± SE of resistance, recovery, resilience, and relative resilience indexes obtained from diploid, triploid, and tetraploid aspen trees in study areas within the AW2 (Pembina) CPP region, in Alberta, Canada. Significant differences at α = 0.05.

Chapter 5. General conclusion

5.1. Summary and synthesis

This study provides new insights into the sex performance and genetic diversity of aspen seedlings and mature trees in Alberta. My research goals were to investigate if there are differences in the performance of female and male aspen seedlings before the onset of costs of reproduction (on seedlings), and after reproduction (on mature trees), based on the theory that in dioecious plants females incur higher energy costs from the production of seeds than males do from the production of pollen. In addition, this study explored if sexes showed differential responses under drought conditions, looking separately at aspen seedlings and mature trees. Climate was explored as a potential driver of aspen sex distribution on the landscape. Genetic diversity and clonal structure were described for aspen stands within the AW2 (Pembina) active aspen forest management region in Alberta using phenotypic assessments and microsatellite markers. My findings increase the knowledge of the characteristics of aspen during the seedling stage and in mature stands in wild populations. Such information can be used by aspen tree improvement programs, to support policies for clonal deployment and associated regulatory standards for aspen, and on the management of aspen stands in Alberta. My study contributes new information on the ecology of aspen stands, providing insights into clonal structure and sex distribution that can be useful for the survival of the species under future climate conditions.

Chapter 2 investigated if parent tree origin (i.e., provenances of lower vs. higher climate moisture index; CMI), family variability, and sex (i.e., female or male) of aspen seedlings had an influence on seedling performance under well-watered and drought conditions. The hypothesis that seedlings from the boreal region would present reduced performance under a drought stress treatment when compared to seedlings from the parkland region was rejected. An exploration of climate on the study sites for two different normal periods 1961-1990 and 1991-2018 showed that the historical pattern of positive CMI at the boreal region and negative CMI at the parkland region has changed in the past 30 years, and the 1991-2018 climate normal period CMI was negative in both regions. A shift in patterns of water availability is likely to change vegetation communities within those regions. While seedlings from provenances of lower and higher CMI showed the same level of plasticity to drought to most traits (except SLA) under our study conditions, seedlings from the parkland (lower CMI) show signs of adaptation to drought

conditions. Seedlings may also be adapted to the levels of light and resource availability of their place of origin. As hypothesized, family performances were variable, with higher variability under well-watered conditions and lower variability in performance under drought. Exploring the drought strategies of different families allowed low and high-productivity genotypes to be identified. Low-productivity genotypes showed advantageous strategies to survive under extreme drought conditions, such as allocation of resources to root growth under drought conditions, increased stomata closure, and higher water-use efficiency. High-productivity genotypes can be a good option for selection in tree improvement, as they present high productivity under optimal environmental conditions and a desirable level of plasticity under drought stress, however, trade-offs between growth and hydraulic safety and growth and defense must be considered. Families showed differential sex responses to drought. The hypothesis of differential performance of sexes on aspen seedlings under drought stress was rejected for growth but supported for water-use efficiency. Female seedlings had higher $_{i}$WUE under well-watered conditions, and male seedlings had higher $_{i}$WUE under drought conditions, however, biomass growth did not differ between the sexes.

Chapter 3 explored patterns of aspen sex distribution, growth, wood resistance, and drought responses in mature trees within the AW2 region in south-central Alberta. Female-biased sex ratios were found across most of the study sites, while males and females had similar diameter growth and wood density. Such findings suggest differential energy allocation in female and male aspen associated with different modes of reproduction, in which females may be investing more in asexual reproduction, spreading roots and forming more ramets, while males may be investing more energy in stem growth and sexual reproduction. Contrary to my hypotheses, an association between the distribution of sexes and CMI was not found for the period studied, similarly, mature aspen trees did not respond differently in radial growth before, during, or after a drought event.

Chapter 4 includes a description of aspen clonal structure and genetic diversity in natural populations within the AW2 region. Aspen populations were originally delineated based on phenotypic assessments and the genetic diversity within those populations was then explored using microsatellite markers. Additionally, aspects and patterns of ploidy in aspen and the relationship of ploidy with climate were explored. As hypothesized, high levels of genetic diversity were found in the study populations, with most of the populations containing small multi-ramet clones and unique genotypes interspersed among a few larger multi-ramet clones with distances between ramets ranging from 600 to 1700 m. New genotypes appear to be

established in the studied aspen populations either via seed establishment, genotype banks in the root systems, or most likely through a combination of both strategies. The genetic diversity found in aspen populations is further complicated by somatic mutations, which can accumulate over cycles of stem dieback and regrowth. The distribution of trees of different ploidies was not significantly influenced by CMI, yet there was a pattern of areas with low CMI (i.e., less available moisture) having the highest proportions of polyploids (tetraploids and triploids). Polyploids had greater diameter growth, while wood density was not different between cytotypes. Additionally, diploids had greater resistance to drought, while triploids had greater recovery after drought. Tetraploids had higher diameter growth, resistance, and recovery to drought when compared to diploids, showing a high adaptive potential.

5.2. Management implications and policy impacts

Changes in climatic conditions, such as an increase in temperature, a reduction in moisture availability, and longer growing seasons, have been reported since the 20th century (Keenan 2015; Hynes and Hamann 2020; Eum et al. 2023). The province of Alberta, in Canada, is getting warmer and drier due to climate change (Gray and Hamann 2015; Eum et al. 2023). In the face of this reality, forest managers need to adapt their strategies to consider future climatic conditions. The findings of Chapter 2 highlight the potential that aspen seedlings have to persist and survive under a changed future climate, by relying on plasticity and genetic diversity. The growth of seedlings was negatively affected by drought and aspen seedlings from regions of higher and lower water availability showed similar levels of plasticity to drought, while seedlings from the parkland region had lower SLA, and consequent higher water use efficiency, which is an adaptive trait to drought. High levels of plasticity in aspen may allow the species to survive abiotic stress (Goessen et al. 2022), however, the capacity to reach their full genetic potential may be limited under drought. The variability in performance in studied traits, shown by different families, displays the importance of knowing the characteristics of clones used for selection and breeding. While some clones grew better under both well-watered and drought conditions, which would make them preferred choices for selection to be used on a tree improvement program, other clones show potential for survival and growth under severe drought conditions, which may become the norm for some areas in Alberta and Canada in the near future (Hynes and Hamann 2020). I recommend that future clonal testing and selection of genotypes for the aspen improvement program in Alberta include, in addition to clonal selection for gains in height and volume, selection for traits that maximize survival and growth under drought conditions,

such as the traits described in Chapter 2. Interestingly, the lack of difference in total biomass and growth between female and male aspen seedlings under both well-watered and drought conditions shows that aspen does not present sex differential energy costs before reproduction. Nevertheless, female and male clones appear to have different water-use strategies under well-watered and drought conditions, consequently, females may ultimately be more sensitive to drought and cavitation (Olano et al. 2017; Liu et al. 2022; Goessen et al. 2022) due to higher hydraulic efficiency (Liu et al. 2022). Further studies are needed to explore wood characteristics and hydraulic efficiency in aspen with an emphasis on sex differential performance under drought.

Chapter 3 challenged the premise of costs of reproduction associated with the production of seed vs. pollen in aspen. Aspen seeds are small and have little to no endosperm (Einspahr and Winton 1977; Jelinski and Cheliak 1992) and therefore the energy costs to produce pollen and seed may not be very different. Instead, the differences in presence on the landscape between mature female and male aspen trees may be associated with differential reproductive strategies (i.e., sexual or vegetative reproduction), production of defence compounds, investment in stem density, or higher allocation of growth to either above or below-ground parts. The Alberta Forest Genetic Resource Management and Conservation Standards (FGRMS 2016) regulates that seed or vegetative materials produced from controlled parentage programs may be deployed for the purposes of rehabilitation and intensive deployment, subject to the condition that for deployment in a continuous area of 10 ha or more, at least 20% of deployed clones must be known to be male and 20% known to be female. Based on the findings of Chapter 3, considering that female aspen trees may invest more energy into the production of roots and consequently into asexual reproduction, while males may invest more energy into wood density, it may be a good approach to deploy a higher percentage of known male clones, for higher gains in wood quality, while maintaining a minimum known percentage of female clones. Deploying a known percentage of both sexes is important as it allows for sexual reproduction and genetic diversity to be maintained in the population. Despite having different water-use strategies under drought when seedlings (Chapter 2), mature female and male aspen trees and seedlings showed similar growth under drought. In general, sex performance does not appear to influence the growth or productivity of aspen seedlings under drought conditions, therefore I do not recommend the deployment of a specific sex for wetter or drier areas.

The complex diversity of aspen genotypes, as found in Chapter 4, speaks to the plasticity that this tree species has in its means of reproduction, as it can reproduce sexually

and asexually. While it is difficult to find natural seedling establishment of aspen (Namroud et al. 2005), especially in mature stands, the input of new genotypes is still occurring. Evidence points to the existence of genotype banks stored in the root systems of aspen stands (Jelínková et al. 2009), in addition to post-disturbance seedling establishment (Mitton and Grant 1980; Landhäusser et al. 2019) and the variability brought about by somatic mutations and its effects on gene expression (Van de Peer et al. 2021). Multiple unique genotypes were found in stands expected to be comprised of a single or few multi-ramet clones (based on phenotype, e.g., bark colour and texture, and branching pattern), at the same time, ramets of some multi-ramet clones were found in stands expected to be comprised of different clones (over the transects in different aspen populations). To avoid collecting wild material from the same clones while considering them to be different, FGRMS (2016) regulates that collections of seed or vegetative materials from stands of native species must be separated by at least 500 m. In the current study, ramets were sampled in stands separated by at least 500 m, still, ramets of the same aspen clone were found in distances ranging from 600 to 1700 m. Realistically, the chances of collecting material from the same clone when stands are separated by 500 m may be very low, considering the high levels of diversity and the presence of unique genotypes within populations. However, to reduce the likelihood of collecting material from the same clone, while expecting them to be from different genotypes, the distance between point collections could be increased from 500 m to 1000 m. In my study, only one multi-ramet clone extended beyond 1000 m. Lastly, the superior growth and drought responses of polyploids, compared to diploids, showed the potential of using polyploid aspen in tree improvement programs. I recommend the selection of tetraploid clones for trials aiming at genetic gains in volume and resistance to drought. Additionally, I recommend clonal propagation of triploids in areas exposed to recurrent drought stress, as this cytotype showed better growth performance post-drought. Similarly, the ploidy of plus trees selected for improvement should be known, so managers can associate their performance and gain with potential ploidy effects. Further research could explore the growth and survival of diploids, triploids, and tetraploids of aspen when subjected to successive drought events, to better understand cytotype responses before, during, and after drought.

5.3. Ecological implications and study limitations

Foundation species are abundant in an ecosystem; they form the base of a network of ecological interactions, provide structural support to other species, and regulate fluxes of energy and nutrient flow through the system (Ellison 2019). Aspen is a foundation species of great

ecological importance in Canada, and Alberta, due to its widespread distribution and its capacity to support biodiversity and provide ecosystem services (Rogers et al. 2020). In the northern hemisphere, stands of different aspen species have been associated with increased landscape biodiversity, where the decline in aspen has resulted in a decline in the diversity of animal and plant species in some areas (Kouki et al. 2004; Rogers and Ryel 2008; Griffis-Kyle and Beier 2003).

My study showed how drought negatively influences growth in aspen and limits seedlings from reaching their full genetic potential. The aspen range in Alberta is limited by drought where the southern range of the species, in the parkland region, coincides with a threshold of negative to positive water availability (based on climate moisture index; CMI), while the boreal region has historically higher water availability, allowing the maintenance of a closed canopy forest (Hogg 1994). Climate data for the collection points used in Chapter 2 showed that this pattern of available moisture is starting to shift, as study sites in the parkland and boreal region had negative CMI for the past 30 years. In dry and cold climates, such as in Alberta, frequent periods of drought occurring over consecutive years can leave a prolonged effect of reduction in the growth and productivity of aspen (Hogg et al. 2013). Additionally, widespread aspen mortality was observed after a severe drought event from 2000 to 2003, and the effects of this drought resulted in increased aspen mortality that extended until 2011 (Worrall et al. 2010; Worrall et al. 2013; Anderegg et al. 2013).

The increase in frequency and intensity of drought events in Alberta (Gray and Hamann 2015; Keenan 2015; IPCC 2022; Eum et al. 2023) may negatively impact aspen stands in the province. The southern range of the species could shift north compared to the current range, while boreal aspen stands could change structurally and functionally, becoming more similar to the parkland region today (e.g., small patches of aspen trees, low growth, and higher proportion of edge habitats; Hogg and Hurdle 1995). Such changes may result in reduced biodiversity of animal and plant species that rely on the ecosystem services provided by the aspen stands (Kouki et al. 2004; Rogers and Ryel 2008). The effects of drought are further complicated by the inhibition of the genetic potential of aspen under drought, which limits the plasticity of traits such as growth under severe conditions. On the other hand, the potential adaptive capacity of polyploids seen in Chapter 4, including better resistance and recovery to drought, suggests that aspen stands may continue to persist on the landscape, even under future scenarios of severe drought. Further research and conservation efforts on wild aspen stands should explore ploidy in relation to drought resilience and adaptive potential. Lastly, previous studies found a

differential distribution of female and male *P. tremuloides* driven by environment, where males were predominantly found at higher elevations or in drought-prone environments (Grant and Mitton 1979; Goessen et al. 2022). My study did not find an association between the distribution of sexes and environment, still, the hypothesis of sex differential performance under stress should not be discarded. The study sites used in Chapters 3 and 4 were located within the southern range of the AW2 region. All the study sites had positive climate moisture index (CMI > 0) for the climate normal period 1960-1990. Positive CMI values for an area, where CMI equals precipitation minus potential evapotranspiration, indicate that conditions are sufficiently wet to sustain a closed canopy forest (Hogg 1997). Future studies on the subject should explore the distribution of female and male aspen in relation to the environment, more specifically to drought, in climatically different regions (CMI < 0 vs. CMI > 0). Sex differential distribution driven by environmental stress would shift aspen sex ratios, potentially towards male-biased ratios in dry regions, this, in turn, may negatively affect sexual reproduction in aspen populations, influencing rates of adaptation, and reducing the effective population size across the landscape (Petry et al. 2016).

The research experiment and observational studies included in this thesis were performed with a substantial amount of resources and financial support, however, my study has potential limitations derived from available time, space, financial resources, and due to the COVID-19 pandemic. The study design in Chapter 2 included one female and one male aspen seedling of each family, per treatment, on each block. The study included 19 families, two treatments, and eight blocks, for a total of 608 seedlings. 608 is a large sample size, developing and running a drought greenhouse experiment of such dimensions was a difficult and time-consuming task. Finding a greenhouse space available to run an experiment with 608 seedlings was also a difficult task. Nevertheless, for the statistical analyses, it would have been better to include a larger sample size of sex by family and treatment, to better represent the population. However, increasing sex sample size would reduce the number of blocks, as the number of seedlings genotyped by sex within each family was limited, and increase the potential bias caused by variation in the data. In the end, the approach used seemed to be the most effective in terms of space and resource use. In addition, it would have been beneficial for the greenhouse experiment if it was run for a longer period of time. However, the experiment was performed in 2020, the year of COVID-19. The COVID-19 pandemic slowed down research, with the closure of universities and businesses all over the country. Additional time and effort were required to have all the paperwork, hiring, and training of people organized in a closed

university, by the time we could plant the seedlings in the greenhouse it was already May 2020. After that, another three weeks were needed to acclimate the seedlings before the beginning of the experiment in June 2020. By the end of July, the seedlings were setting buds, so they were not actively growing anymore, and the experiment ended. Under different circumstances, it would have been better to perform the greenhouse experiment for a longer time. Additionally, the families included in Chapter 2 were not genotyped for clonal fingerprinting. In this case, the distances between parent tree selections were used to ensure, as much as possible, that parent trees were different clones/genotypes. Families F7 and F8 were separated by 9 m (with a wetland in between the trees), and families F11 and F12 were separated by 8 m (on a gravel road). When the parent trees were selected for the greenhouse experiment, we did not have the results from Chapter 4. Considering the results of Chapter 4, families F7 and F8 might be one single family, and the same is applicable to families F11 and F12. The parent trees selected for the remaining families included in Chapter 2 were separated by larger distances and are unlikely to be the same clones.

The greatest limitation of Chapter 3 was the sampling of sites only within the AW2 region, for the exploration of sex distribution as a response to drought stress, as discussed earlier. In Chapter 4, the delineation of aspen populations could have been done with a standardized plot and sample size for all study populations. Standardizing, however, would not have accounted for variation between populations, in terms of stand density and phenotype, but it would have made the sampling more consistent. In addition, one of the primer sets (PTR4), did not amplify well, reducing the number of markers available for clonal fingerprinting in our study. We could not have predicted that a microsatellite marker would not amplify, but it would have been safer to use more microsatellite markers in the analyses, to increase the accuracy of results even under unpredictable scenarios. Finally, cytology is a very difficult method to work with, as it is complicated to perform, it is time consuming, and the results obtained do not have high accuracy. The resolution of the images obtained from the root tip spreads was not particularly sharp and it was generally difficult to see all the chromosomes, as expected as aspen chromosomes are generally very small. It was possible, however, to identify chromosome 1 in some cells on each genotype, but it was not possible to identify all chromosomes, even following the original methods used in previous studies. Although other methods, such as flow cytometry, are available to verify molecular results, those methods also have their own drawbacks.

Researching trembling aspen is not a trivial task. It is very challenging to understand the patterns of distribution and stand and clonal establishment in a species that propagates vegetatively through root suckers. The study of sex differences is complex as the mechanisms behind sex expression are not yet completely understood. Lastly, the study of genetic diversity of aspen stands is further complicated when taking into consideration the effects of somatic mutations and ploidy. Ultimately, future research will be essential in answering many of the questions that remain regarding the patterns of sex expression and genetic diversity in trembling aspen.

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Appendices

Appendix 1. Trembling aspen controlled parentage programs in Alberta

As specified by the Alberta Forest Genetics Resource Management and Conservation Standards (FGRMS; 2016):

> *"Controlled parentage program (CPP)* seed movement guidelines and *deployment* rules differ from natural stand seed movement guidelines because seed or *vegetative propagule* production is carried out under several production scenarios involving various *production sites*, *production populations* and *production units*.
>
> *Deployment* of improved varieties is controlled by a *CPP region* system. Each *CPP region* is based on a target *deployment* area for a single species. *Seed orchards* are mostly developed on agricultural land outside the forest zone to reduce outcrossing with contaminating pollen. Repeated collections are made from the same trees, and control is exercised on genetic composition, genetic quality and *genetic diversity* of seedlots. *CPP regions* are not necessarily coincident with *seed zones*, as they are based on a target *deployment* area for a single species, and are generally accompanied by provenance, progeny and/or clonal testing.
>
> *Controlled parentage program regions* are initially delineated and mapped based on general genetic and ecological information, as well as administrative boundaries, and are reviewed and modified on the basis of further progeny and provenance testing. *Controlled parentage program regions* are also delineated on the basis of testing for adaptiveness of genetic material."

The currently approved CPP regions in Alberta include two trembling aspen regions: AW1 and AW2 (Figure A1-1 and Table A1-1).

Figure A1-1. Two trembling aspen regions in the Alberta Controlled Parentage Program. *Image from*: Gray and Hamann (2015).

Table A1-1. Area, latitudes, elevation, and description of approved trembling aspen improvement regions in Alberta's Controlled Parentage Program (CPP). Adapted from FGRMS (2016).

Species	CPP region	Area (ha)	Latitudinal range (°N)	Elevation operational range (m)	Description
Trembling aspen	AW1	6,594,381	56.00 – 58.00	350 – 750 north of 57˚N and 450 – 850 south of 57˚N	Lower boreal highlands Central mixedwood Dry mixedwood Peace River parkland
Trembling aspen	AW2	5,416,633	52.50 - 55.50	650 - 1050	Lower foothills Central mixedwood Dry mixedwood Upper foothills

Appendix 2. Chapter 2: greenhouse experiment, pre-experiment trials

Soil mixture

A trial to determine which soil mix would be used in the greenhouse experiment was established from April 16th, 2020, to May 6th, 2020. Four different soil combinations were applied to 24 pots to help determine what soil mixture would be best suited for the experiment's purposes and design. The objective was to find a soil with a mix:sand ratio that would allow drainage, but also would keep water available to the roots for a practical amount of time. The soil mixture also could not become hydrophobic in drought conditions, as the trees included in the drought experiment would be watered from a tray in the bottom, therefore the soil needed to absorb water available at the bottom of the pots through capillarity. The four different soil combination options were: 100% mix (Sunshine Mix #4); 1:1 mix:sand; 2:1 mix:sand; 1:2 mix:sand. This trial was based purely on soil mixture characteristics, but not on their interaction with the plant, as there were no plants on the pots.

Each soil mixture was added into six individual 4L square plastic pots: three for well-watered and three for drought treatments. All pots were weighed and had EC-5 (Decagon Devices, Inc.) soil moisture sensor raw readings measured twice a day (once in the morning and once in the evening). A percent weight reduction was calculated based on the pots' first weight at saturation. Pots belonging to the control treatment were watered every day and pots belonging to the drought treatment were not watered until they had reached the lowest observed weight reduction (15 days without watering).

The soil mixture considered to be best suited for the purposes of this experiment was 1:1 mix-sand, as it was slower to lose percent weight with water loss when compared to pure mix and 2:1 mix-sand, but it was quicker than 1:2 mix-sand. Also, the 1:1 mix-sand mixture would not become hydrophobic when dried out, as opposed to pure mix and 2:1 mix-sand. The 1:2 mix-sand could have been used when considering only its capillarity, but there would be greater soil runoff, and since sand is heavier, the percent weight reduction with water loss was minimal (when compared to the other options). Therefore, the 1:1 mix-sand soil mixture was selected for the greenhouse experiment.

Determination of soil moisture levels

Soil moisture levels were measured with the use of a ProCheck probe logger and EC-5 soil moisture sensor (Decagon Devices, Inc.). The EC-5 soil moisture sensor is a frequency domain sensor that is used to infer the volumetric water content (VWC) of soils through the measurement of the dielectric constant of bulk soil, as described by Campbell (2017). The EC-5 sensor senses the bulk dielectric constant and its relationship with soil water content (Campbell 2017). Sensor calibration is needed when a calibration curve is not included in the probe's default menu for the type of soil mixture used. The 1:1 mix-sand soil mixture was not included as a pre-established curve option in the menu of the Procheck handheld meter, so the sensor was calibrated for this specific soil type.

The process of calibration is completely described in Meter Environment (no date). Briefly, the process consists of slowly adding a known percentage of water content to an initially dry soil; controlling for volume and density; and measuring weight and VWC raw readings (EC-5 soil moisture sensor raw readings) for each step, until the soil reaches saturation. During this process, an amount of 1 mL of water for every 10 mL of soil volume must be added to increase VWC by approximately 10% (Meter Environment no date).

After completing the calibration process, the numbers obtained from the calibration samples were then used to derive VWC and water percent weight increase (or loss) for the experiment. For the 1:1 mix-sand soil mixture, every 10% increase in soil water content resulted in an average of 4.89% increase in weight. The same pattern was observed for weight loss. Raw sensor readings were obtained for each point of water content increase, for the creation of a calibration curve. The raw readings, then, were applied to the created curve, to generate VWC readings. The VWC values obtained could then be associated with the percent moisture levels known (based on each point of water content increase; Table A2-1).

The VWC values obtained from the probe's calibration process were then tested in another trial. This final pre-experiment trial included aspen seedlings in the pots and ran from June 5th, 2020 until June 15th, 2020. The trial consisted of 10 trees planted in 1.67 L round 15 cm (6") plastic pots containing an artificial soil mixture of Sunshine Mix #4 and sand (1:1 mix-sand; by volume), and slow-release fertilizer 14-14-14, with the same characteristics as the ones included in the greenhouse experiment. Those pots were in the same greenhouse space as the experimental trees. From those, five pots were assigned to a control treatment and five pots were assigned to a drought treatment, with the same target moisture levels as the ones included in the main experiment.

Pot weight and water content measurements were taken once a day for the duration of the trial. To monitor if percent weight reductions from water loss followed the ones expected for

the experiment (based on the first pre-experiment trial), pots were weighed daily, and their relative weight was obtained compared to weight at saturation. And, to confirm if VWC readings were indicating the moisture levels predicted (with reference to the percent weight reduction caused by water loss), two VWC readings were obtained from each pot, averaged, and associated with pot weight relative to saturation. The VWC at 100% saturation was scored as 100% VWC, and the subsequent reductions in VWC were then compared to 100% VWC, to determine the levels of VWC reduction achieved during the experiment. The results from this trial confirmed the reference moisture levels obtained from the probe's calibration. Those values were used as the reference of moisture levels for the duration of the experiment (reference values available in Table A2-2).

Table A2-1. Data obtained from EC-5 sensor calibration for 1:1 mix-sand soil mixture. Including the different points after a water content increase of 10%; soil was air dry at the first point and have reached saturation at the final point (point 6). Weight of the calibration container is provided after each 10% increase in soil water content. Percent (%) weight increase at a point is relative to the weight before the water content increment (e.g., the calibration container at Point 2 was 5.68% heavier than it was when soil was air dry). EC-5 sensor raw readings were collected at each point and converted into volumetric water content (VWC) levels after applied to the calibration curve; VWC numbers were provided as reference.

Points	Calibration container weight (g)	Soil water content increase (%)	Weight increase (%)	Sensor 1 (raw)	Sensor 2 (raw)	VWC 1 ($m^3.m^{-3}$)	VWC 2 ($m^3.m^{-3}$)
Air dry	4000.8	0	0	552	535	0.028	0.018
Point 2	4228.0	10	5.68	695	680	0.114	0.105
Point 3	4450.3	10	5.26	775	778	0.162	0.164
Point 4	4665.6	10	4.84	903	931	0.239	0.256
Point 5	4882.4	10	4.65	1164	1179	0.395	0.404
Point 6	5079.8	10	4.04	1222	1230	0.430	0.435
		Average	4.89				

Table A2-2. Reference moisture level values obtained from the second pre-experiment trial. Relative weight (%) is the pot weight at a determined moment relative to its own weight at saturation. The count represents the number of samples included in a specific group (or bin). Relative weights were separated into bins of 5% (from 100% to 70%). The values shown for sensors' raw readings and volumetric water content (VWC) are an average of the samples included in those bins. The mean of both VWC measurements on each pot is shown as the VWC mean, the percentage of VWC based on 100% saturation is shown at VWC %, and finally, the percent reduction in VWC is shown.

Relative weight (%)	Count	Sensor 1 (raw)	Sensor 2 (raw)	VWC 1 ($m^3.m^{-3}$)	VWC 2 ($m^3.m^{-3}$)	VWC mean ($m^3.m^{-3}$;)	VWC % ($m^3.m^{-3}$)	Reduction in VWC (%)
96 - 100	34	1042	1056	0.322	0.331	0.326	100.0	0.0
91 - 95	34	918	899	0.248	0.237	0.242	74.2	25.8
86 - 90	14	788	786	0.170	0.169	0.169	51.9	48.1
81 - 85	9	732	723	0.136	0.131	0.134	41.0	59.0
76 - 80	8	675	665	0.102	0.096	0.099	30.4	69.6
70 - 75	1	637	617	0.079	0.067	0.073	22.5	77.5

Appendix 3. Chapter 2: greenhouse experiment, supplemental data

This appendix contains miscellaneous supplemental data from the greenhouse experiment to complement the methods of chapter 2.

Table A3-1. Straight line distances between female parent trees for families 2 to 22, in the Peace River (boreal region) and Camrose regions (parkland region).

Families	Region	Distance
F2 to F3	Peace River	36.0 km
F3 to F5	Peace River	6.5 km
F5 to F6	Peace River	5.3 km
F6 to F7	Peace River	19.5 km
F7 to F8	Peace River	9.0 m
F8 to F9	Peace River	136.0 m
F9 to F10	Peace River	114.0 m
F10 to F11	Peace River	21.0 km
F11 to F12	Peace River	8.0 m
F13 to F14	Camrose	6.1 km
F14 to F15	Camrose	12.8 km
F15 to F16	Camrose	6.6 km
F16 to F17	Camrose	1.3 km
F17 to F18	Camrose	1.1 km
F18 to F19	Camrose	10.9 km
F19 to F21	Camrose	6.2 km
F21 to F22	Camrose	8.5 km

Table A3-2. Female parent tree information at catkin collection site, including parent tree identifier, region of collection, elevation, date of seed collection, and DBH of parent tree.

Parent tree (family)	Region	Elevation (m)	Date of seed collection	DBH (cm)
2	Boreal	806	23-May-18	24.5
3	Boreal	820	23-May-18	12.9
5	Boreal	701	23-May-18	25.3
6	Boreal	650	24-May-18	28.1
7	Boreal	649	24-May-18	31.2
8	Boreal	643	24-May-18	6.6
9	Boreal	645	24-May-18	39.4
10	Boreal	639	24-May-18	15.7
11	Boreal	498	24-May-18	10.2
12	Boreal	506	24-May-18	8.8
13	Parkland	737	29-May-18	15.5
14	Parkland	724	29-May-18	21.0
15	Parkland	720	29-May-18	9.5
16	Parkland	721	29-May-18	19.7
17	Parkland	726	29-May-18	28.1
18	Parkland	724	29-May-18	11.5
19	Parkland	761	29-May-18	21.1
21	Parkland	770	29-May-18	23.2
22	Parkland	819	29-May-18	22.6

Table A3-3. Results from seed germination tests and equilibrium relative humidity (eRH) measurements. The Alberta Seed Testing Standards (2016) categories of germination were used as follows: *i*, germinated, radical visible and at least 4x length of seed; *ii*, low vigour, radicle visible but not at least 4x length of seed, and; *un*, ungerminated seed, no radical.

Family	Seeds planted	Seeds germinated (*i*)	Seed germination with low vigour (*ii*)	Total seeds germinated (*i*+*ii*)	Ungerminated (*un*)	Germination (%)	eRH (%)
2	100	96	0	96	4	96	17.2
3	100	98	2	100	0	100	15.7
5	100	100	0	100	0	100	19.0
6	100	100	0	100	0	100	19.9
7	100	99	1	100	0	100	23.5
8	100	100	0	100	0	100	12.9
9	100	100	0	100	0	100	12.2
10	100	99	0	99	1	99	17.3
11	100	100	0	100	0	100	17.0
12	100	100	0	100	0	100	18.9
13	100	52	45	97	3	97	18.4
14	100	100	0	100	0	100	17.7
15	100	99	0	99	1	99	15.9
16	100	97	3	100	0	100	14.5
17	100	90	8	98	2	98	17.3
18	100	42	55	97	3	97	25.3
19	100	95	4	99	1	99	21.7
20	100	92	6	98	2	98	21.1
21	100	97	3	100	0	100	21.1
22	100	92	8	100	0	100	20.6

Table A3-4. Number of females and males identified in each family from DNA screening for sex obtained from 48 seedlings. The number of seedlings used in the experiment per sex was determined by the lowest number of seedlings available for a specific sex on a family. The number of blocks was determined based on the seedlings used in the experiment divided by two, as seedlings were allocated to two treatments (control and drought).

Family	Number of females	Number of males	Seedlings used in experiment (per sex)	Number of blocks
F2	22	26	16	8
F3	27	21	16	8
F5	26	22	16	8
F6	29	19	16	8
F7	28	20	16	8
F8	26	22	16	8
F9	29	18	16	8
F10	21	27	16	8
F11	18	30	16	8
F12	26	22	16	8
F13	28	20	16	8
F14	25	23	16	8
F15	25	23	16	8
F16	21	27	16	8
F17	27	21	16	8
F18	26	22	16	8
F19	29	19	16	8
F21	27	21	16	8
F22	31	17	16	8
Total number of seedlings used			608	

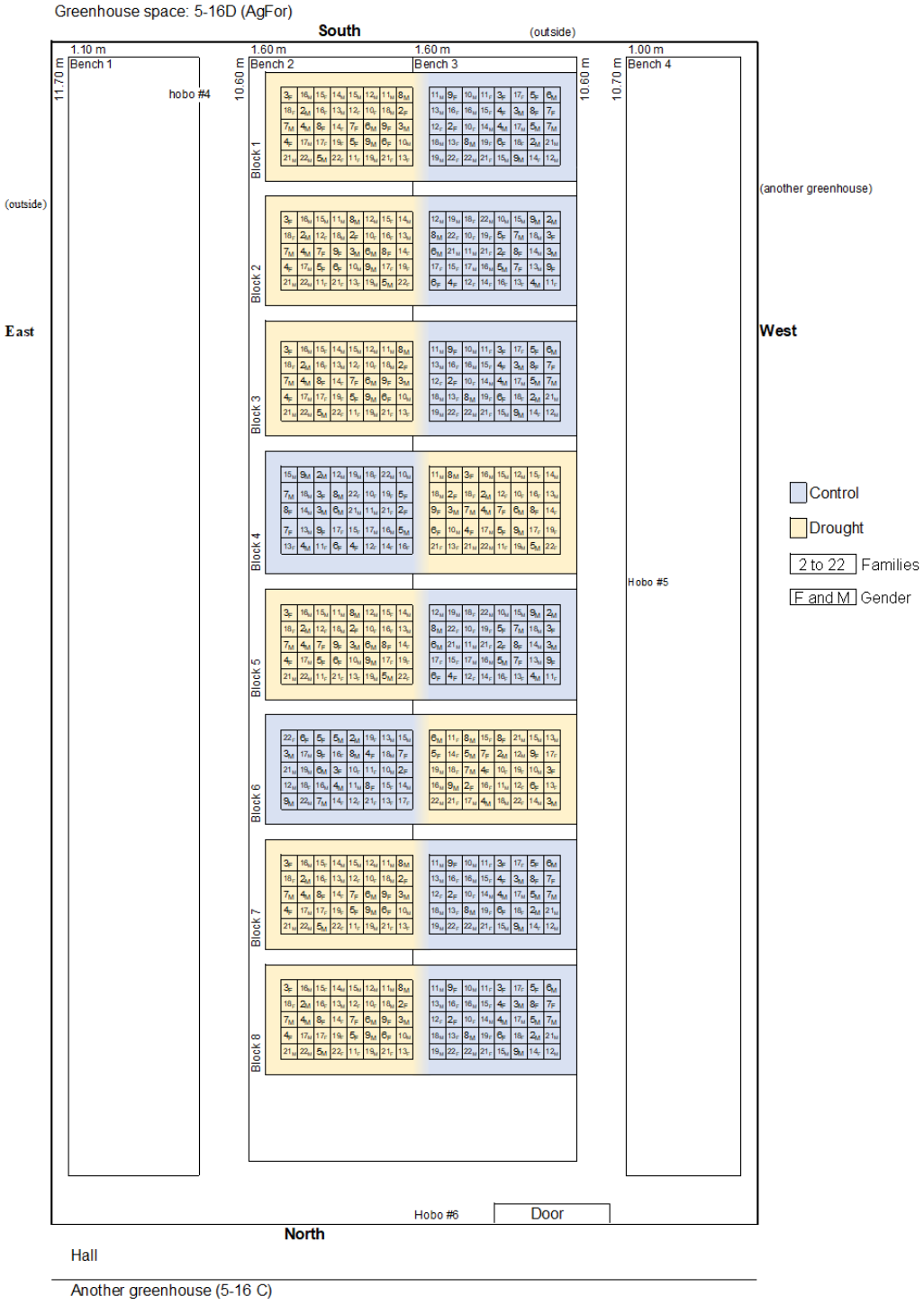

Figure A3-1. Schematic representing experimental design of the split-plot blocked design in the greenhouse showing treatments within blocks, positions of hobos in the greenhouse, and each individual pot identified based on its family and sex. Block and treatment positions remained static while pot position within each block and treatment was changed a minimum of every two weeks.

Appendix 4. Chapter 4: phylogenetic trees

The following UPGMA trees with $(\delta\mu)^2$ microsatellite distance were obtained from DNA analysis performed for Chapter 4. Bootstrapping of 100,000 UPGMA trees pairs the members with the smallest distance between them into a cluster and repeats that process until a rooted tree is created. Trees are built from data on each of the 12 aspen populations and the transects connecting the three populations within each area. With all trees care must be taken when adding outlier populations, as these tend to skew that dataset and lead to erroneous trees being produced. Hence, a tree was produced using only the clones of interest. Subsequently, two separate trees were created one adding in the outside trees, and a second tree adding in the transect trees sampled between two putative clones.

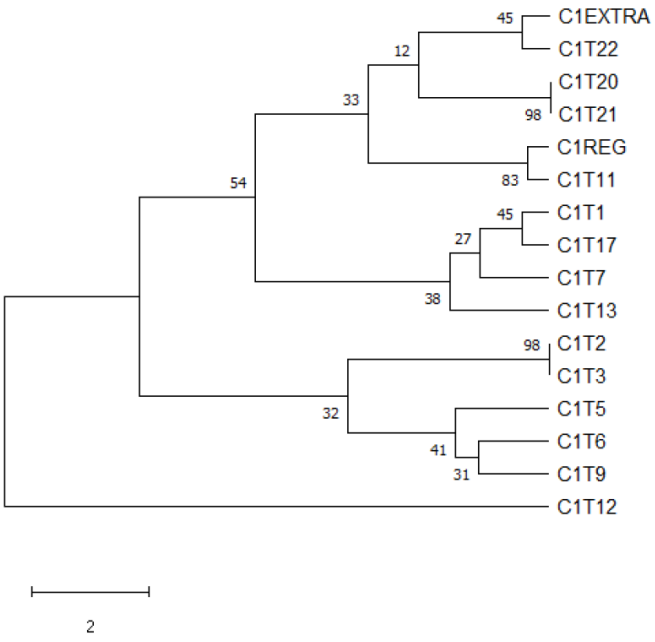

Figure A4-1. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 1. Tree identifiers include C for putative clone, 1 for putative clone identifying number, T for tree, and a unique identifier number for each tree, EXTRA refers to extra sample for testing, and REG refers to regenerating tree sampled on stand.

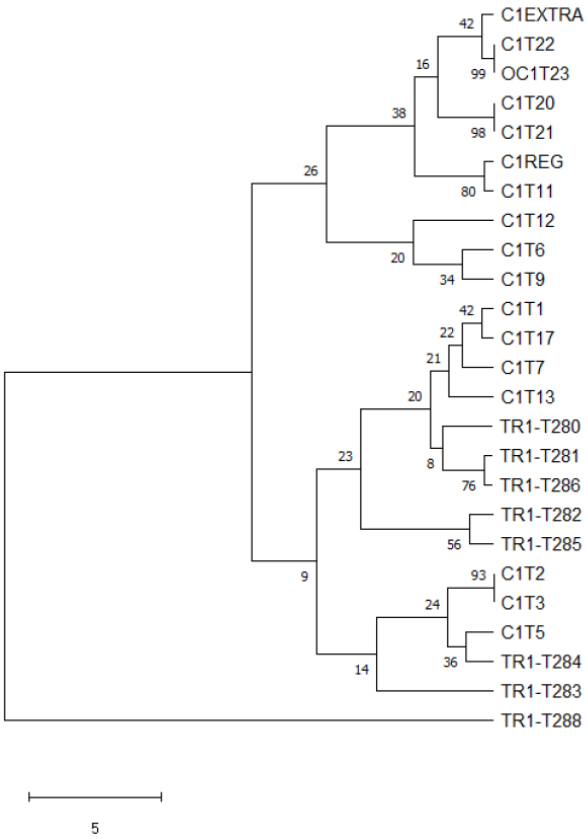

Figure A4-2. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 1 including outside trees and transect 1. Tree identifiers include C for putative clone, 1 for putative clone identifying number, T for tree, and a unique identifier number for each tree, EXTRA refers to extra sample for testing, and REG refers to regenerating tree sampled on stand, O refers to outside tree, and TR refers to transect.

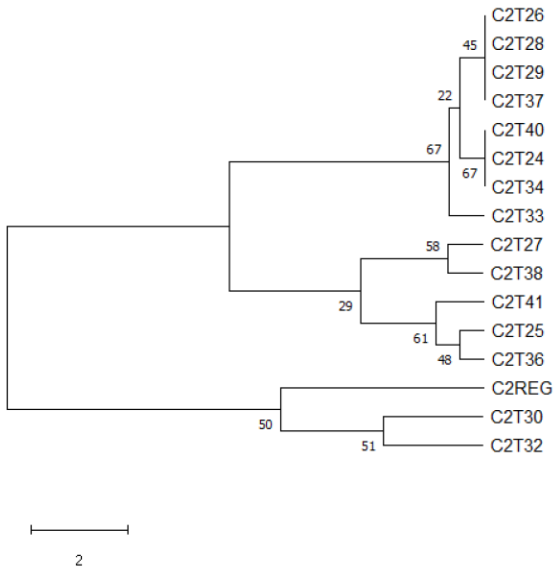

Figure A4-3. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 2.

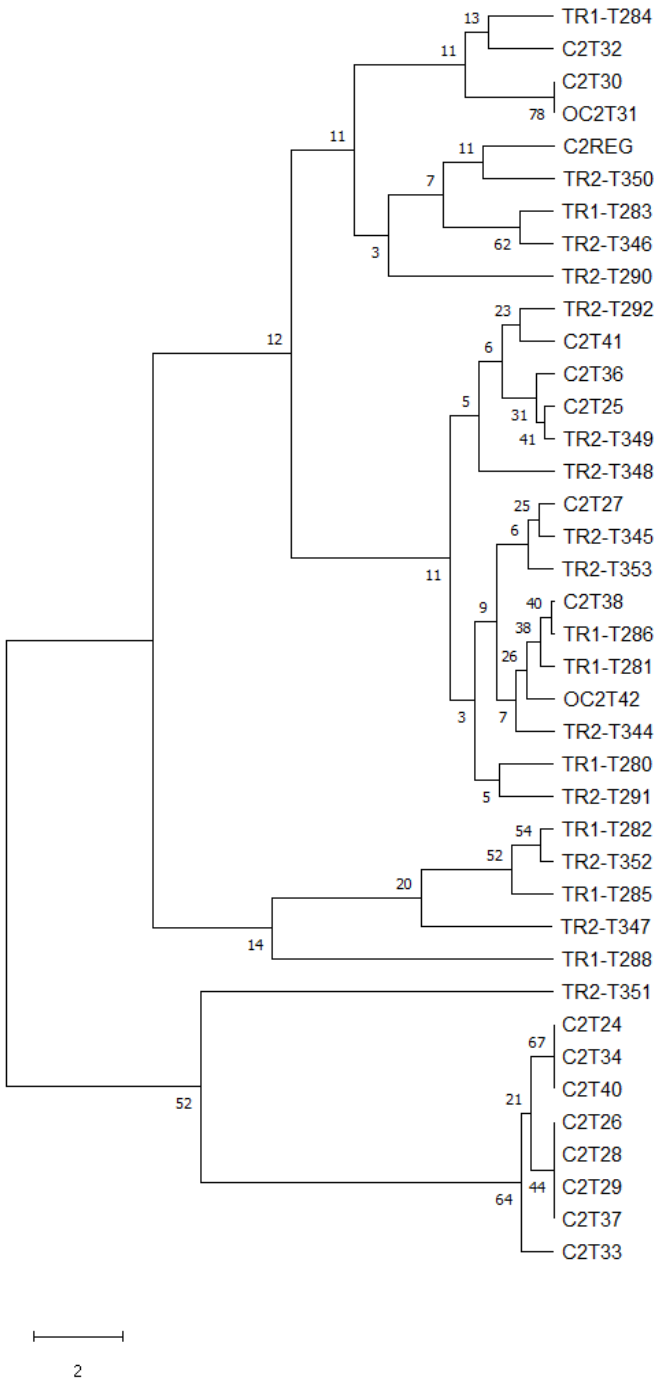

Figure A4-4. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 2 including outside trees, transect 1, and transect 2.

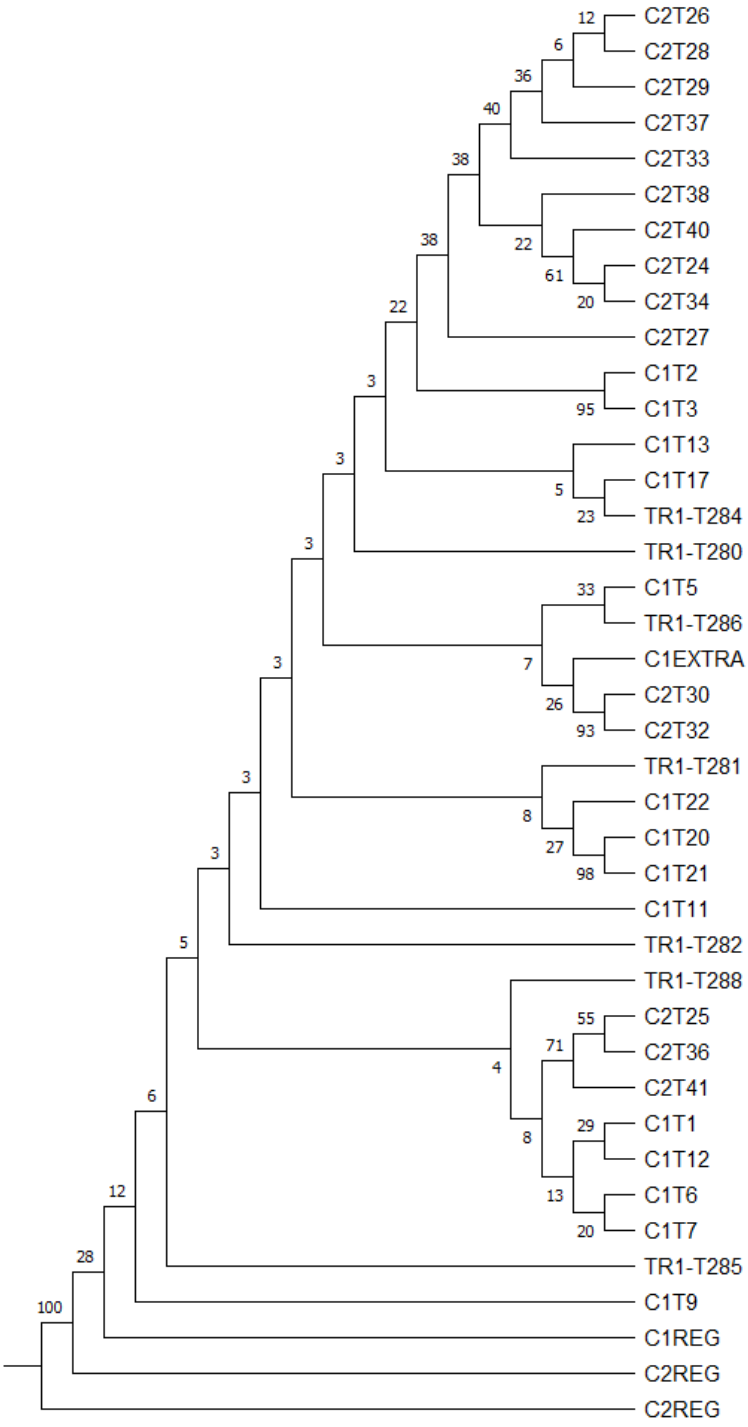

Figure A4-5. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clones 1 and 2 including transect 1.

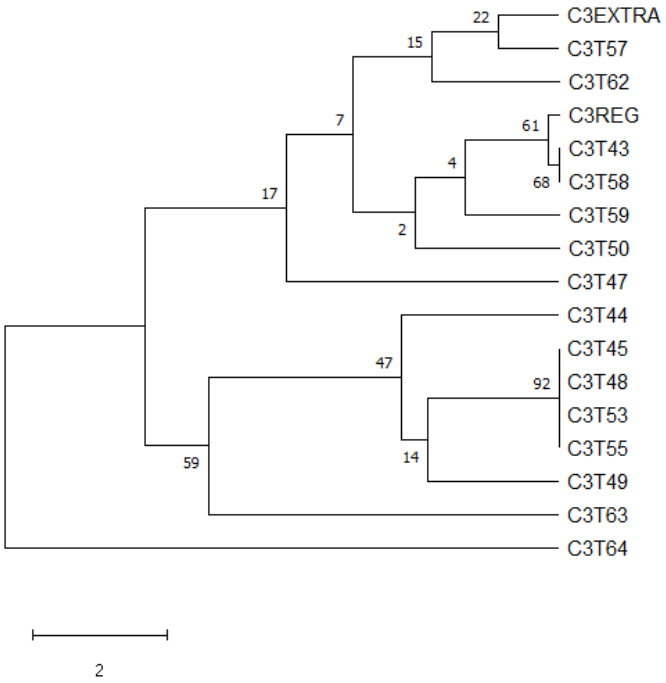

Figure A4-6. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 3.

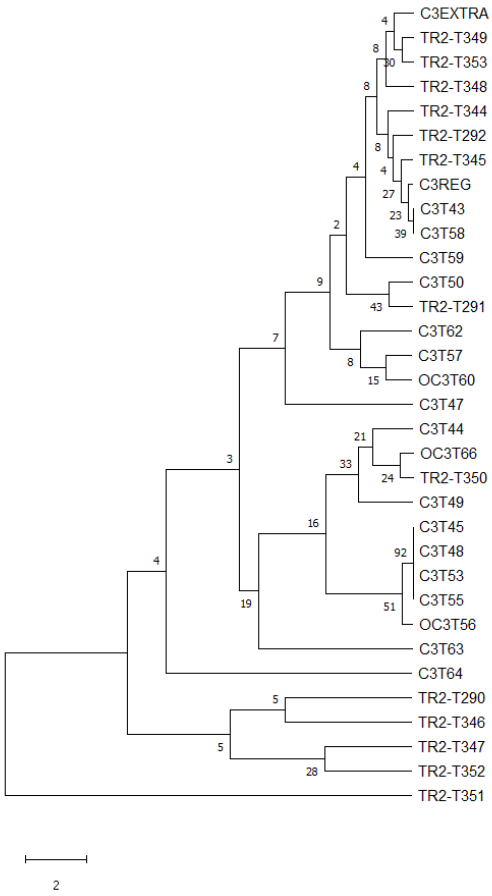

Figure A4-7. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 3 including outside trees and transect 2.

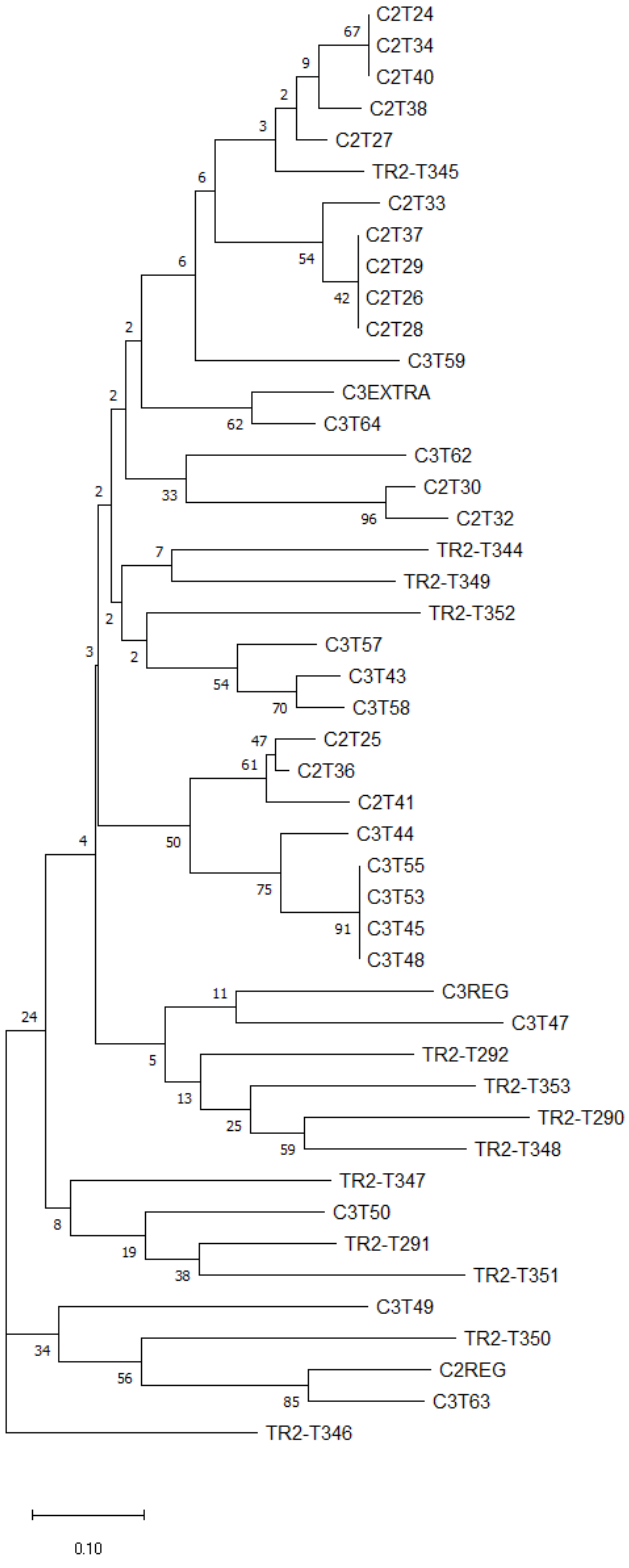

Figure A4-8. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clones 2 and 3 including transect 2.

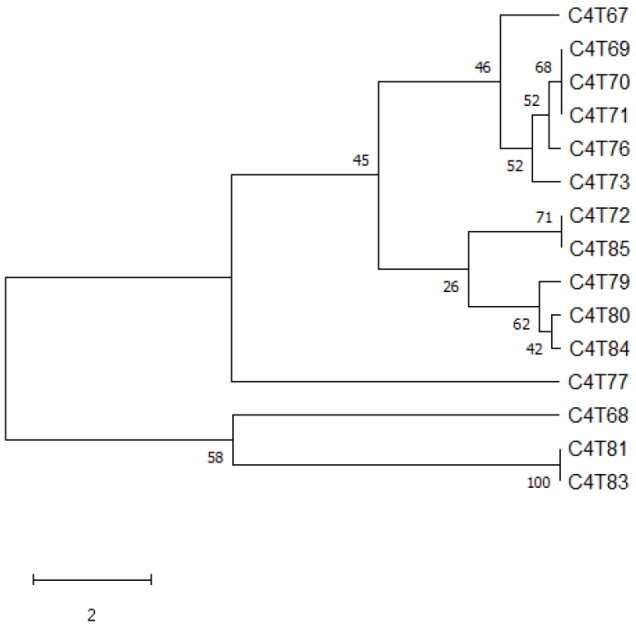

Figure A4-9. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 4.

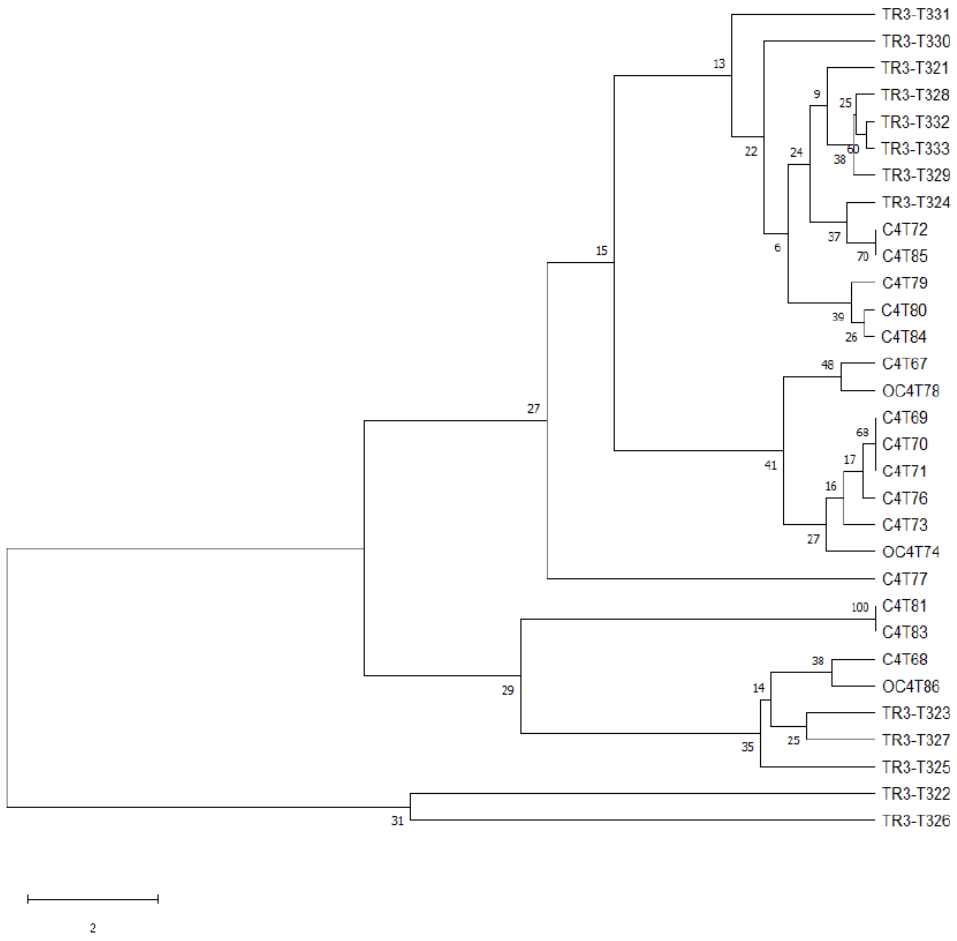

Figure A4-10. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 4 including outside trees and transect 3.

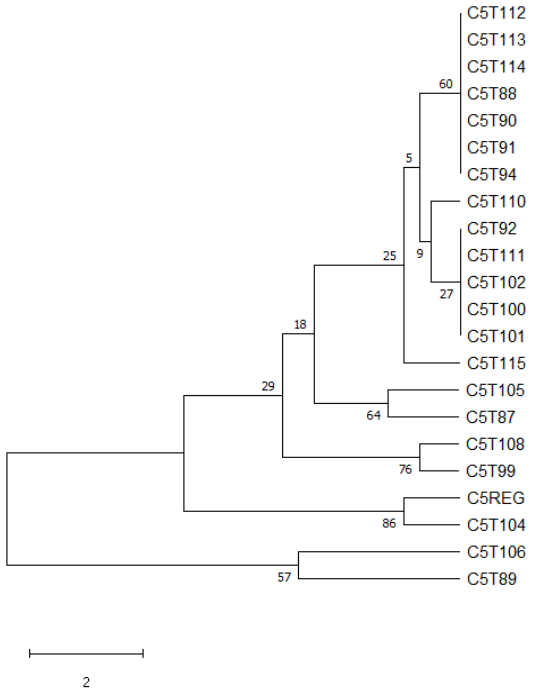

Figure A4-11. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 5.

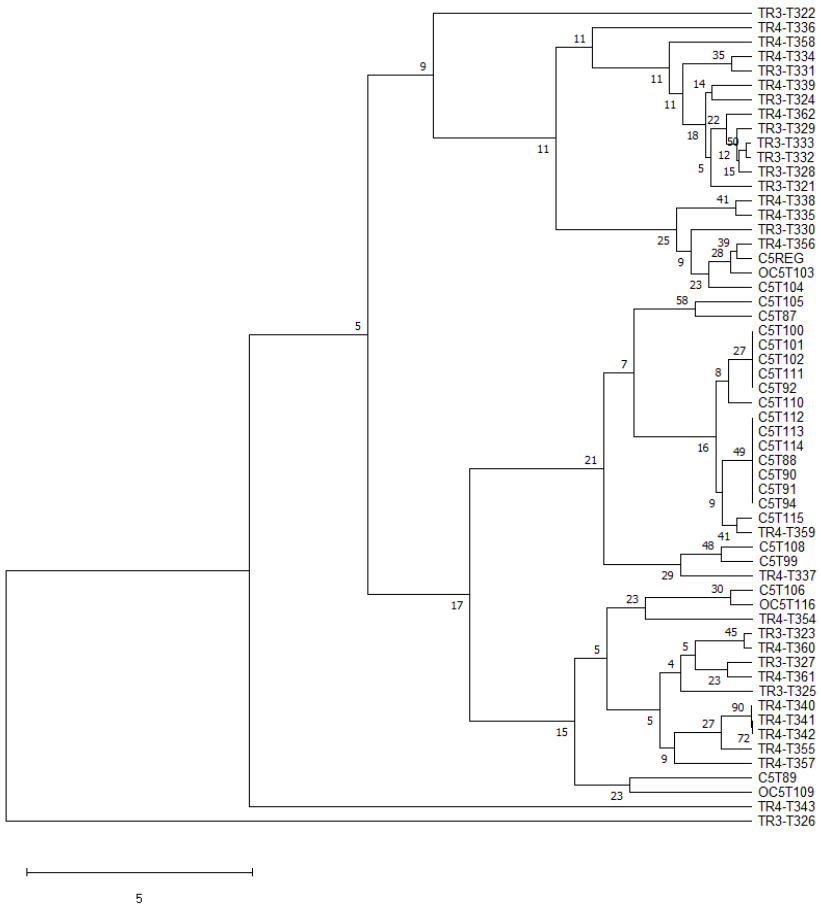

Figure A4-12. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 5 including outside trees and transects 3 and 4.

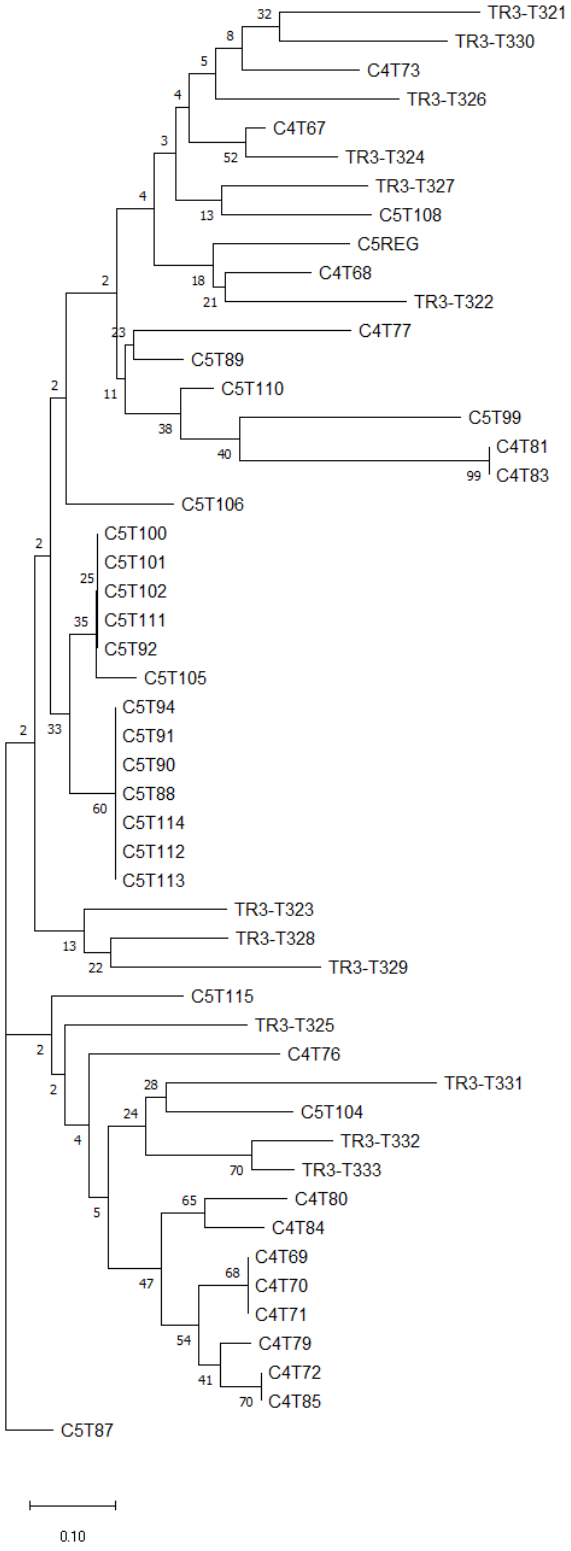

Figure A4-13. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clones 4 and 5 including transect 3.

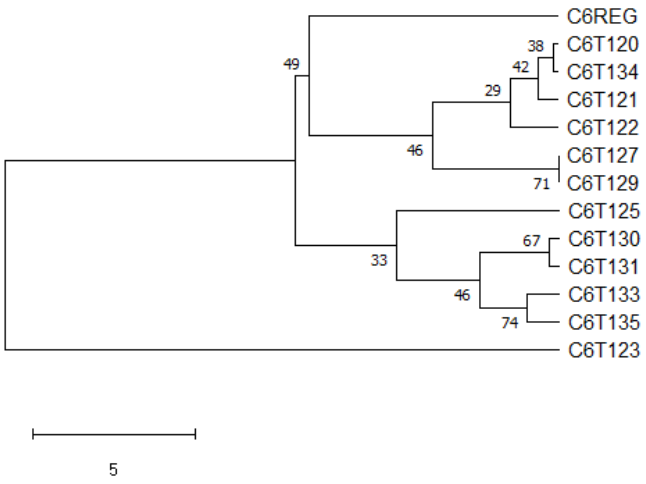

Figure A4-14. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 6.

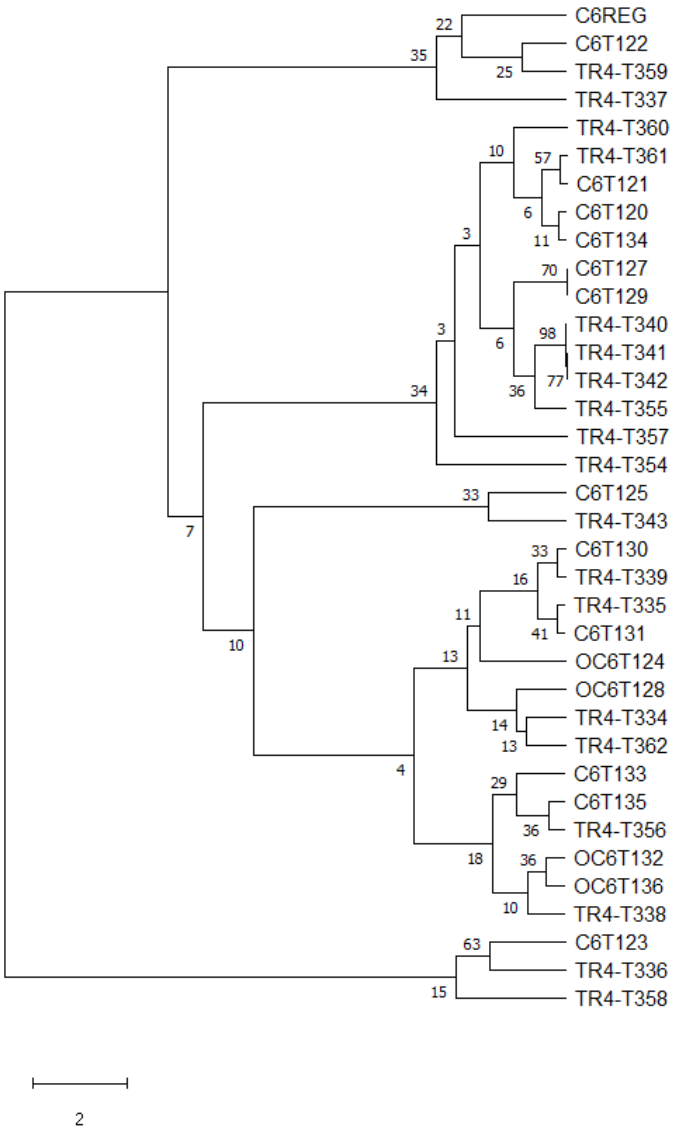

Figure A4-15. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 6 including outside trees and transect 4.

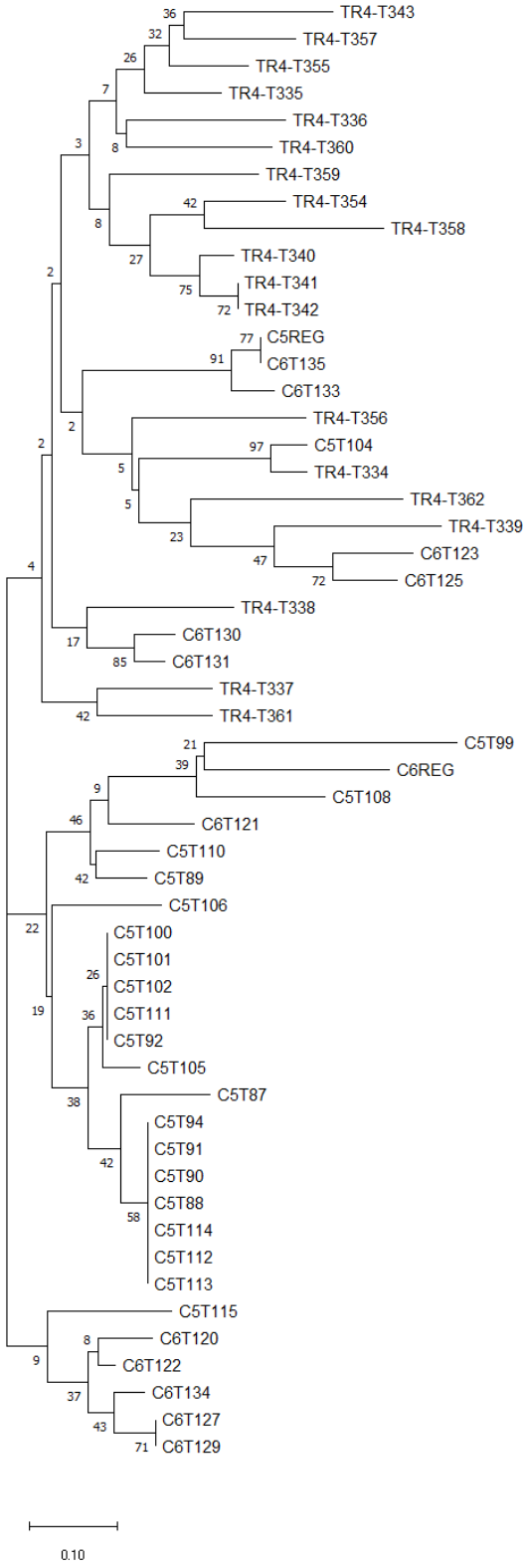

Figure A4-16. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clones 5 and 6 including transect 4.

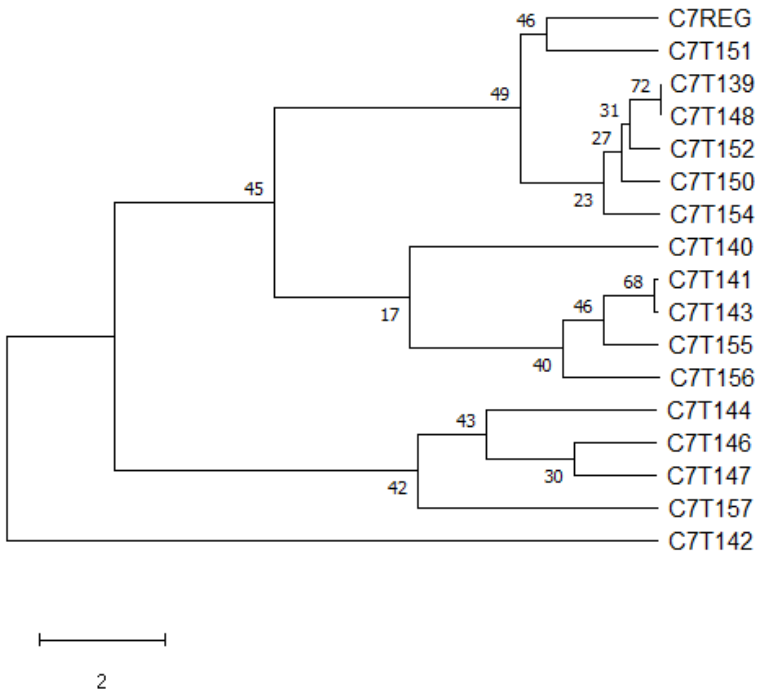

Figure A4-17. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 7.

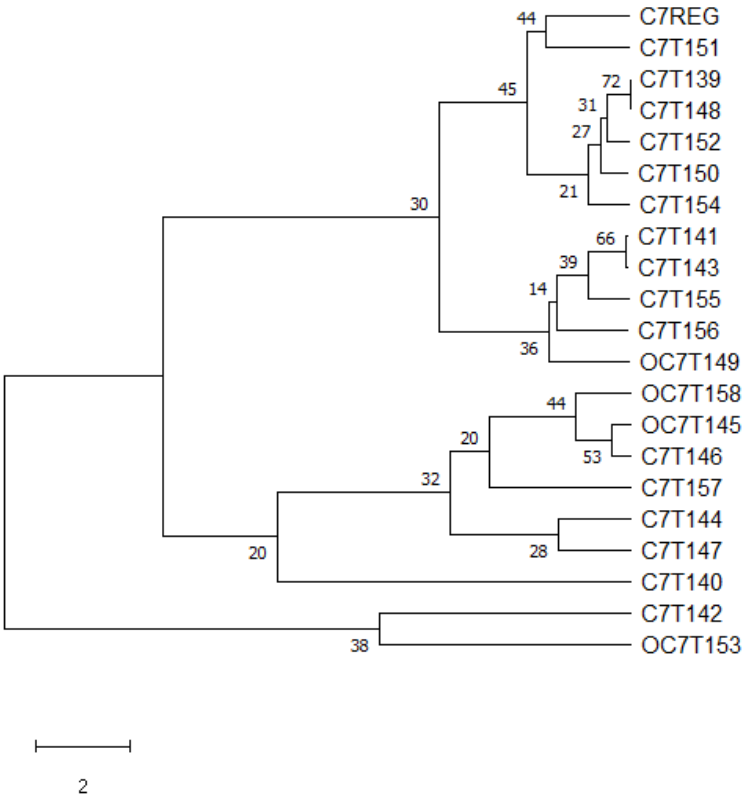

Figure A4-18. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 7 including outside trees.

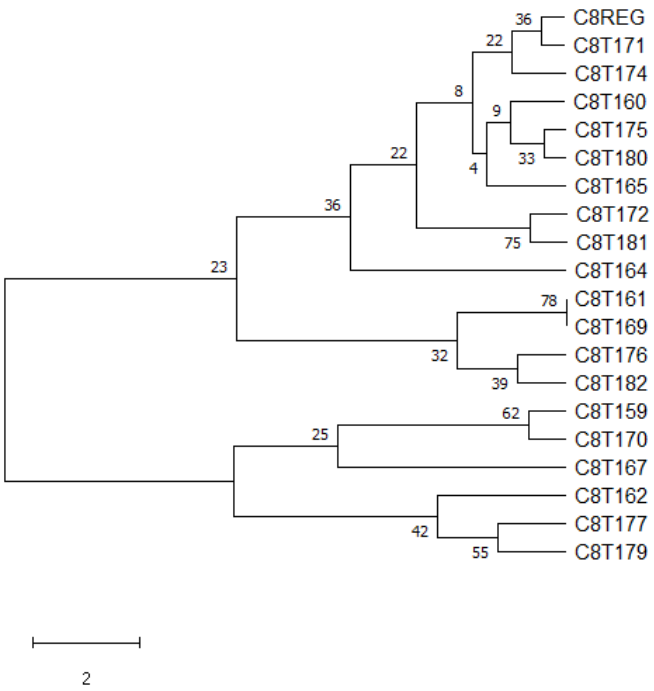

Figure A4-19. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 8.

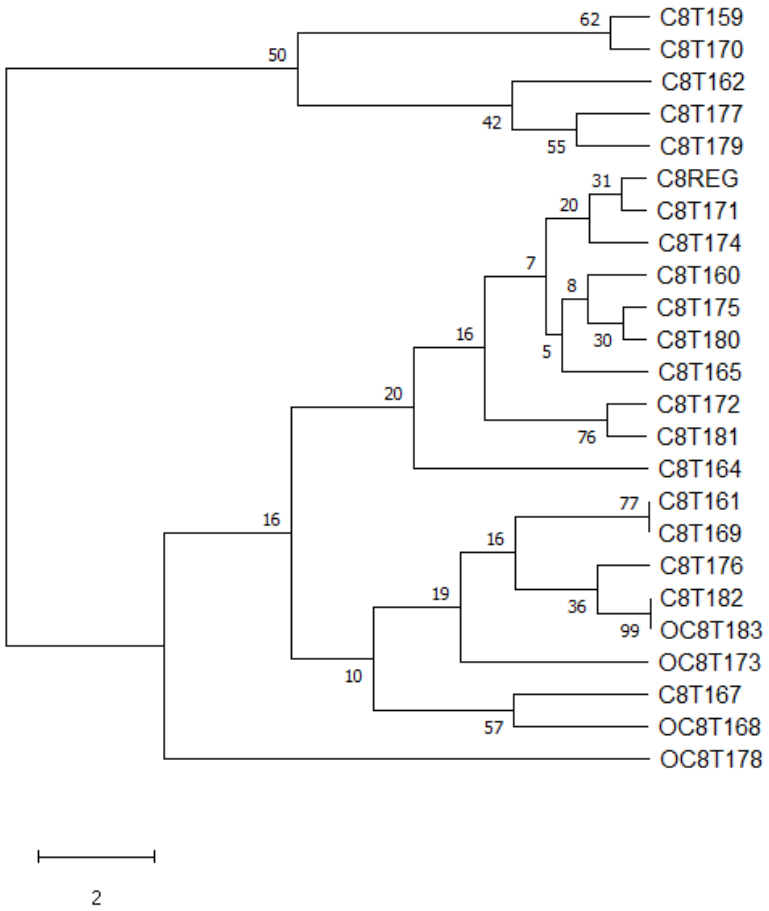

Figure A4-20. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 8 including outside trees.

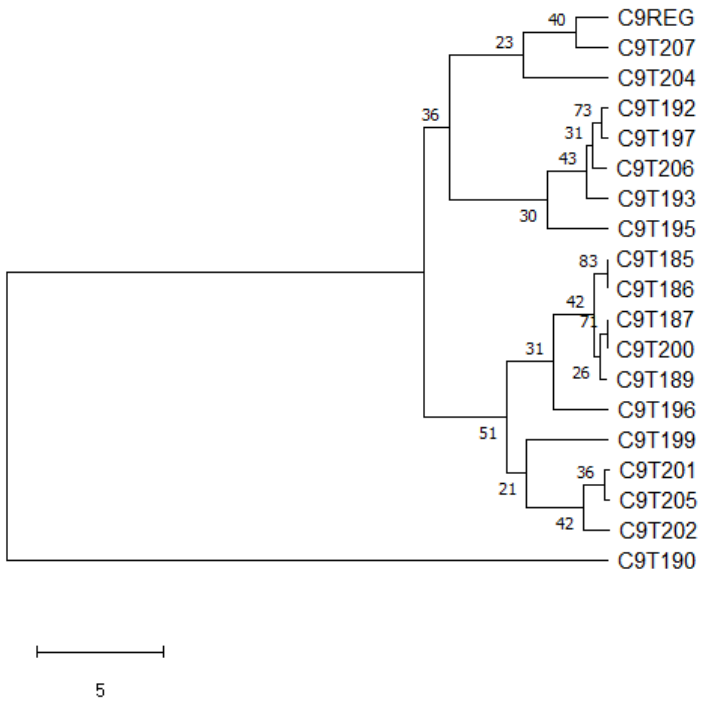

Figure A4-21. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 9.

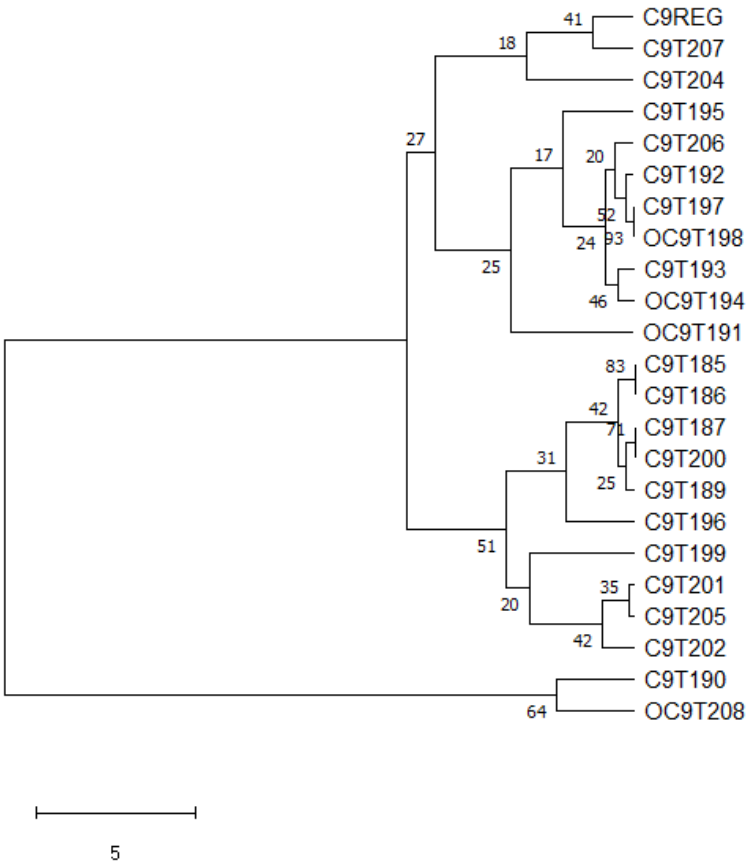

Figure A4-22. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 9 including outside trees.

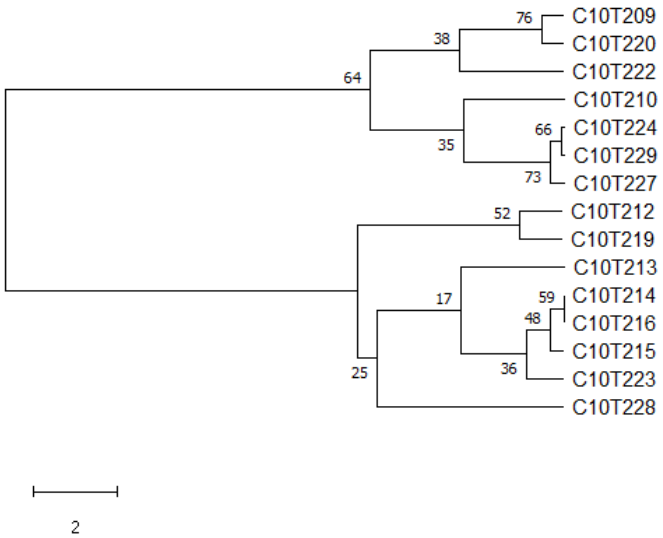

Figure A4-23. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 10.

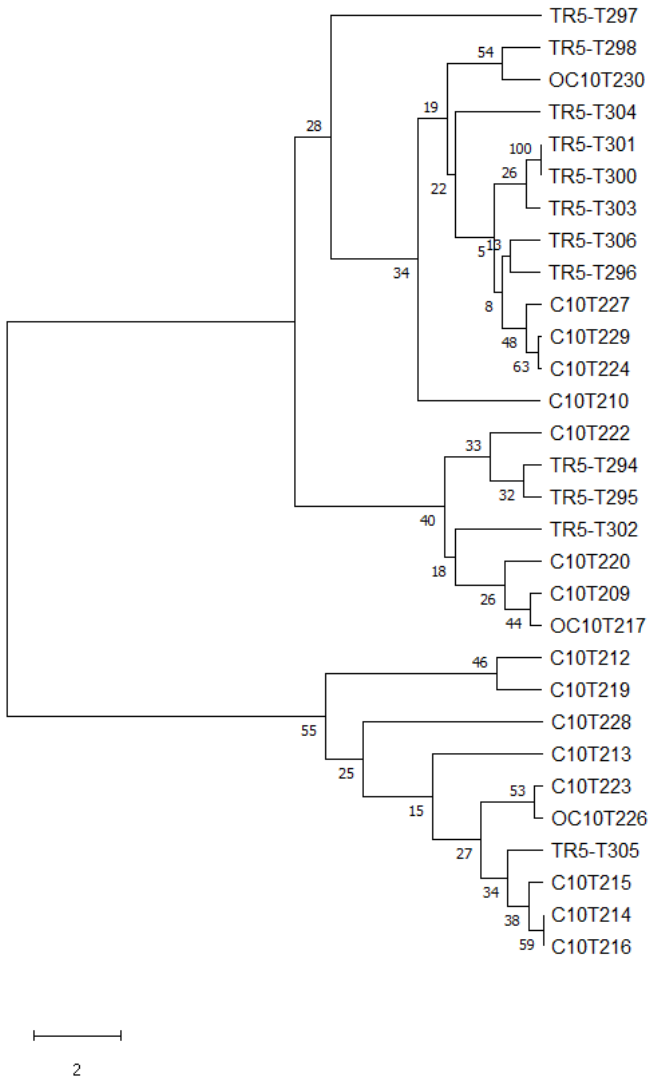

Figure A4-24. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 10 including outside trees and transect 5.

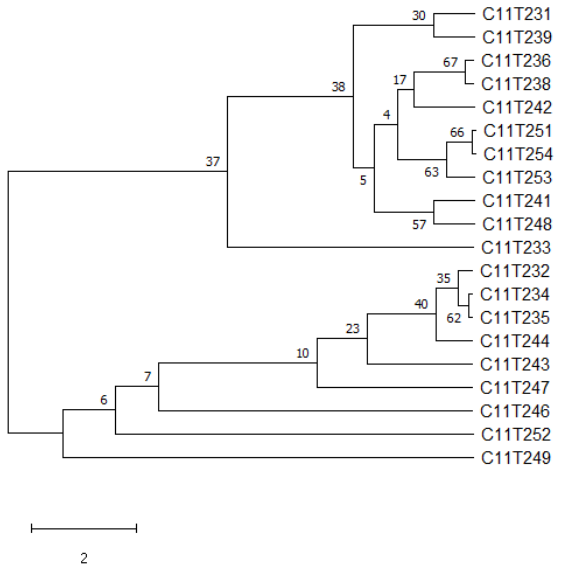

Figure A4-25. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 11.

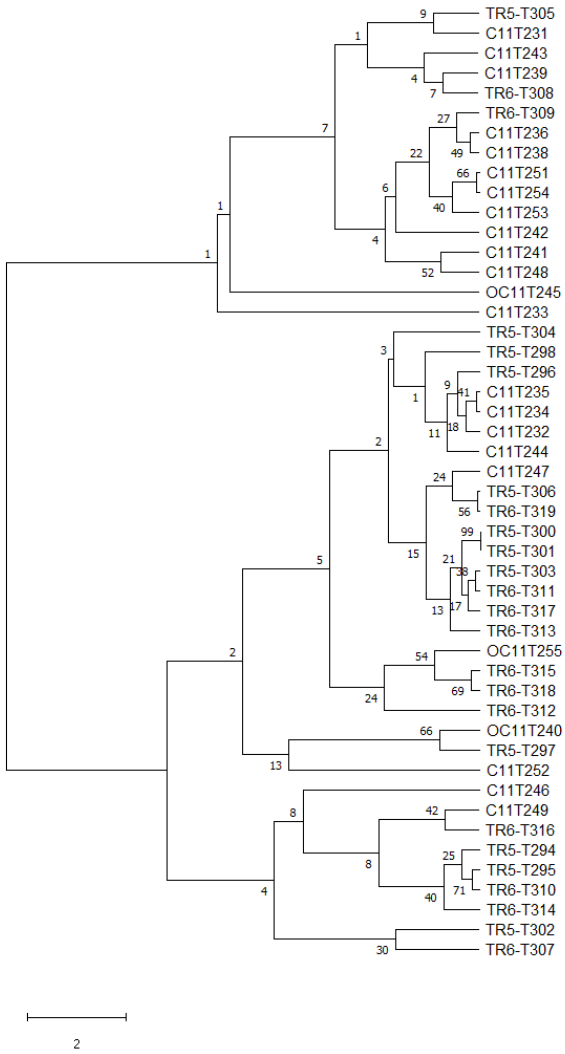

Figure A4-26. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 11 including outside trees and transects 5 and 6.

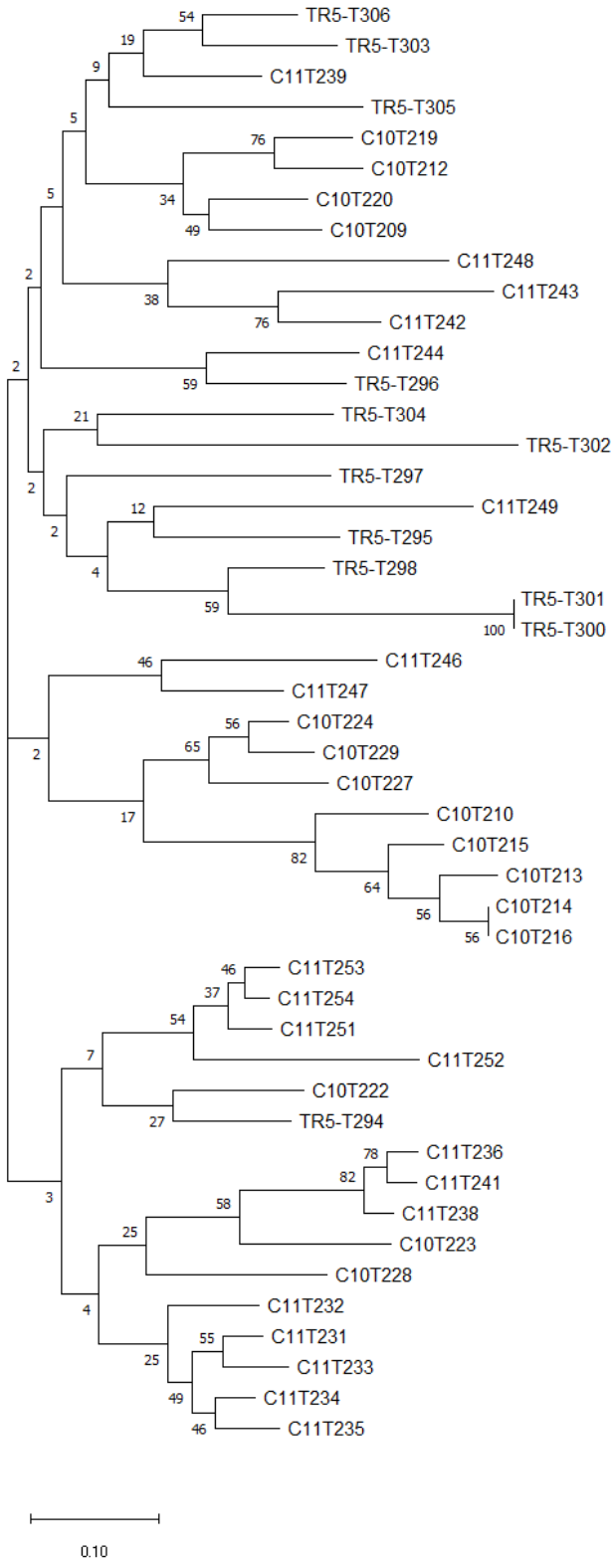

Figure A4-27. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clones 10 and 11 including transect 5.

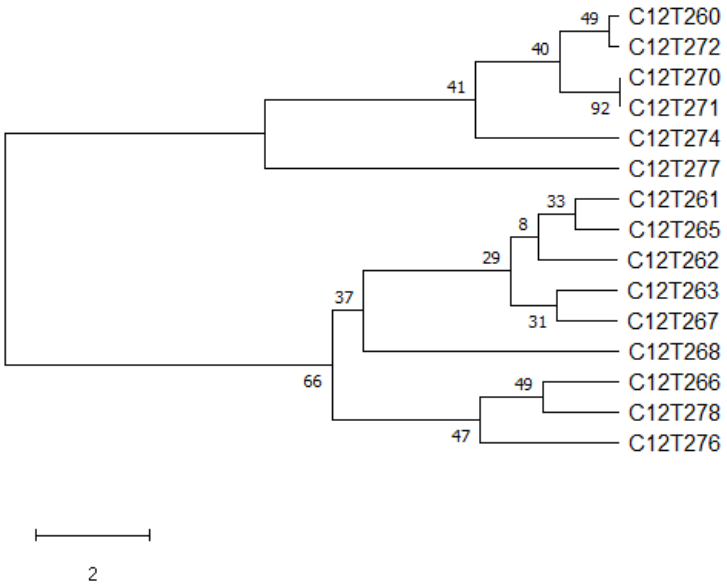

Figure A4-28. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 12.

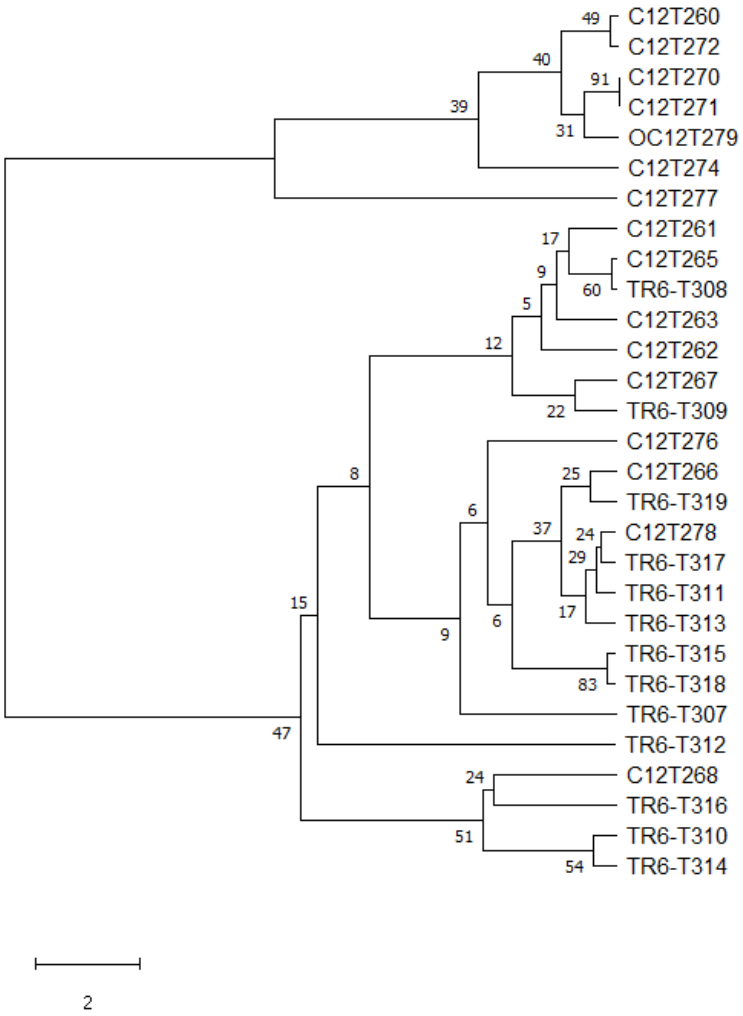

Figure A4-29. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clone 12 including outside trees and transect 6.

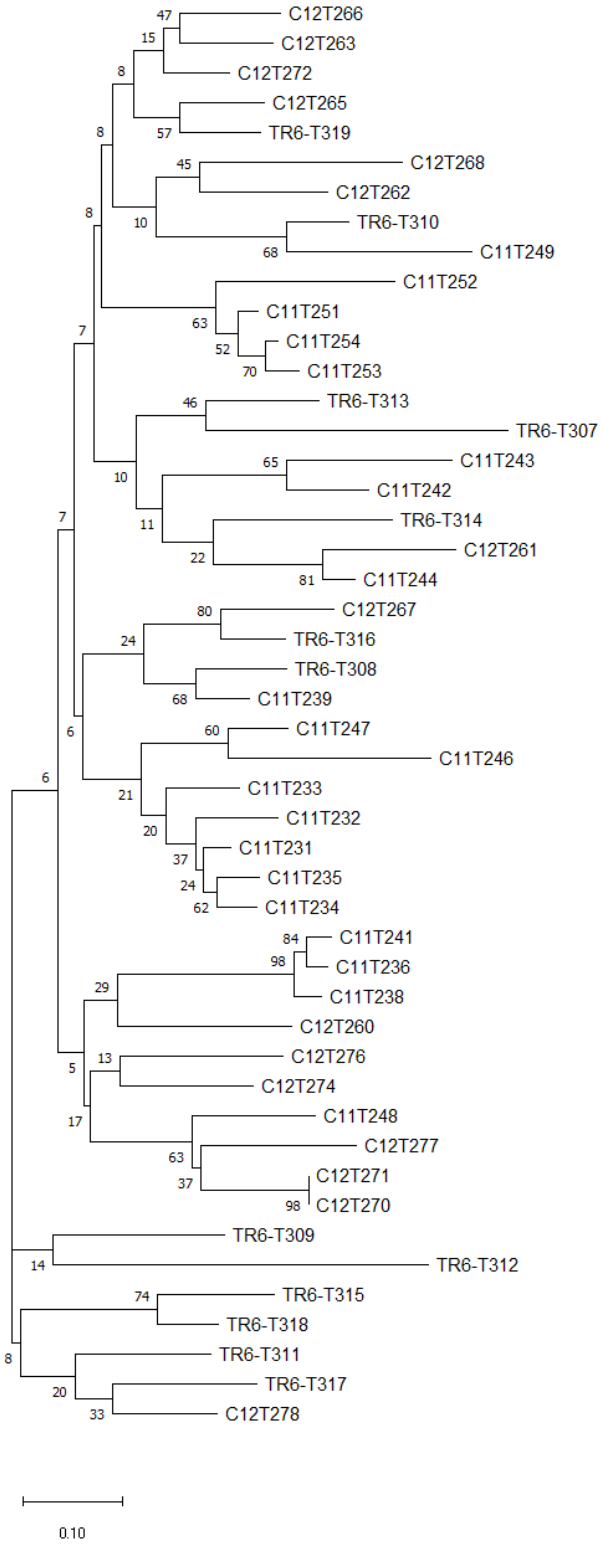

Figure A4-30. Aspen UPGMA tree $(\delta\mu)^2$ microsatellite distance, bootstrap 100,000 for putative clones 11 and 12 including transect 6.

Appendix 5: Chapter 4: clonal structure and spatial distribution maps

The following maps of the structure and spatial distribution of aspen clones within the AW2 (Pembina) tree improvement region were created with the use of GPS coordinates on the ArcMap (ESRI, Redlands, CA) software. After obtaining the results from clonal fingerprinting, sex screening, and ploidy, each tree was assigned its specific clonal, sex, and ploidy information, which was reflected on the maps of spatial distribution of trees within populations.

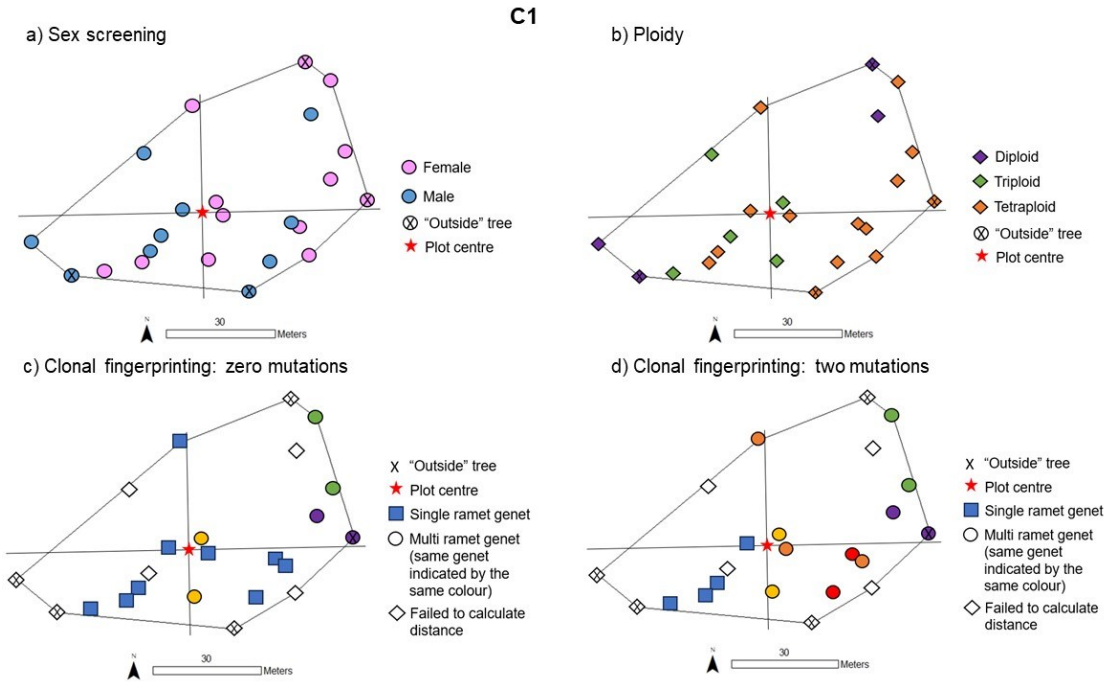

Figure A5-1. Spatial distribution and clonal structure of a study aspen population (C1) within the AW2 (Pembina) tree improvement region. Each symbol represents a sampled tree, an 'X' inside a circle represents a tree initially marked as "outside" of a putative clone, and the red star represents plot centre, including: a) sex screening, where pink circles represent females, and blue circles represent males; b) ploidy, diamond shapes of different colours represent different ploidies, where purple is diploid, green is triploid, and orange is tetraploid; and c and d) clonal fingerprinting, where blue squares represent single ramet genets, circles represent multi-ramet genets, with the same colour indicating the same clone, and the white diamond shapes represent trees for which a distance was not calculated. Trees were considered to belong to the same clone when the total genetic distances between them were equal to zero (c) or two (d) mutations.

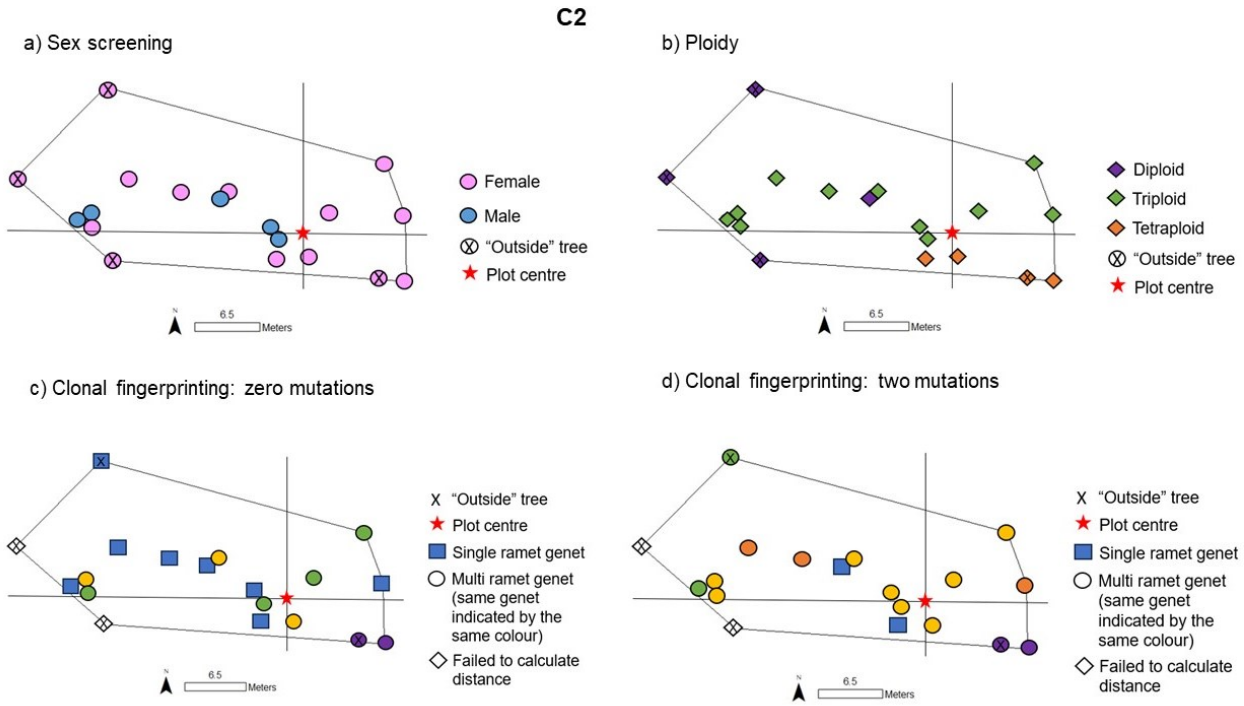

Figure A5-2. Spatial distribution and clonal structure of a study aspen population (C2) within the AW2 (Pembina) tree improvement region. Each symbol represents a sampled tree, an 'X' inside a circle represents a tree initially marked as "outside" of a putative clone, and the red star represents plot centre, including: a) sex screening, where pink circles represent females, and blue circles represent males; b) ploidy, diamond shapes of different colours represent different ploidies, where purple is diploid, green is triploid, and orange is tetraploid; and c and d) clonal fingerprinting, where blue squares represent single ramet genets, circles represent multi-ramet genets, with the same colour indicating the same clone, and the white diamond shapes represent trees for which a distance was not calculated. Trees were considered to belong to the same clone when the total genetic distances between them were equal to zero (c) or two (d) mutations.

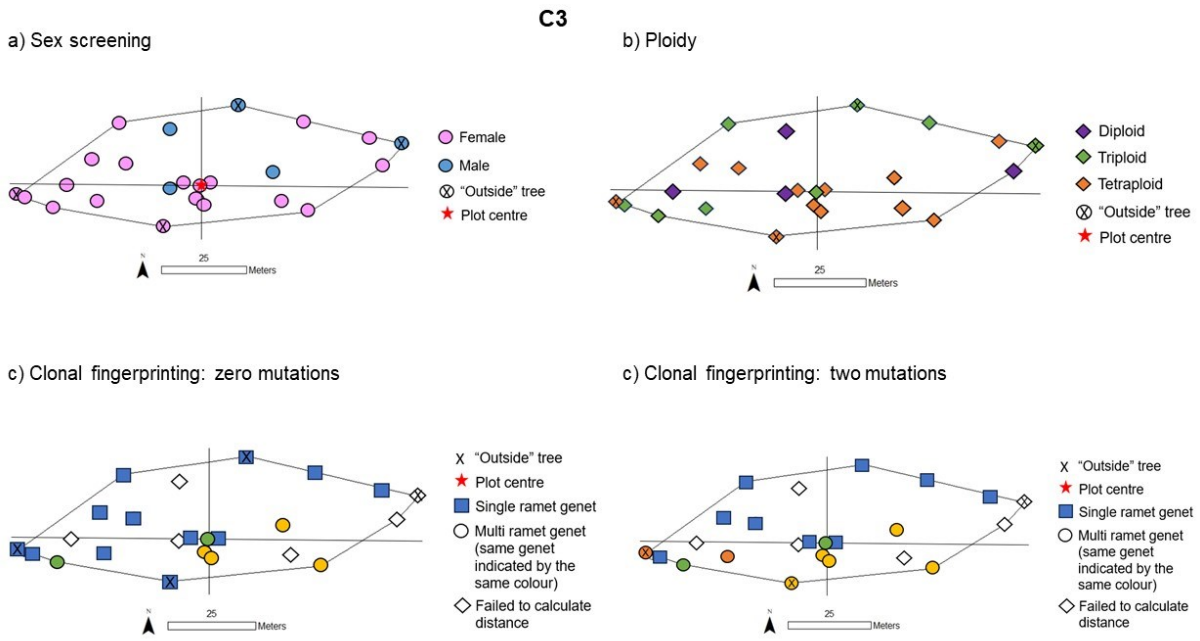

Figure A5-3. Spatial distribution and clonal structure of a study aspen population (C3) within the AW2 (Pembina) tree improvement region. Each symbol represents a sampled tree, an 'X' inside a circle represents a tree initially marked as "outside" of a putative clone, and the red star represents plot centre, including: a) sex screening, where pink circles represent females, and blue circles represent males; b) ploidy, diamond shapes of different colours represent different ploidies, where purple is diploid, green is triploid, and orange is tetraploid; and c and d) clonal fingerprinting, where blue squares represent single ramet genets, circles represent multi-ramet genets, with the same colour indicating the same clone, and the white diamond shapes represent trees for which a distance was not calculated. Trees were considered to belong to the same clone when the total genetic distances between them were equal to zero (c) or two (d) mutations.

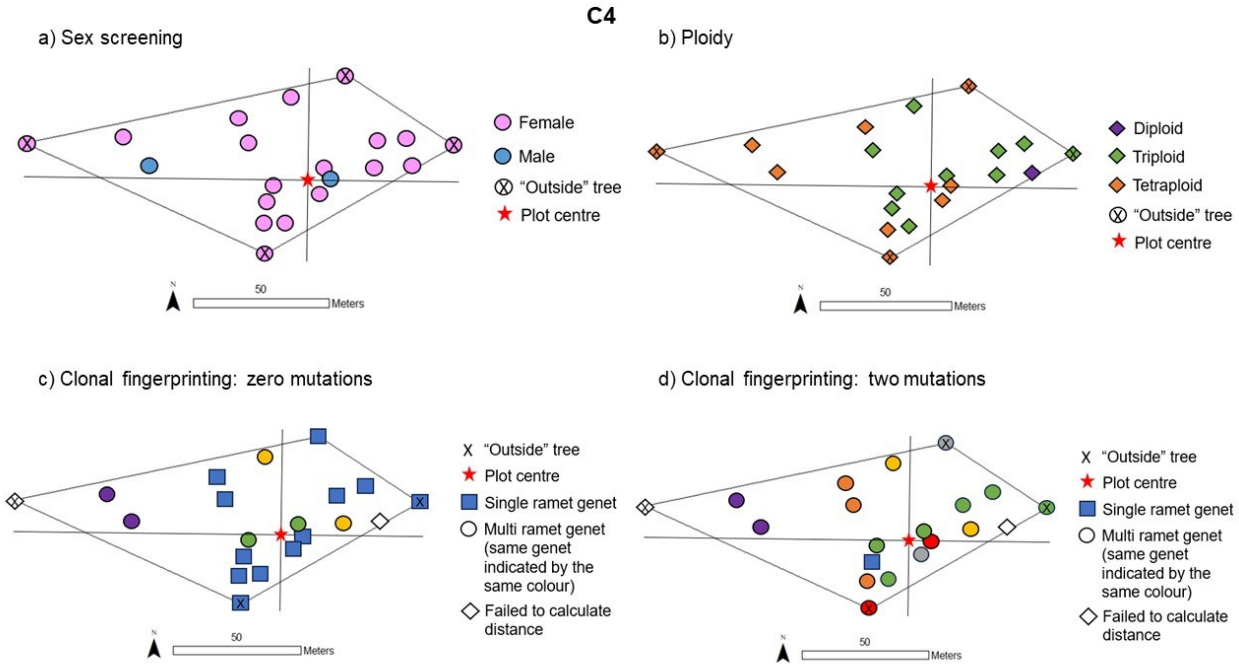

Figure A5-4. Spatial distribution and clonal structure of a study aspen population (C4) within the AW2 (Pembina) tree improvement region. Each symbol represents a sampled tree, an 'X' inside a circle represents a tree initially marked as "outside" of a putative clone, and the red star represents plot centre, including: a) sex screening, where pink circles represent females, and blue circles represent males; b) ploidy, diamond shapes of different colours represent different ploidies, where purple is diploid, green is triploid, and orange is tetraploid; and c and d) clonal fingerprinting, where blue squares represent single ramet genets, circles represent multi-ramet genets, with the same colour indicating the same clone, and the white diamond shapes represent trees for which a distance was not calculated. Trees were considered to belong to the same clone when the total genetic distances between them were equal to zero (c) or two (d) mutations.

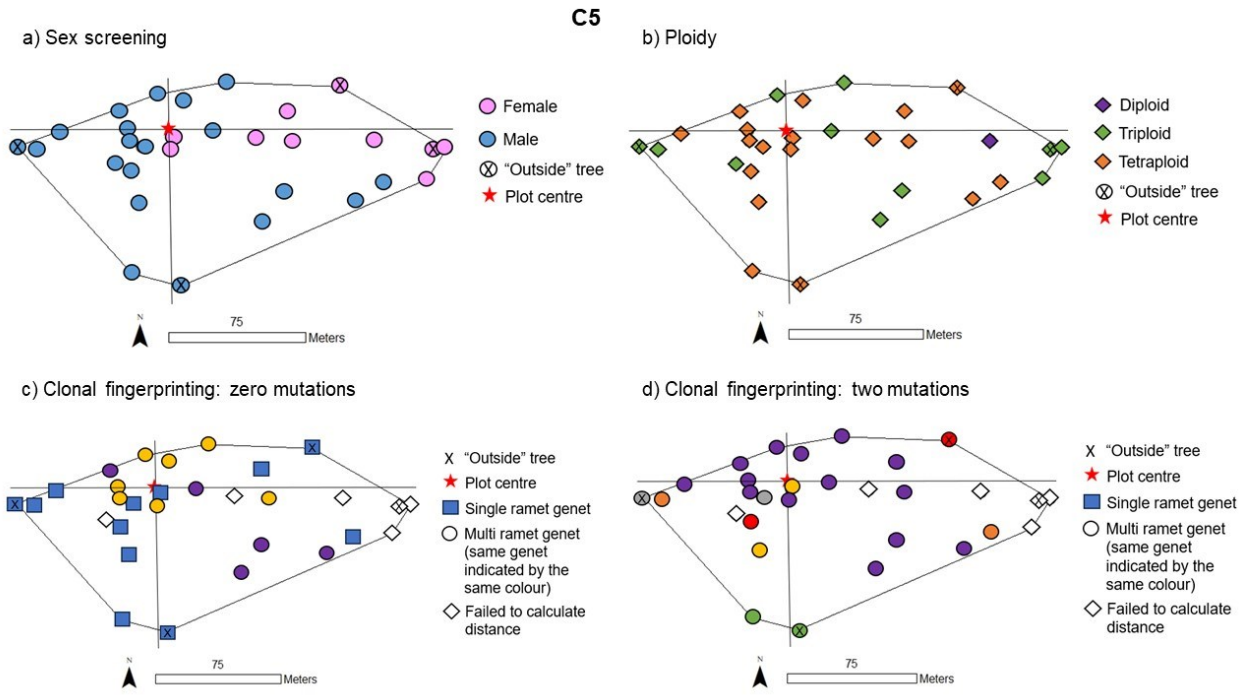

Figure A5-5. Spatial distribution and clonal structure of a study aspen population (C5) within the AW2 (Pembina) tree improvement region. Each symbol represents a sampled tree, an 'X' inside a circle represents a tree initially marked as "outside" of a putative clone, and the red star represents plot centre, including: a) sex screening, where pink circles represent females, and blue circles represent males; b) ploidy, diamond shapes of different colours represent different ploidies, where purple is diploid, green is triploid, and orange is tetraploid; and c and d) clonal fingerprinting, where blue squares represent single ramet genets, circles represent multi-ramet genets, with the same colour indicating the same clone, and the white diamond shapes represent trees for which a distance was not calculated. Trees were considered to belong to the same clone when the total genetic distances between them were equal to zero (c) or two (d) mutations.

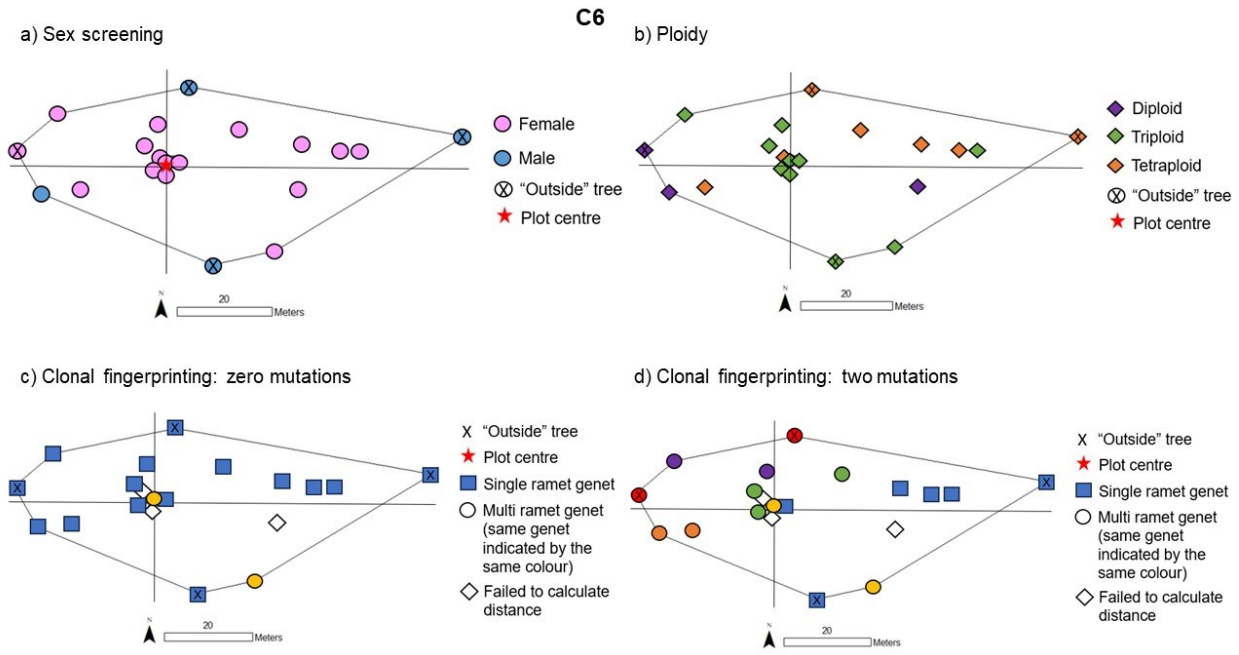

Figure A5-6. Spatial distribution and clonal structure of a study aspen population (C6) within the AW2 (Pembina) tree improvement region. Each symbol represents a sampled tree, an 'X' inside a circle represents a tree initially marked as "outside" of a putative clone, and the red star represents plot centre, including: a) sex screening, where pink circles represent females, and blue circles represent males; b) ploidy, diamond shapes of different colours represent different ploidies, where purple is diploid, green is triploid, and orange is tetraploid; and c and d) clonal fingerprinting, where blue squares represent single ramet genets, circles represent multi-ramet genets, with the same colour indicating the same clone, and the white diamond shapes represent trees for which a distance was not calculated. Trees were considered to belong to the same clone when the total genetic distances between them were equal to zero (c) or two (d) mutations.

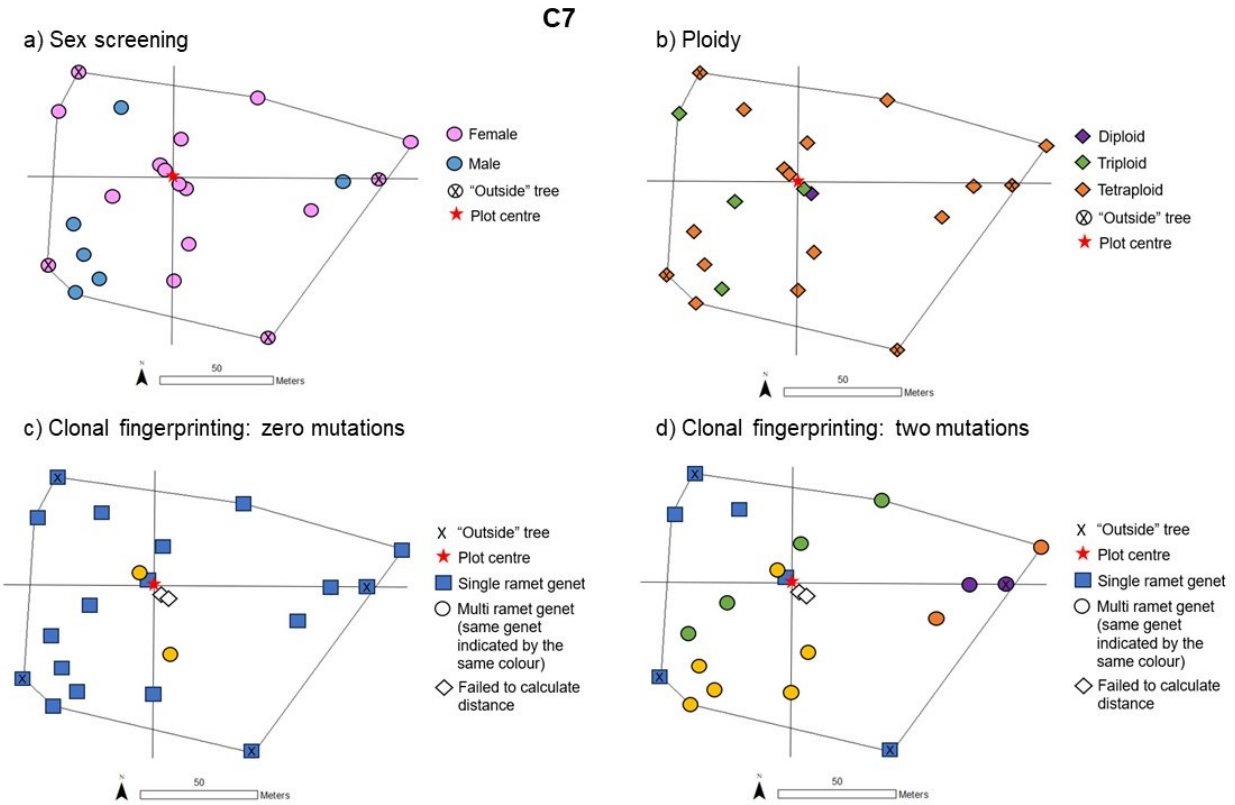

Figure A5-7. Spatial distribution and clonal structure of a study aspen population (C7) within the AW2 (Pembina) tree improvement region. Each symbol represents a sampled tree, an 'X' inside a circle represents a tree initially marked as "outside" of a putative clone, and the red star represents plot centre, including: a) sex screening, where pink circles represent females, and blue circles represent males; b) ploidy, diamond shapes of different colours represent different ploidies, where purple is diploid, green is triploid, and orange is tetraploid; and c and d) clonal fingerprinting, where blue squares represent single ramet genets, circles represent multi-ramet genets, with the same colour indicating the same clone, and the white diamond shapes represent trees for which a distance was not calculated. Trees were considered to belong to the same clone when the total genetic distances between them were equal to zero (c) or two (d) mutations.

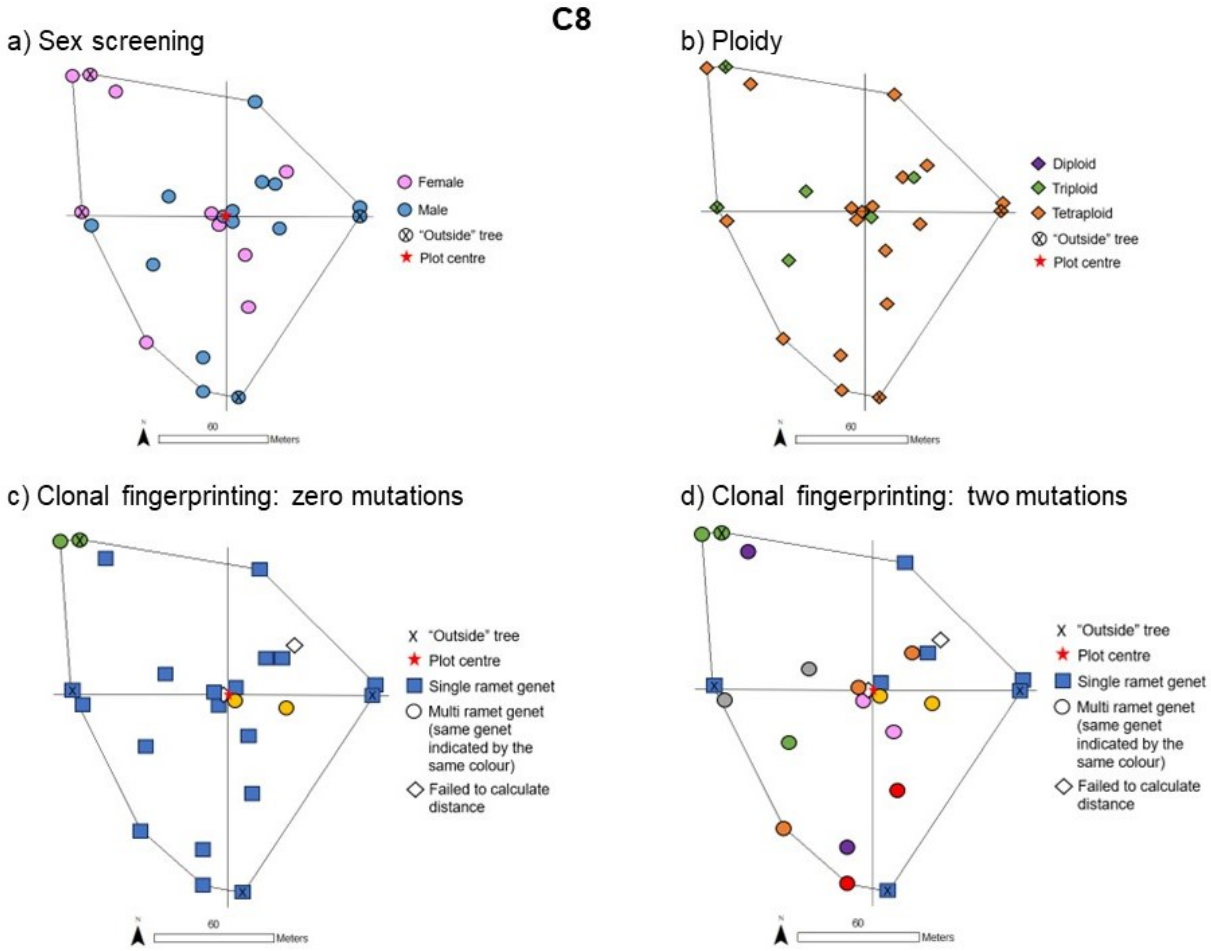

Figure A5-8. Spatial distribution and clonal structure of a study aspen population (C8) within the AW2 (Pembina) tree improvement region. Each symbol represents a sampled tree, an 'X' inside a circle represents a tree initially marked as "outside" of a putative clone, and the red star represents plot centre, including: a) sex screening, where pink circles represent females, and blue circles represent males; b) ploidy, diamond shapes of different colours represent different ploidies, where purple is diploid, green is triploid, and orange is tetraploid; and c and d) clonal fingerprinting, where blue squares represent single ramet genets, circles represent multi-ramet genets, with the same colour indicating the same clone, and the white diamond shapes represent trees for which a distance was not calculated. Trees were considered to belong to the same clone when the total genetic distances between them were equal to zero (c) or two (d) mutations.

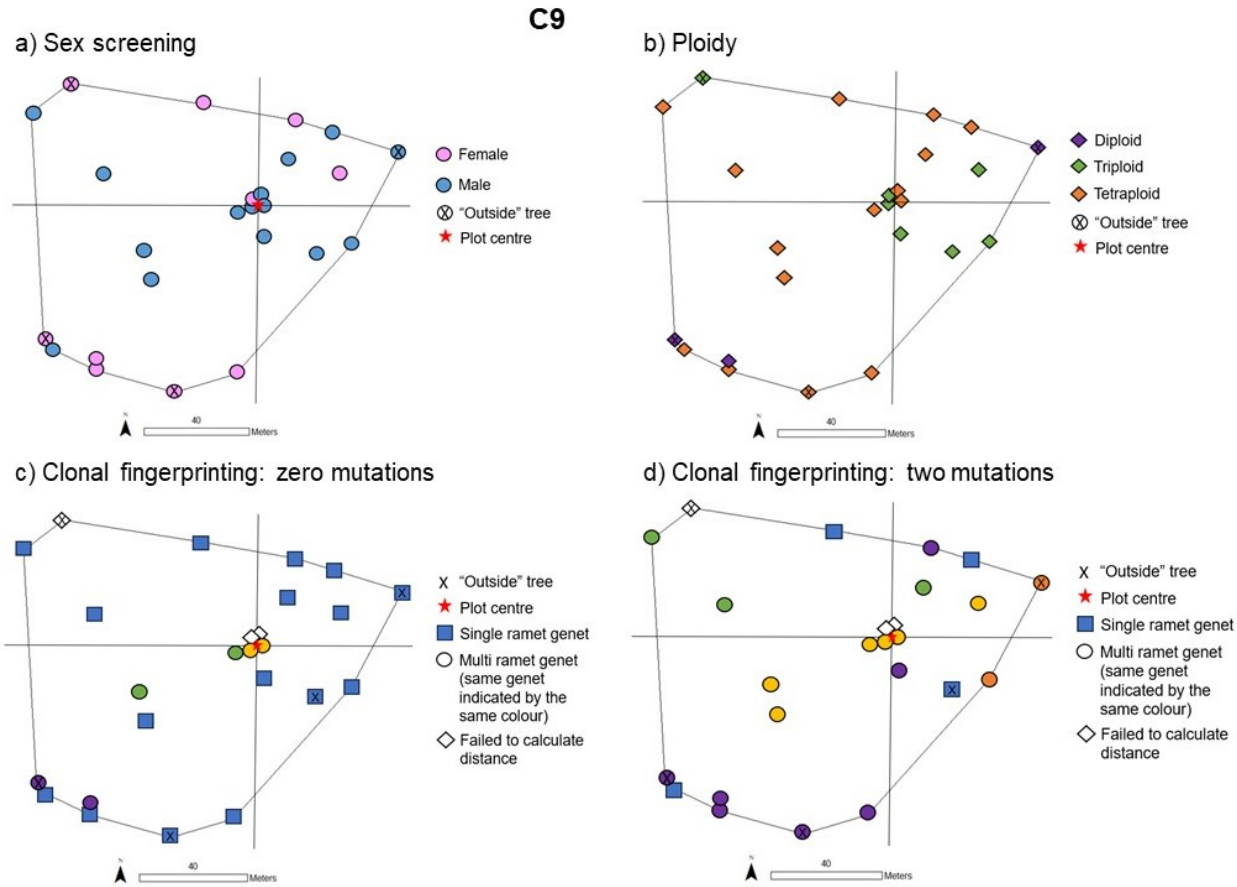

Figure A5-9. Spatial distribution and clonal structure of a study aspen population (C9) within the AW2 (Pembina) tree improvement region. Each symbol represents a sampled tree, an 'X' inside a circle represents a tree initially marked as "outside" of a putative clone, and the red star represents plot centre, including: a) sex screening, where pink circles represent females, and blue circles represent males; b) ploidy, diamond shapes of different colours represent different ploidies, where purple is diploid, green is triploid, and orange is tetraploid; and c and d) clonal fingerprinting, where blue squares represent single ramet genets, circles represent multi-ramet genets, with the same colour indicating the same clone, and the white diamond shapes represent trees for which a distance was not calculated. Trees were considered to belong to the same clone when the total genetic distances between them were equal to zero (c) or two (d) mutations.

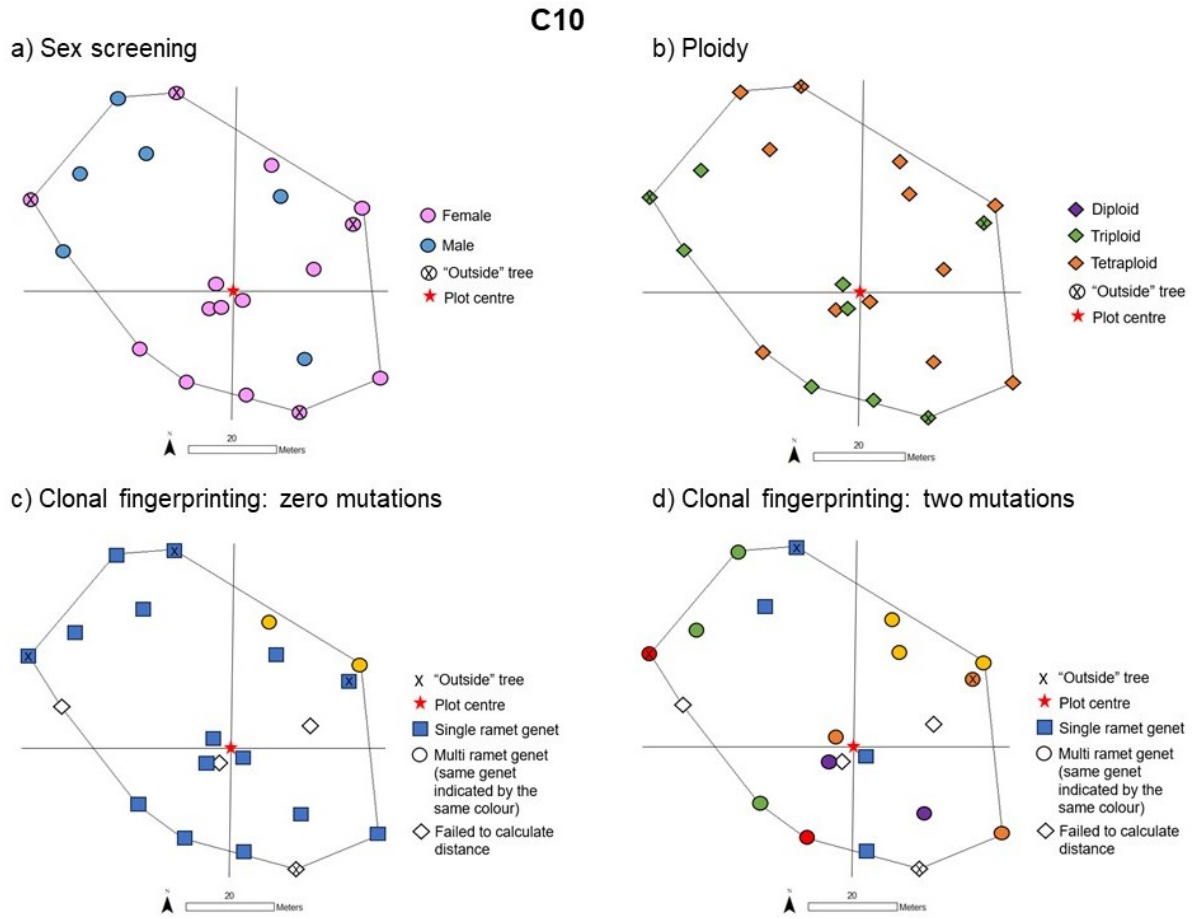

Figure A5-10. Spatial distribution and clonal structure of a study aspen population (C10) within the AW2 (Pembina) tree improvement region. Each symbol represents a sampled tree, an 'X' inside a circle represents a tree initially marked as "outside" of a putative clone, and the red star represents plot centre, including: a) sex screening, where pink circles represent females, and blue circles represent males; b) ploidy, diamond shapes of different colours represent different ploidies, where purple is diploid, green is triploid, and orange is tetraploid; and c and d) clonal fingerprinting, where blue squares represent single ramet genets, circles represent multi-ramet genets, with the same colour indicating the same clone, and the white diamond shapes represent trees for which a distance was not calculated. Trees were considered to belong to the same clone when the total genetic distances between them were equal to zero (c) or two (d) mutations.

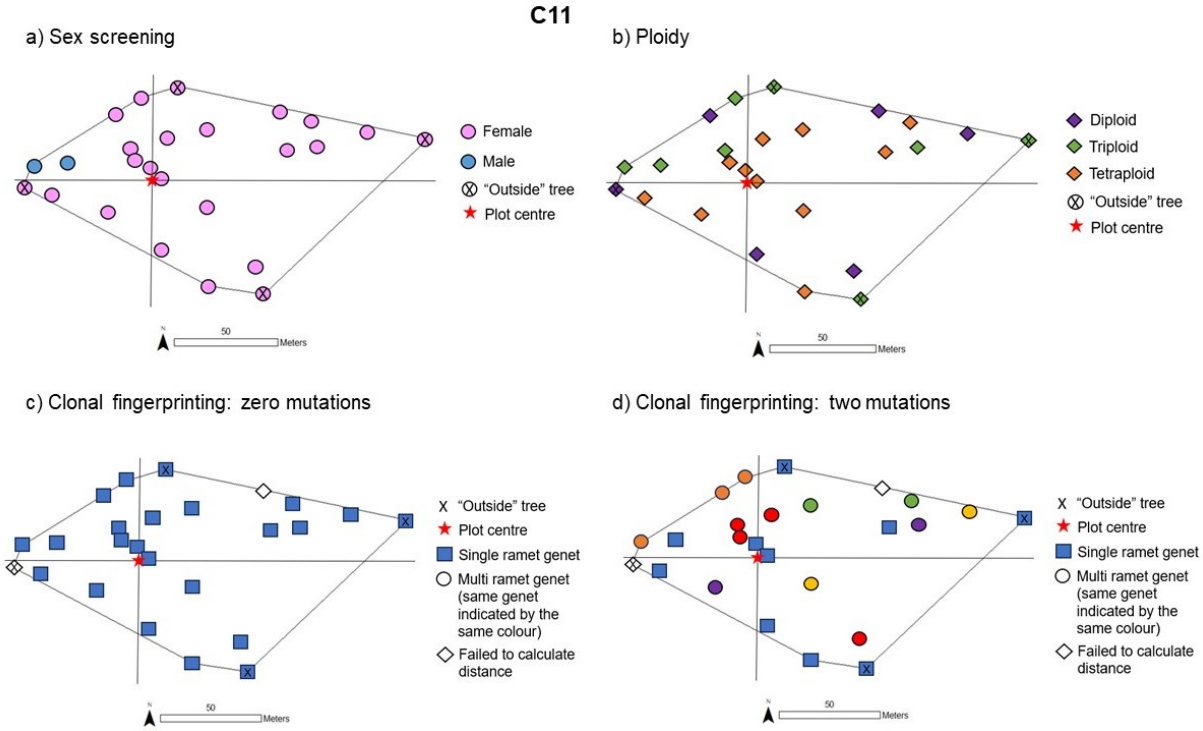

Figure A5-11. Spatial distribution and clonal structure of a study aspen population (C11) within the AW2 (Pembina) tree improvement region. Each symbol represents a sampled tree, an 'X' inside a circle represents a tree initially marked as "outside" of a putative clone, and the red star represents plot centre, including: a) sex screening, where pink circles represent females, and blue circles represent males; b) ploidy, diamond shapes of different colours represent different ploidies, where purple is diploid, green is triploid, and orange is tetraploid; and c and d) clonal fingerprinting, where blue squares represent single ramet genets, circles represent multi-ramet genets, with the same colour indicating the same clone, and the white diamond shapes represent trees for which a distance was not calculated. Trees were considered to belong to the same clone when the total genetic distances between them were equal to zero (c) or two (d) mutations.

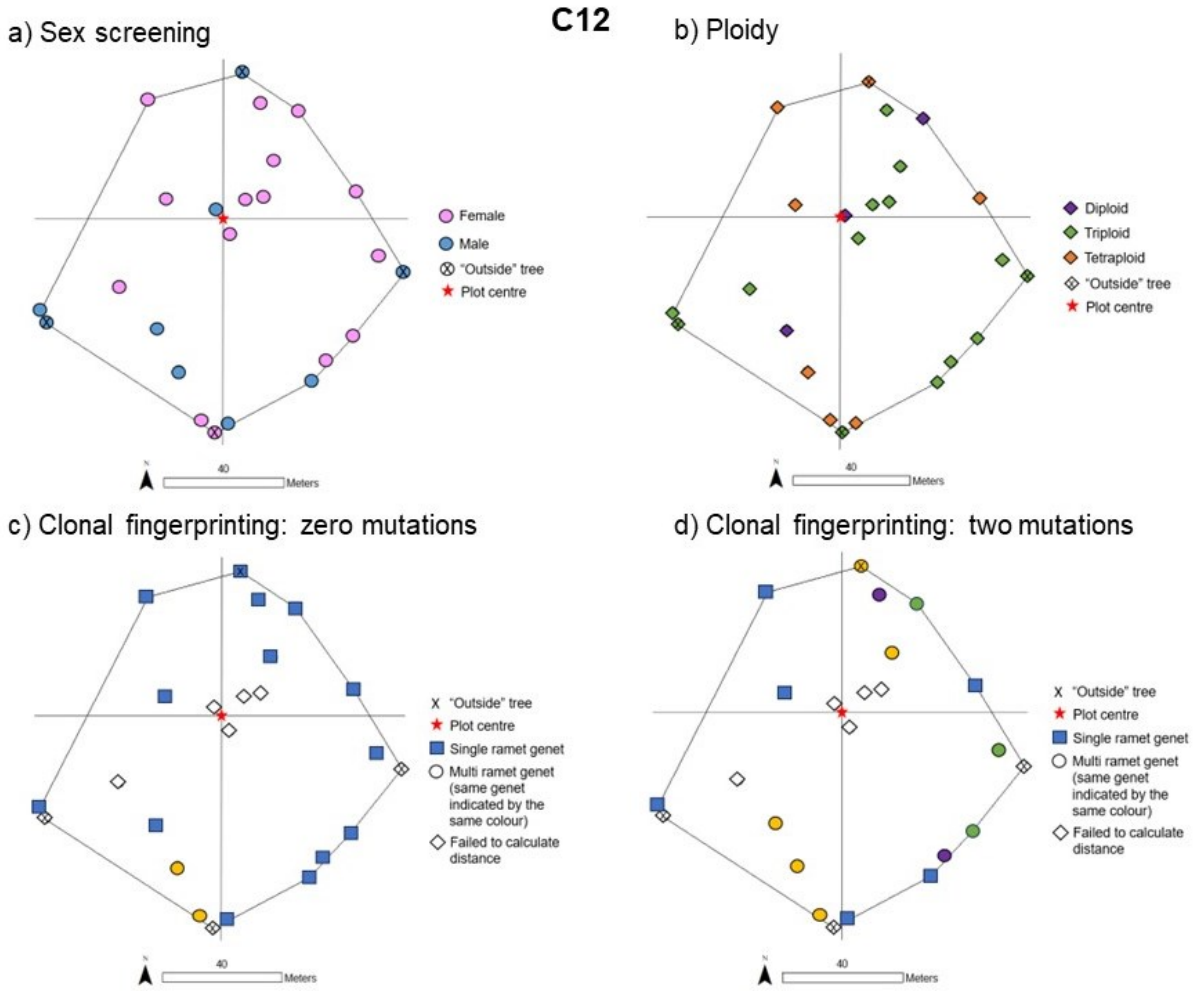

Figure A5-12. Spatial distribution and clonal structure of a study aspen population (C12) within the AW2 (Pembina) tree improvement region. Each symbol represents a sampled tree, an 'X' inside a circle represents a tree initially marked as "outside" of a putative clone, and the red star represents plot centre, including: a) sex screening, where pink circles represent females, and blue circles represent males; b) ploidy, diamond shapes of different colours represent different ploidies, where purple is diploid, green is triploid, and orange is tetraploid; and c and d) clonal fingerprinting, where blue squares represent single ramet genets, circles represent multi-ramet genets, with the same colour indicating the same clone, and the white diamond shapes represent trees for which a distance was not calculated. Trees were considered to belong to the same clone when the total genetic distances between them were equal to zero (c) or two (d) mutations.