The Genetic Diversity and Conservation Potential of Eastern Hemlock (*Tsuga canadensis* (L.) Carrière) in Minnesota

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Dedication

for my family

Abstract

Eastern hemlock (*Tsuga canadensis* (L.) Carrière) is a slow-growing and long-lived conifer in the Pinaceae family. Its range extends from Nova Scotia west into Wisconsin and Minnesota and south along the Appalachian Mountains, Northern Georgia, and Alabama with outlier populations along the western range limits in Minnesota, Ohio, Indiana, Georgia, Alabama, Kentucky, and Tennessee. Eastern hemlock is a foundation species across its range that has transformational effects on its surrounding ecosystem. As of 2013, eastern hemlock has been listed as near threatened due to the presence of an invasive insect, the hemlock woolly adelgid (*Adelges tsugae* Anaand.), which is destroying populations in the eastern United States. Eastern hemlock has historically existed in Minnesota in disjunct and marginal populations and it is listed as endangered in the state. Additionally, trees of known native provenance at the Minnesota Landscape Arboretum were collected from a now extirpated population near Mille Lacs Lake, Minnesota and there are additional trees of unknown provenance in state and municipal parks and public gardens. The objectives of this research were to understand the propagation potential and genetic diversity of native and unknown provenance eastern hemlock in Minnesota with the aim of using this information to inform conservation strategies. Field site visits revealed that there are less than 40 known native mature eastern hemlock trees in Minnesota, with scattered seedlings and saplings. Information on individual trees and herbarium specimens including details on height, diameter at breast height (DBH), location, and notes on tree health, are included in the supplementary spreadsheet Appendix B. Using previously published microsatellite markers (SSRs) derived from eastern hemlock, we observed inbreeding in disjunct Minnesota native trees when compared with trees in the main range. Hemlock Ravine was the most genetically distinct from all other sites sampled, as were native origin trees

at the Minnesota Landscape Arboretum. Interestingly, neither of these two sites were similar to the other Minnesota disjunct site, West Duluth. The West Duluth trees were more genetically similar to populations sampled in Wisconsin and Michigan. Seedlings grown from native Minnesota trees also displayed inbreeding. From paternity analyses, we found that trees at the Minnesota Landscape Arboretum are potentially outcrossing with non-native trees. Additionally, trees in Minnesota of both native and non-native origin can be propagated successfully via seed. Trees at the Minnesota Landscape Arboretum in particular are amenable to seed propagation, but had little overall success when propagated vegetatively, with the exception of a singular accession (MLA19). These discoveries can be used to inform conservation practices in Minnesota. We recommend that land managers continue in situ preservation of sites across Minnesota and continue ex situ maintenance of eastern hemlock trees in parks and gardens. We also recommend that land managers focus on native Minnesota trees when sourcing material for propagation, planting, and seed-banking in national and local repositories.

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CHAPTER 1:

Literature Review of Eastern Hemlock (*Tsuga canadensis* (L.) Carriére): Perspectives from its Northwestern Range Limit

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INTRODUCTION AND HISTORY

A genus of woody conifers in the Pinaceae family, *Tsuga* is comprised of ten species globally, along with four intraspecific taxa (Table 1-1). Four species are native to North America, *Tsuga canadensis* (L.) Carrière (eastern or Canadian hemlock) and *Tsuga caroliniana* Engelm. (Carolina Hemlock), which are found in eastern North America, and *Tsuga heterophylla* (Raf.) Sarg. (western hemlock) and *Tsuga mertensiana* (Bong.) Carrière (mountain hemlock), which are found in western North America. Eastern, western, and mountain hemlock have large longitudinal and elevational ranges which suggest that they can grow in a variety of habitats (Barbour, Ruth, & Karrfalt, 1980). Carolina hemlock, however, has a small range which falls completely within the range of eastern hemlock (Figure 1-1). A hybrid species, *Tsuga* x *jeffreyi* (A.Henry) A.Henry (*Tsuga mertensiana* x *T. heterophylla*), was unknown in the wild until evidence of hybrid colonies were discovered south of Mount Rainier, Washington (Swartley, 1984). The remaining species are native to Asia.

The name *Tsuga* is generally accepted as being Japanese for “tree mother” (Swartley, 1984). The Chinese characters for the name describe “tree with hanging branches”, a fitting description. Generally, hemlocks have branches with a loose and pendulous habit, with spirally arranged evergreen leaves on twigs and pendant cones of various sizes. The flexible and pendulous nature of their branches help prevent snow and ice damage in winter months (Heptin, 1971).

Eastern hemlock (*Tsuga canadensis*) is a slow-growing, long-lived and late successional conifer. Its range extends from Nova Scotia west into Wisconsin and south along the Appalachian Mountains, Northern Georgia, and Alabama (Little, 1971; Potter et al., 2012). There are also outlier populations along the western range limits in Minnesota, Ohio, Indiana, Georgia, Alabama, Kentucky, and Tennessee. Of the four North American species, eastern hemlock is the most widely variable and is cultivated as a landscape ornamental. It was introduced to Europe in 1736 and it is estimated that there have been over 280 cultivars of dwarf, weeping, prostrate, and variegated varieties (Swartley, 1984). Horticulturally, they are used in a variety of settings as specimen plantings and as hedges. Eastern hemlock has long been considered a worthy tree for the landscape; the 18th century botanist, Thomas Meehan, wrote, “It would not be an exaggeration to pronounce this the most beautiful evergreen in cultivation…” (Swartley, 1984). There are records that indicate Thomas Jefferson planted hemlocks on his property (Swartley, 1984).

Eastern hemlock has furrowed, brown bark, pendulous and drooping branches, a nodding top which often faces away from prevailing winds, and the smallest cones of all the hemlock species (Figure 1-2a, b). Their small, linear leaves have two stomatal bands on the abaxial side of the leaf and leaves are spirally arranged on the branch and bent at

the petioles, with some being appressed along the stem (Figure 1-1c, d). Cones are small (13-19 mm long) with small, winged seeds.

Early accounts of hemlock describe it being used in a variety of ways across its range. Native Americans steeped needles in water to make tea and harvested the inner bark to make bread. Post-European settlement, eastern hemlock was used as a source for lumber, especially between 1890 and 1910 (Godman & Lancaster, 1990). The wood grain of eastern hemlock is reported as being uneven, brittle, and dry, but the species was nonetheless used in the paper-pulping industry and for building material used in making boxes, crates, flooring, roofing, railroad ties and sleeper cars (Smith, 2008; Swartley, 1984). Additionally, the bark of eastern hemlock is comprised of 7 – 12% naturally occurring tannins, which in the 1800s to early 1900s were extracted and commonly used to tan leather, imparting a sought-after reddish-brown color (Heptin, 1971; Swartley, 1984).

ECOLOGY

Eastern Hemlock as a Foundation Species

Foundation species function to regulate the processes of their surrounding ecosystems (Ellison et al., 2005). These processes include water, nutrient, and energy cycling and flux, and community compositional dynamics. They are generally locally abundant and regionally common, although their effects can be larger than expected based on sheer abundance (Dayton, 1972).

Eastern hemlock is considered a late successional foundation species in ecosystems across its range. They create damp, cool microclimates due to the deep shade they cast from their dense canopy cover. This shade, in addition to the tannin-rich, acidic leaf-litter, creates vegetatively depauperate understories and unique associated species

assemblages. Eastern hemlock occurs in up to 29 forest cover types as described by the Society of American Foresters, within four of which they are integral components: white pine-hemlock, eastern hemlock, hemlock-yellow birch, and yellow-poplar-eastern hemlock (Eyre, 1980; Godman & Lancaster, 1990).

Eastern hemlock is incredibly shade-tolerant and has the ability to survive in as little as 5% full sunlight. It can withstand overstory suppression for up to 400 years, waiting to capitalize on a break in the canopy. Trees less than 1 inch in diameter have been documented to be 100 years old and it is typical for trees in natural stands to be suppressed for between 25 and 200 years (Fowells, 1965).

Eastern hemlock's extensive range, especially its large North-South gradient (N 48°18'57" to N 33°40'58"; Figure 1-1) demonstrates that it can grow in a variety of habitats (Kessell, 1979). Eastern hemlocks generally grow in moist temperate climates (USDA Plant Hardiness Zone 3-7) between 600 – 1,800 meters in elevation. They are most often found on moist or rocky ridges as well as north-facing slopes and valleys and along streams (Calcote, 1987; Eckenwalder, 2009; Godman & Lancaster, 1990). Moisture is often the limiting factor in hemlock establishment and growth. However Kessel (1979) found two apparent ecotypes across the range; one that prefers mesophytic sites and is highly sensitivity to moisture but not to temperature and one that prefers mesic and subxeric slopes that is highly sensitive to temperature but not to moisture. Eastern hemlocks grow in a variety of soils from sandy and/or silty loams to rocky, glaciated and fluvial soils (Fowells, 1965).

The effects of eastern hemlock on these ecosystems are comprehensive, involving a wide range of ecosystem dynamics. For example, eastern hemlock affects stream dynamics by stabilizing thermal and hydrologic regimes (Snyder et al. 2002; Brantley et al. 2015). Within streams, they can influence the species composition of fish

and macroinvertebrate communities, which can be indicators for the amount of nutrient and carbon processing in the ecosystem (Ross et al., 2003; Snyder et al., 2002). Under the forest floor, eastern hemlock has recorded associations with ectomycorrhizal fungi (O'Brien, Gomola, & Horton, 2011; Poznanovic, Lilleskov, & Webster, 2014; Vendettuoli, Orwig, Krumins, Waterhouse, & Preisser, 2015).

Although the dense hemlock canopies reduce associated vegetation, they can create extensive habitat for mammals, birds, and insects. In the canopy, eastern hemlock provides habitat for birds; with some species such as the black-throated green warbler being specifically associated with hemlock (Tingley, Orwig, Field, & Motzkin, 2002). They also support diverse and dynamic insect and arachnid communities (Dilling, Lambdin, Grant, & Buck, 2007; Mallis & Rieske, 2011).

Ecosystem Challenges

There are a number of biotic stresses, life history, and climatic factors that affect the longevity and ornamental value of eastern hemlock. According to pollen records, about 4,800 years ago, eastern hemlock suffered a relatively swift range-wide decrease, followed by a rebound 1,000 to 2,000 years later (Davis, 1976). This decrease could have been caused by a number of factors, including climate, disease, and pest pressures, with disease and pest pressures often cited as being the most likely causes (Bhiry & Filion, 1996; Booth, Brewer, Blaauw, Minckley, & Jackson, 2012; Davis, 1976; Foster, Oswald, Faison, Doughty, & Hansen, 2006).

Today, certain life history traits, climate pressures, diseases, and pests are a continual concern. Eastern hemlocks have shallow root systems, which makes them susceptible to windthrow (Godman & Lancaster, 1990). Frelich and Reich (1995) found that hemlock-dominated forests that experience intense fire after windthrow events will have trouble regenerating and generally convert to early successional paper birch

(*Betula papyrifera* Marshall) and aspen (*Populus tremuloides* Michx.) forests. Wind also causes radial stress cracks known as wind shake or ring shake (Fowells, 1965). Young eastern hemlocks are particularly susceptible to fire and moisture depletion and even in mature trees, prolonged drought through the upper soil horizon is problematic (Fowells, 1965; Tubbs, 1975).

Eastern hemlock has been reported to withstand temperatures as low as -76°F and does not usually experience fatality from cold alone, as late onset spring growth decreases the chance of spring frost injury (Heptin, 1971). They are more likely to suffer from a combination of cold temperatures, frozen ground, and water loss through transpiration (Fowells, 1965). The Intergovernmental Panel on Climate Change (IPCC) has reported that terrestrial species have shifted their ranges and abundances in response to climate change (IPCC, 2014). Climate change has been found to be a factor in the abrupt hemlock decline 5,000 years ago (Foster et al., 2006). It is very likely that there will be more extreme weather events in the future such as drought, fire, heat waves, and heavy precipitation. Models suggest that under the current greenhouse gas emissions, tree species will be under pressure to move their ranges, which has the potential to disturb ecosystem dynamics (Iverson, Prasad, Matthews, & Peters, 2008).

Eastern hemlock has been known to be vulnerable to a multitude of common fungal and bacterial pathogens. They are susceptible to collar, foot, and crown rots (*Phytophthera* Bary spp.), wetwood and slime flux bacterial infections (Dreisadt, Clark, & Flint, 1994), and various needle blights and diseases (Heptin, 1971). There are few fatally damaging root rots for mature trees, although root rots and damping-off incited by fungal pathogens such as *Pythium debaryanum* R. Hesse, *Rhizoctonia solani* J. G. Kühn, *Cylindrocladium scoparium* Morgan, *Rhizina undulata* Fr., and *Botrytis cinerea* Pers. are common problems in growing seedlings in the nursery trade (Heptin, 1971).

One of the more economically important diseases is cone rust incited by *Melampsora abietis-canadensis* C.A. Ludw., which alternates hosts between *Populus* L. spp. and eastern hemlock and can cause newly-formed cones to abort (Heptin, 1971).

Eastern hemlock provide dense winter protection for a variety of mammals, including white-tailed deer (*Odocoileus virginianus* Zimmerman). Deer prefer hemlock forests due to the wind and snow protection the trees provide and as a winter food source. Browsing by white-tailed deer causes observed decline in eastern hemlock forests and affects the ability for regeneration, even more so than a changing climate and poor seedbed conditions (Anderson & Loucks, 1979; Faison, DeStefano, Foster, & Plotkin, 2016; Frelich & Lorimer, 1985; Rooney, 2001). Porcupines (*Erthizon dorsatum* Linnaeus) and other small mammals have also been known to browse on eastern hemlock in the winter months when other herbaceous understory vegetation is not available (Faison et al., 2016; Griesemer, Fuller, & Degraaf, 1998). Hemlock seedlings have trouble with regrowth after browsing on terminal shoots (Anderson & Loucks, 1979).

Eastern hemlock is associated with a range of insect pests including the hemlock sawfly (*Neodiprion tsugae* Middleton), hemlock scale (*Hemiberlesia ithacae* Ferris), hemlock rust mite (*Nalepella tsugifoliae* Keifer), and the hemlock borer (*Melanophila fulvoguttata* Harris). Hemlock looper (*Lambdina fiscellaria* Guen.) is one of the more damaging pests. They defoliate trees and affect not just eastern hemlock, but also balsam fir (*Abies balsamea* (L.) Mill) (Fowells, 1965; Martineau, 1984). The hemlock borer is a secondary pest, which damages previously stressed trees (Cranshaw, 2004; Fowells, 1965). However, as of 2017, arguably the most pressing challenge for eastern (and Carolina) hemlock is the presence of an invasive insect, the hemlock woolly adelgid (*Adelges tsugae* Annand.).

Hemlock Woolly Adelgid (Adelges tsugae Annand.)

In the last few decades, populations of eastern hemlock in the eastern United States have suffered substantial decline due to the outbreak of an invasive insect, the hemlock woolly adelgid (*Adelges tsugae* Annand., hereafter referred to as HWA) (Homoptera: Adeligidae) (McClure, 1991). The presence of HWA is a contributing factor to the listing of eastern hemlock as a near-threatened species with a decreasing population trend, according to the International Union for Conservation of Nature (IUCN) (Farjon, 2013). HWA is an aphid-like insect native to Asia. Genetic studies indicate that HWA introduced to the eastern United States originated in Japan. A total of eight distinct lineages were present in the United States in 2016 (Havill et al., 2016).

HWA was first discovered in North America in British Columbia in 1924 and then in Oregon in 1928 (R. F. Young, Shields, & Berlyn, 1995). However, it was found on the east coast in 1951 in Virginia, where it has proven to be an invasive problem. It destructively feeds on both eastern hemlock and Carolina hemlock, although it has not been a problem on the mountain hemlock or western hemlock (Havill et al., 2016; McClure, 1991; R. F. Young et al., 1995). As of 2015, HWA has been found in 19 eastern states from northeastern Georgia to southeast Maine and as far west as Michigan (Preisser, Oten, & Hain, 2014).

HWA are parthenogenic, meaning that they can produce two asexual generations in a single year. This life cycle contributes to the speed and voracity of their infestations (Havill et al., 2016; McClure, 1991). They insert their piercing and sucking mouthparts (stylets) near the center of the abscission layer of the adaxial sides of needles. They feed almost exclusively on the xylem ray parenchyma cells, which are cells that store and transfer nutrients throughout the tree (R. F. Young et al., 1995). In

addition to the stylet damage, evidence suggests a possible toxic effect from salivary secretions (R. F. Young et al., 1995) and induction of a hypersensitive response in hemlock trees marked by accumulation of H_2O_2 in tissue (Radville, Chaves, & Preisser, 2011). Feeding and possible toxic effects cause decreased strength and flexibility of twigs and decreased needle strength (Soltis et al., 2014), resulting in desiccation, defoliation and die-back. HWA affects trees of all size and age classes, however they are most often found on smaller trees and new growth (McClure, 1991; Orwig & Foster, 1998). HWA can be spread via wind, birds, deer and humans (McClure, 1990). Eggs and crawlers removed from hemlocks could survive for up to two weeks in ambient conditions, highlighting the need for careful treatment of hemlock timber (McClure, 1990).

Although problems with HWA are currently restricted to the eastern United States due in part to its low cold tolerance, there is a chance that climate change will intensify the effects of HWA (Saladyga & Maxwell, 2015). Several studies have investigated the role of low winter temperature on survival rates of the insect and indicate that insects did not survive temperatures below -30° C (Gouli, Parker, & Skinner, 2000; Parker, Skinner, Gouli, Ashikaga, & Teillon, 1999). Paradis et al. (2008) found that at least half of the remaining unaffected eastern hemlock range in the northeast will likely become infested under low carbon emissions. Paradis et al. (2008) and Trotter and Shields (2009) maintain that most of the eastern range of eastern hemlock will be exposed to infestation.

There is evidence that infestation of HWA is problematic for both hemlock trees and ecosystems generally. Infestation leads to decreased carbon in roots and reduced colonization by rhizosphere bacteria and ectomycorrhizal fungi (Vendettuoli et al., 2015), and higher nitrification and inorganic nitrogen availability and potential nitrogen leaching

(Jenkins, Aber, & Canham, 1999). In addition, infestations lead to the invasion of opportunistic woody species such as *Betula* L. spp. (Davis, 1976; Ellison et al., 2005; Orwig, Foster, & Mausel, 2002), and opportunistic herbaceous and exotic species (Orwig & Foster, 1998), along with other foundational changes in eastern hemlock dominated ecosystems. The removal of eastern hemlock from ecosystems could also impact bird community structure (Tingley et al., 2002). Brantley et al. (2015) found that a decline in hemlock leads to a decrease in total water yield in streams, yet an increase in peakflow events. The Harvard Forest Hemlock Removal Experiment began in 2005 to specifically measure the loss of foundation species on ecosystem processes and dynamics. Kendrick et al. (2015) found that purposeful canopy removal affected decomposition rates and ant assemblages, which in turn changed soil nitrogen availability and Lustenhouwer et al. (2012) observed significant changes in understory microclimate, especially in air temperature.

Control of HWA is challenging. For a fully integrated management strategy, chemical control, biological control, cultural treatments, host plant resistance, and host gene conservation should be considered together (Vose, Wear, Mayfield, & Dana Nelson, 2013). Chemical controls include systemic insecticides such as imidacloprid and dinotefuran, which are generally applied as a soil drench, soil injection, trunk injection, or, with dinotefuran, a basal trunk spray. These chemical controls are effective for small scale adelgid infestations, however they are less practical for forest and ecosystem wide controls due to regulatory and budget considerations (Vose et al., 2013). Since the early 1990s, researchers and professionals have been working on introducing useful biological controls. The most widely used biological control is a lady beetle, *Sasajiscymnus* (formerly *Pseudoscymnus*) *tsugae* Sasaji and McClure, but other possibly predatory species are *Laricobius nigrinus* Fender and *Laricobius osakensis*

Shiyake and Montgomery. Host plant resistance is also a possibility and needs investigation.

The best chances for control of HWA is thorough an integrated pest management program that combines chemical controls, biological controls, and pest resistant germplasm. For a recent, thorough treatment of the challenges facing hemlock from HWA, see Preisser et al. 2014.

Disjunct and Marginal Eastern Hemlock Populations in Minnesota

Disjunct, marginal populations occur throughout the range of eastern hemlock, most notably in Indiana, Ohio, Kentucky, and Minnesota. Spaulding and Rieske (2010) investigated the effects of HWA specifically on the southwestern range of hemlock. Through the combination of vegetation assessments and the USDA FVS (Forest Vegetation Simulator), they found that with HWA, there could be almost a complete loss of hemlock in disjunct and marginal populations within 20 years with a conversion to dense hardwood deciduous forests (Spaulding & Rieske, 2010).

Eastern hemlock has existed in Minnesota in small disjunct populations for at least 1,200 years. These populations are generally between 60-130 kilometers from the main range in Wisconsin (Calcote, 1987). The number of trees has decreased significantly due to logging, fire, herbivorous predation, and poor recruitment (Calcote, 1987; Smith, 2008) and eastern hemlock is now considered endangered in Minnesota (Calcote, 1987; MN DNR, 2013).

There have been sixteen outlier populations recorded in Minnesota by the Minnesota Natural Heritage Program, 8 of which still existed in the 1950s (Calcote, 1987; Zabinski, 1992). The largest stand occurred outside Paupores in St. Louis County in northeastern Minnesota. Known as the Paupores stand, this 280-acre area had more than 5,000 eastern hemlocks of various size and age classes (The North Woods, 1919;

Lawson, 1942). In 1912, trees in this stand were used to construct over 8,000 railroad ties. Realizing the rarity and ecological importance of this stand, foresters called on botanists and historians alike to take note, and even suggested that the area be made into a state park (The North Woods, 1919). However, in October 1918, the combination of a dry autumn and sparks from several trains ignited The Moose Lake-Cloquet Fire. In addition to the loss of 453 lives and 36 communities, it consumed 1,500 acres (Carroll & Raiter, 1983) and reduced the remaining hemlock in the Paupores stand to ashes (Lawson, 1942).

In 1975, eastern hemlock was listed as a “species of special interest” (Moyle, 1975) and was subsequently studied. Calcote (1987) investigated palynological evidence for the persistence of eastern hemlock in Minnesota. Sediment cores were gathered from water sources in Minnesota and those with substantial amounts of eastern hemlock pollen were carbon-dated. Researchers found that eastern hemlock has been in Minnesota for more than 1,200 years and postulate that there were more disjunct populations in northeastern Minnesota than documented in historical records. Results suggested that there was what Calcote (1987) refers to as a “shifting mosaic” of outliers in Minnesota, meaning that trees would occupy sites for one to two generations until climate, logging, fire, or animal herbivory prevented them from expanding (Calcote, 1987).

This is not surprising given that eastern hemlock grows in specific localities in Minnesota, often on moist, north-facing slopes and sheltered valleys near water (Smith, 2008). They are often found in mixed hardwood-conifer forests, growing with *Betula alleghaniensis* Britton (yellow birch), *Thuja occidentalis* L. (northern white cedar), *Pinus strobus* L. (white pine), and *Picea glauca* (Moench) Voss (white spruce). From the 8 remaining outlier populations known from the 1950s, only two exist today, one in West

Duluth and one in Hemlock Ravine Scientific Natural Area and Sanctuary. A Scientific and Natural Area (SNA) Sanctuary designation is the highest level of protection given to state lands by the Minnesota DNR.

In the mid-1980s, Calcote found 29 trees in distinct populations and several isolated trees in Minnesota. Thirty years later, this estimate has not increased. The stand of trees at Hemlock Ravine SNA was probably never any larger than 12 mature trees, according to pollen records (Calcote, 1987). Interestingly, it is rumored that Chippewa Native Americans may have brought seed from Wisconsin and Fond du Lac, near Duluth, planting them along commonly traversed routes as points of wayfinding (Lawson, 1942; G. Steele, personal communication).

In addition to the small number of known native remnant eastern hemlock trees in Minnesota, mature individuals of native, putatively native, and unknown provenance exist in state and municipal parks, public cultivated areas, and on private property. Notably, Jay Cooke State Park near Hemlock Ravine SNA, has one mature native tree. The Minnesota Landscape Arboretum in Chanhassen, MN contains over a dozen trees that are recorded as being derived from seeds collected in the late 1950s from an extirpated population at Mille Lacs Lake, MN. Additionally, there are over 200 trees of unknown, but possibly native origin. McCarthy Beach State Park near Hibbing, MN contains a number of trees that were planted in 1935, possibly by the Civilian Conservation Corps (T. Westbrook, personal communication). Theodore Wirth Park and the Eloise Butler Wildflower Garden in Minneapolis also contain a large number of trees, some of which were received from Anoka, MN in 1909, and from unspecified “Park Board Nurseries” in 1911 and 1914. Interestingly, Glensheen Mansion in Duluth, a nationally registered Historic Place, has several trees in cultivated and uncultivated

areas of the garden that were recorded as being planted in 1907. For more information on the history and source of sites, see Appendix A.

HORTICULTURE

Seed Propagation

Eastern hemlock has generally been propagated in two ways: via seed and vegetative cuttings. Individuals propagated by seed will be genetically distinct from parents and are of interest from a conservation perspective to maintain genetic diversity. Understanding the requirements for effective seed propagation depends on first understanding the reproductive biology of eastern hemlock.

Eastern hemlock trees start producing seed between 20 to 30 years of age (J. A. Young & Young, 1992) and can continue to produce seed until they are at least 450 years old (Barbour et al., 1980). They are monoecious, with male and female strobili that develop on lateral branches of the previous years' growth. Eastern hemlock produces ovoid to oblong female cones every year but will have larger crops every 2 to 3 years (Barbour et al., 1980). They are wind pollinated and seeds are dispersed via wind and gravity. Eastern hemlock has non-micropylar germination, meaning that instead of pollen entering the micropyle to germinate, as with all other species in the Pinaceae, the pollen attaches to the bracts on scales, germinates, and then the pollen tubes enter the micropyle (Barbour et al., 1980; Olson, Stearns, & Nienstaedt, 1959). Eastern hemlock is a diploid organism, with 2n = 24 chromosomes.

Female strobili are receptive to pollen between April and June, cones generally ripen in September and October, and seed is dispersed from October through the winter months. For seed harvesting purposes, cones should be collected when they are transitioning from purple to brown, but have not yet opened. Eastern hemlock has

hygroscopic cone scales which open when dry and close when wet, thus seed is dispersed intermittently in accordance with the weather (Fowells, 1965). Although seed is dispersed gradually throughout the winter, seeds shed in later months are often not viable (Olson et al., 1959). Cones can be difficult to harvest, as they are generally borne at the ends of lateral branches, often high in the tree (Barbour et al., 1980). After cones are dried and seeds extracted, eastern hemlock seeds have various pre-treatment options.

Eastern hemlock seeds have reportedly poor germination rates, with reported values ranging from 15% - 50%. However, these challenges can be overcome with proper seed treatment (Baldwin, 2011; Barbour et al., 1980; Jetton, Whittier, & Dvorak, 2014). Low germination rates may be due to difficulty in separating viable and non-viable seed (Dirr, 2006) or difficulty in replicating their somewhat strict emergence requirements in a nursery, greenhouse or artificial setting. Eastern hemlock seeds germinate best with considerable stratification (Baldwin, 2011; Dirr, 2006; Duchesne, Mueller-Rowat, Clark, & Pinto, 1999; Jetton et al., 2014; J. A. Young & Young, 1992). Cold-moist stratification, typically in a sterile mixture of sand/peat between 1° C and 5° C for between 6 and 10 weeks increases the germination speed and rates of eastern hemlock (Baldwin, 2011; Jetton et al., 2014). Olson et al. (1959) found success with 10 weeks of stratification for a variety of seed collected across North America, but there were considerable fungal pathogen problems noted and in-stratification germination occurred when stratification timeframes extended over 20 weeks.

Germination of stratified and un-stratified seed has been found to vary with photoperiod and temperature (Olson et al., 1959). A thorough review of eastern hemlock pre-treatment, germination, growth, and storage was published by the Connecticut Experiment Station (Olson et al., 1959). In general, stratified seeds germinated

sufficiently when temperatures fluctuated between 12° C at night and 21° C during the day. Olson et al. (1959) collected seed from 30 sources and found that for northern seed sources, 8-hour night intervals yielded the best germination and growth. There were also differences in stratification needs by seed source. Northern sourced seed was affected to a greater degree by stratification, whereas southern sourced seed was more dependent on photoperiod. When grown, northern sourced seedlings also went dormant earlier and had lower growth rates, indicating that seed source is important for survival in different environments.

Other conditions affecting germination include media and fertilization requirements. Coffman (1978) found that seeds germinated better on decomposed birch logs with low light compared to filter paper and A1 horizon soil, probably due to good moisture retention. Pollen, seeds, and seedlings are all sensitive to drying (Coffman, 1978; Olson et al., 1959). Post germination, eastern hemlocks are notoriously slow-growers and have trouble in nursery settings due to sun exposure and frost-heaving if sowed directly in the field (Barbour et al., 1980).

Vegetative Propagation

Vegetative propagation is the most common propagation technique used for eastern hemlock in the landscape nursery industry (Dirr, 2006), seeing as seed viability in the species is low and cuttings create genetic clones, which is important if certain ornamental characteristics such as dwarfism or variegation are desired (Hartmann, 2011). Eastern hemlock does not generally layer vegetatively in wild stands (Fowells, 1965). The rooting success of horticultural cuttings is dependent on many variables, including the time of year cuttings are taken, the age of the cutting, the age of the tree and its genotype, and the type and concentration of rooting hormones used.

Winter hardwood cuttings are used preferentially to summer softwood cuttings (Dirr, 2006; Jetton, Frampton, & Hain, 2005; Mitsch, 1975). Jetton et al. (2005) found that summer softwood cuttings had a lower rooting rate than reported for dormant or semi-dormant winter cuttings. However, successfully rooted summer softwood cuttings may put on new growth earlier than winter hardwood cuttings (Del Tredici, 1985).

There is no conclusive evidence that first or second year wood at the basal end of the cutting is better for producing cuttings. Several studies have noted that first year growth cuttings root better than older growth cuttings (Doran, 1952; Zak, 1958). However, Jetton et al. (2005) found that 6 cm cuttings had higher mortality rates but, if successful, had longer and more abundant roots than smaller, 3 cm cuttings. Studies using second year growth have reported success in rooting (Del Tredici, 1985; Gray, 1958; Waxman, 1985). If first year growth is small, it may be necessary to use second year growth.

Waxman (1985) noted that 5-year old trees showed greater rooting percentages on average than 12-year old trees, but genotype differences were also noted. However, Del Tredici (1985) found that a 120-year old weeping specimen still rooted successfully. Del Tredici (1985) also noted that all the genotypes in the study behaved differently, leading to the conclusion that different genotypes may be more or less suited to vegetative propagation.

Rooting hormones are beneficial for rooting eastern hemlock cuttings (Del Tredici, 1985; Doran, 1952; Fordham, 1971; Gray, 1958; Jesinger & Hopp, 1967). Common auxin treatments used in rooting hemlocks are indol-3-butyric acid (IBA) and the synthetic auxin, 1-napthaleneacetic acid (NAA). Fordham et al. (1971) found that the use of IBA and NAA together was the most successful in rooting eastern hemlock winter hardwood cuttings. However, rooting percentages decreased with increasing NAA

concentrations in softwood cuttings (Jetton et al., 2005). Wounding the cuttings has been purported to be beneficial to softwood cuttings (Dirr, 2006).

Winter dormant cuttings have been kept on bottom heat between 21° C and 24° C (Dirr, 2006; Mitsch, 1975) in a polyethylene chamber due to the high humidity that conifer cuttings require (Del Tredici, 1985; Fordham, 1971; Zak, 1958).

Although much literature has been published on the vegetative propagation techniques of eastern hemlock in commercial production settings, little is known about the vegetative propagation potential of older native and cultivated trees in Minnesota. There is evidence that genotype (Del Tredici, 1985; Ky-dembele et al., 2016) and age (Stuepp, de Bitencourt, Wendling, Koehler, & Zuffellato-Ribas, 2017) impact rooting response. This information will be critical for restoration and conservation efforts.

CONSERVATION

Introduction

In 1993, biologists from across the world came to the consensus that humans need to take considerable action to conserve the world's biodiversity. They began the Convention on Biological Diversity, an international treaty that outlined the ways the international community can combat biodiversity loss in all forms of biological life (CBD, 2017). It is now estimated that between 80,000 and 100,000 of the world's seed-bearing plants, about 25% of all flora, are under threat of extinction (BGCI, 2017). In Minnesota alone, there are 85 endangered plant species and as many threatened plant species (MN DNR, 2013). Common causes of species loss are deforestation, habitat degradation and fragmentation, pollution and the introduction of invasive plants, diseases, and pests. The results of these actions are exacerbated by climate change, which is likely to affect the range and survival of species (BGCI, 2017).

In Situ vs. Ex Situ Resources of Eastern Hemlock

Generally, there are two kinds of conservation strategies: in situ or on-site conservation and ex situ or off-site conservation. The preferred method of conservation is in situ, or preserving plants in their natural, native habitat (Reichard, 2011). In situ conservation efforts attempt to either preserve tracts of land with important habitat for flora and fauna or restore degraded ecosystems, a practice known as ecological restoration (Galatowitsch 2012; Havens et al., 2006; Reichard, 2011). Eastern hemlock is preserved in situ on protected lands in state and national parks and natural areas. As discussed previously, eastern hemlock is classified as an endangered species in Minnesota. The protection of lands, such as those at Hemlock Ravine Scientific and Natural Area and Sanctuary, are examples of in situ conservation (Northern Institute of Applied Climate Science, 2016).

Ex situ conservation is becoming increasingly important and there are several strategies available including seed-banking, propagation, and the preservation of genetic resources in cultivated landscapes and seed orchards. Seed-banking is a successful ex situ conservation strategy and is described as the drying, freezing, and saving of seeds in long-term storage. About 90% of all genetic resources saved for future use are held in seed banks (Pritchard, 2004). Eastern hemlock has orthodox seed, which is amenable to long-term storage in seedbanks, as opposed to unorthodox or recalcitrant seed which cannot be stored. A search of the USDA-ARS Germplasm Resources Information Database (GRIN) National Plant Germplasm System (NPGS) provides 231 active accessions of eastern hemlock from across North America, 8 of which are available to order (GRIN-Global, 2017).

A novel approach to conservation propagation includes somatic embryogenesis and cryopreservation. A study by Merkle et al. (2014) showed that embryogenesis and

cryopreservation of eastern hemlock are possible. However, few somatic seedlings survived the potting and hardening off phase, indicating a need for more research if cryopreservation is to be considered a long-term storage technique. In some situations, vegetative cuttings may be the only available propagation tool when seeds are unable to be seed-banked, not viable or mature, or there are too few to collect without damaging a population (Sugii & Lamoureux, 2004).

Another method used in ex situ conservation is the display of cultivated conservation species. It has sometimes been referred to as the 'ark' model referencing Noah's Ark, which refers to the thought that cultivated landscapes could be safe-havens for species during times of extreme habitat loss (Maunder et al., 2004). They can be grown in traditional garden environments, conservatories, nurseries, or natural landscapes and preserved through horticultural techniques. Eastern hemlock is a popular landscape plant and is seen in many collections in parks, botanical gardens and arboreta. For example, the Arnold Arboretum of Harvard University has at least 98 living accessions of eastern hemlock, many of which are wild collected from various sites in North America (Arnold Arboretum, 2017) and the Minnesota Landscape Arboretum has at least 16 trees that were wild collected from an extirpated population near Mille Lacs Lake, MN. In addition, seed orchards are viable ex situ alternatives that are established to bridge the gap between breeding and reforestation and nursery practices (Boyle et al., 2000). Camcore, an international tree breeding program headquartered at North Carolina State University, plan to establish experimental ex situ conservation plots of eastern and Carolina hemlock to maintain genetic diversity (Potter et al., 2008). At least one ex situ site in Santa Catarina, Brazil has been established for eastern hemlock (Jetton et al. 2013).

Conservation and Genetics of Eastern Hemlock

The objectives of conservation genetics are to understand how genetic processes affect genetic variation and to synthesize information from many disciplines including genetics, systematics, ecology, sociology, and economics (Boyle et al., 2000). The end goal of conservation genetics is the meaningful conservation of species, achieved by maximizing genetic diversity, reducing inbreeding depression, and understanding life history characteristics to better inform decision-making. Studying genetics can help define boundaries for species and units for conservation (DeSalle & Amato, 2004) and many studies have focused on understanding the genetics of small or disjunct populations (Kramer & Havens, 2009).

Disjunct and marginal populations that are geographically separated from their main range have been studied by ecologists and conservationists for decades. These populations often arise from either range contractions or range expansions. Disjunct populations are often considered to be of conservation value due to unique characteristics or alleles that arise from local adaptations and the loss of neutral alleles (Potter et al., 2012; Yang & Yeh, 1992), but also due to increased genetic differentiation from main range populations due to genetic drift and reduction in gene flow (Eckstein, O'Neill, Danihelka, Otte, & Koehler, 2006; Fang, Chung, Chiang, Chang, & Chen, 2013; Slatkin, 1987). Additionally, small sized populations can result in genetic consequences, such as genetic erosion that leads to inbreeding depression and a reduction in fitness (Hedrick, Savolainen, & Ka, 1999; Kramer & Havens, 2009).

Several studies have investigated the genetic diversity of eastern hemlock throughout its range using a variety of techniques. An early isozyme marker study from the University of Minnesota compared Minnesota disjunct populations with main range populations and found that Minnesota populations exhibited a reduction in

heterozygosity, indicating inbreeding, and few polymorphic loci (Zabinski, 1992). Chloroplast DNA markers revealed little evidence for among-population differentiation across the range of eastern hemlock (Lemieux, Beaulieu, & Bousquet, 2011; Wang, Perlin, Van Stockum, Jr., Hamilton, & Wagner, 1997) and lower within-population genetic diversity (Lemieux et al., 2011).

Microsatellite markers known as simple sequence repeats (SSRs) are commonly used in population genetics studies because they are highly variable and assumed to be selectively neutral (Schlötterer, 2000; Selkoe & Toonen, 2006). At least 21 SSRs polymorphic in populations of eastern hemlock have been published (Josserand, Potter, Echt, & Nelson, 2008; Shamblin, Faircloth, Josserand, Nelson, & Nairn, 2008). Potter et al. (2012) used 13 SSRs across 60 populations and found moderate inbreeding throughout the range of eastern hemlock. They also report low genetic diversity in marginal disjunct populations, but high differentiation (Potter et al., 2012). Likewise, Hobbs (2013) used 7 SSRs across 17 disjunct and 7 main range populations and found low levels of heterozygosity, especially in disjunct populations, and high differentiation. A more recent microsatellite study investigating the recovery of eastern hemlock in post-agricultural forests in Massachusetts found high genetic diversity and low genetic differentiation and inbreeding, emphasizing the importance of gene flow (Lumibao, Gaskill, Flood, & Mclachlan, 2016). The most recent studies have not investigated disjunct populations in Minnesota, likely due to the extremely small population size and its endangered status in the state.

OBJECTIVES

Eastern hemlock is experiencing a decreasing population trend due to the impacts of a foreign pest, the hemlock woolly adelgid (HWA), and an increasingly uncertain future in the face of climate change. There is a clear need for conservation

efforts for eastern hemlock, which is evident from the recent symposia devoted specifically to HWA (Onken & Reardon, 2010) and research on eastern hemlock ex situ resources (Jetton et al. 2013). The status of eastern hemlock in Minnesota is more perilous than ever. The focus of this research is to provide a framework for conservation for eastern hemlock in Minnesota. The information gained from this study can be broadly applied to conservation programs throughout the United States.

The specific objectives of this study are four-fold. 1) Determine the variation in seed and vegetative propagation efficiency for native eastern hemlock trees and eastern hemlock trees of unknown provenance. 2) Using SSRs, determine the genetic diversity and differentiation in Minnesota native and unknown provenance trees and seedlings compared to trees within the species' main range in Wisconsin, Michigan, and North Carolina. 3) Determine the paternity of eastern hemlock trees in the Minnesota Landscape Arboretum and in known Minnesota native populations to determine which individual trees are contributing to the next generation seedlings and estimate genetic purity of potential seed sources. 4) Based on findings in the first three objectives, develop a set of recommendations for the management and restoration potential of eastern hemlock for land managers at the Minnesota Department of Natural Resources, the Minnesota Landscape Arboretum, Theodore Wirth Park, and the Eloise Butler Wildflower Garden.

TABLES

Table 1-1: Origin and conservation status of *Tsuga* species and hybrids (Farjon, 2013).

Native Range	Species	Common Name	Conservation Status
Eastern North America	*Tsuga canadensis* (L.) Carrière	Eastern Hemlock	Near Threatened*
Southeastern North America	*Tsuga caroliniana* Engelm.	Carolina Hemlock	Near Threatened
China, Taiwan, Northeastern Vietnam	*Tsuga chinensis* (Franch.) Pritz.	Chinese Hemlock	Least Concern
	Tsuga chinensis var. *oblongisquamata* W.C.Cheng & L.K.Fu		
	Tsuga chinensis var. *robusta* W.C.Cheng & L.K.Fu		
Northern Japan	*Tsuga diversifolia* (Maxim.) Mast.	Northern Japanese Hemlock	Least Concern
Himalayan Mountains	*Tsuga dumosa* (D.Don) Eichler	Himalayan Hemlock	Least Concern
Southwestern China	*Tsuga forrestii* Downie	Forrest's Hemlock	Vulnerable A2cd
Northwestern North America	*Tsuga heterophylla* (Raf.) Sarg.	Western Hemlock	Least Concern
	Tsuga × *jeffreyi* (A.Henry) A.Henry		
Northwestern North America	*Tsuga mertensiana* (Bong.) Carrière	Mountain Hemlock	Least Concern
	Tsuga mertensiana subsp. *grandicona* Farjon		
Southern Japan, South Korea	*Tsuga sieboldii* Carrière	Southern Japanese Hemlock	Near Threatened*
	Tsuga sieboldii var. *nana* (Endl.) Carrière		

*indicates a decreasing population trend

FIGURES

Figure 1-1: Eastern hemlock (grey) and Carolina hemlock (black) North American ranges (Clemson Center for Geospatial Technologies, 2016).

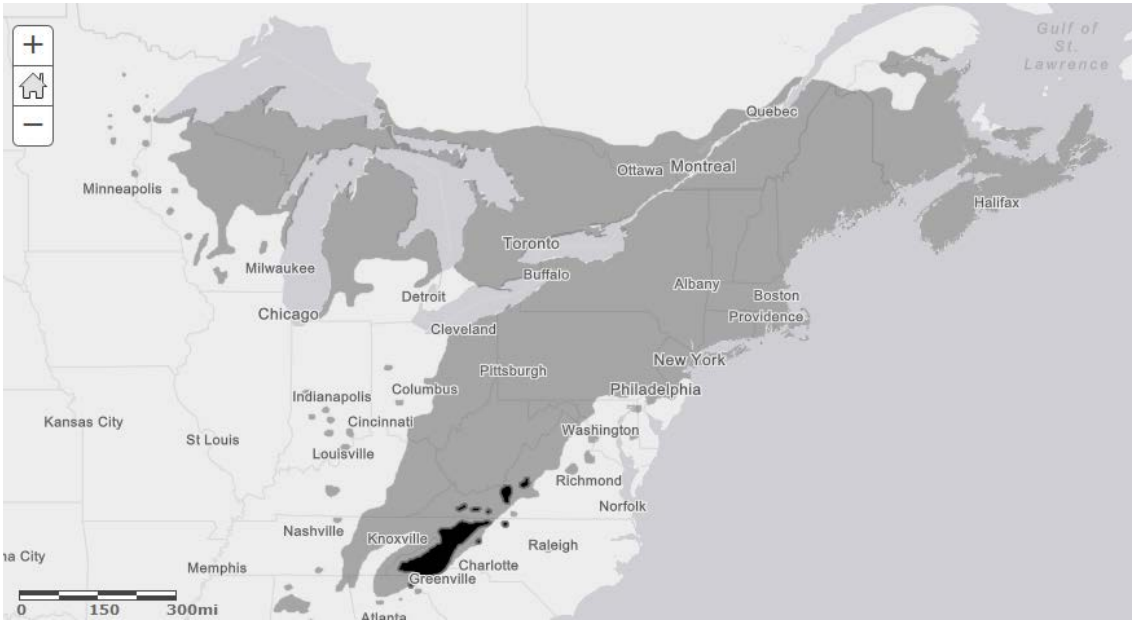

Figure 1-2: Photographs of morphological features of eastern hemlock. A) Eastern hemlock female reproductive cones. Closed, green cones are the current year's crop and open, brown cones are the previous year's crop. B) Nodding terminal top of an eastern hemlock. C) Stomatal bands on abaxial sides of leaves. D) Appressed leaves along the length of the stem.

CHAPTER 2:

The Genetic Diversity of Native and Cultivated Eastern Hemlock (*Tsuga canadensis* (L.) Carrière) in Minnesota

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INTRODUCTION

Microsatellite markers, especially simple sequence repeat markers (SSRs), are widely used in ecological studies and population genetics because they represent selectively neutral variation and are highly variable, or polymorphic (Schlötterer, 2000; Selkoe & Toonen, 2006). SSRs are used to measure, among other things, variation within and among populations, which can give insights into the evolutionary history of populations, as well as the current genetic characteristics of these populations (Wright, 1951). A common measure of genetic differentiation among populations for microsatellite markers is R_{st}, which parallels Wright's well-known fixation index, F_{st}, and measures the amount of genetic variance that can be explained by population structure using a step-wise mutation model (Slatkin, 1995; Wright, 1951). SSRs have been useful in measuring genetic differentiation in trees (Leonarduzzi, Piotti, Spanu, & Vendramin, 2016), as well

as measuring pollen contamination and assigning paternity (Koch, Carey, & Mason, 2010; Slavov, Howe, & Adams, 2005).

The genetic diversity and origin of disjunct populations of trees have been widely studied. Disjunct peripheral populations are separated from main range populations as a result of long distance dispersal events and range expansions (Petit et al., 2003). On one hand, this separation can have major genetic consequences for organisms. For example, trees, especially conifers, that are isolated will often have less within-population differentiation and may experience inbreeding depression (Franklin, 1970). Inbreeding depression signifies a reduction in fitness due to the aggregation of deleterious alleles (Charlesworth & Charlesworth, 1987). On the other hand, populations that are disjunct can contain greater among-population differentiation due to genetic drift and reduction in gene flow (Dantas et al., 2015; Gapare, Aitken, & Ritland, 2005; Pandey & Geburek, 2010). Disjunct populations may be of conservation value due to unique characteristics or alleles that arise from local adaptations and the loss of neutral alleles (Petit, Mousadik, & Pons, 1998; Potter et al., 2012; Yang & Yeh, 1992).

It is estimated that around 25% of the world's flora are under threat of extinction due to deforestation, habitat degradation, fragmentation, pollution, and the introduction of invasive plants, pests, and diseases (BGCI, 2017). Conservation genetics is a discipline of study that applies theories from traditional population genetics to species that are under threat of extinction (Boyle et al., 2000). In addition to the preservation of in situ resources, ex situ resources, such as resources in parks, gardens, and seed-banks, provide support for conservation efforts (Havens, Vitt, Maunder, Guerrant, & Dixon, 2006).

Eastern hemlock (*Tsuga canadensis* (L.) Carrière), a slow-growing and long-lived conifer, is an example of a species with a wide distribution with disjunct, peripheral populations. Its extensive range in North America reaches from Nova Scotia, south along the Appalachian Mountains to northern Georgia and Alabama, and west to Wisconsin. Disjunct, fragmented populations occur in Indiana, Ohio, Tennessee, Kentucky, and Minnesota. It is also a horticultural crop, with resources in the nursery trade and in parks and gardens across North America. Currently, eastern hemlock is experiencing a range-wide decrease in population size due to the invasion of an insect from Asia, the hemlock woolly adelgid (*Adelges tsugae* Anaand.), and eastern hemlock is considered "near-threatened" according to the International Union for Conservation of Nature (Farjon, 2013).

In Minnesota, eastern hemlock has always existed in disjunct populations but is now considered endangered, with just two remaining known native populations and fewer than 40 known mature native trees and scattered saplings. Trees of known native provenance at the Minnesota Landscape Arboretum originated from seed collected from a now extirpated small population near Mille Lacs Lake, Minnesota in 1957 and 1960. There are over 200 additional trees of unknown provenance in state and municipal parks and public gardens.

Numerous studies have focused on the genetic diversity of eastern hemlock in its main range and disjunct populations. Notably, an early study investigated disjunct Minnesotan eastern hemlock using isozyme markers and found that Minnesota disjunct populations had a low number of heterozygotes, indicating inbreeding (Zabinski, 1992). A chloroplast DNA (cpDNA) study comparing disjunct outlier populations and main range populations included trees from Duluth, MN. This work discovered polymorphic cpDNA

loci in Minnesota, and found low differentiation among populations (Wang et al., 1997). Since then, disjunct Minnesota populations have not been included in population genetic studies, likely due to eastern hemlock's state status as an endangered tree. Although not including Minnesota trees, a more recent chloroplast study across the main range of eastern hemlock also found low differentiation among populations and evidence for glacial refugia in southeastern United States (Lemieux et al., 2011). A 2012 study using SSRs also found evidence for glacial refugia and high diversity in the southeastern United States, as well as high differentiation among populations (Potter et al., 2012). The authors also found moderate inbreeding and low genetic diversity in disjunct populations. Likewise, Hobbs (2013) found low levels of heterozygosity and high levels of genetic distinctivenss in disjunct populations and high overall genetic differentiation. With these studies in mind, investigating the genetic diversity of Minnesota disjunct eastern hemlock using genomic derived SSRs can increase our knowledge of the genetics of eastern hemlock across its range and inform conservation strategies.

The objectives of this study were to 1) determine the genetic diversity of Minnesota native trees, trees of unknown provenance, and seedlings using previously published SSR markers (Josserand et al., 2008; Shamblin et al., 2008) and compare these results with trees within the main range of the species in the Great Lakes region (Wisconsin and Michigan) and in the center of species diversity (North Carolina); 2) determine the likelihood that reproductive age trees growing in Minnesota and at the Minnesota Landscape Arboretum are of native origin; and 3) determine the paternity of eastern hemlock trees in the Minnesota Landscape Arboretum and in known Minnesota native populations to determine which individual trees are contributing to the next generation seedlings and estimate genetic purity of potential seed sources. With this

information, we can better understand the genetics of eastern hemlock in Minnesota which can inform conservation and restoration practices across the state.

MATERIALS AND METHODS

Sample Identification and Collection

Eastern hemlock sites across Minnesota were identified using the Minnesota Department of Natural Resources (DNR) Rare Features Database and information from individuals at the Minnesota DNR (MN DNR, 2014). Foliage was collected from the lower crown of 204 trees, each over 1 meter tall, across 10 sites in Minnesota and from 61 trees from three sites outside of Minnesota between October 2014 and February 2017 (Table 2-1, Figure 2-1, and Appendix B). Samples from individual trees comprise one to two 15 cm shoot tips. The descriptions of sites are variable and addressed in Table 2-2. Shoot samples were also collected from the Great Lakes region (Wisconsin and Michigan) and North Carolina from a minimum of 20 trees separated by at least 100 meters, a strategy that ensures sufficient sampling of genetic diversity (Potter et al. 2012). Tissue samples were either placed in silica bead gel desiccant in the field or in a cooler with dry ice (when collected from seedlings in the greenhouse) and transported to the University of Minnesota St. Paul Campus, where they were stored at -80°C until lyophilization. Herbarium specimens were also used as a source of genetic material. Between 15 and 30 dried needles were collected from herbarium specimens from the Bell Museum of Natural History and the Minnesota Landscape Arboretum Herbarium (Table 2-3). Herbarium samples were placed in coin envelopes and stored at room temperature.

Additionally, female cones were collected from 22 trees across seven sites in Minnesota. Trees were selected for sampling based on the presence of ripe cones and

their accessibility. Permits from the Minnesota DNR allowed collection of up to 20% of the cones from each tree greater than 10 inches (about 25 cm) diameter at breast height (DBH) with a maximum of 200 cones from any particular tree. Cones were dried and seeds extracted in the fall of 2014 and 2015, cold-moist stratified, germinated, and grown for sampling for genetic analysis at the Horticultural Research Center in Chanhassen, Minnesota (see Chapter 3 for more on seed treatment).

Herbarium specimens were collected as seed voucher specimens. Herbarium specimens were comprised of 30 cm of foliage sample with seasonally available reproductive parts, such as male and female strobili and/or cones. Samples were pressed in the field, dried, and mounted at the Bell Museum of Natural History Herbarium and the Minnesota Landscape Arboretum Herbarium. Other data collected include DBH (cm), height (m), reproductive status, GPS coordinates, and notes on health and habitat. Full field notes and information on herbarium specimens are presented in Appendix B.

DNA Isolation, PCR Optimization and SSR Marker Analyses

For DNA isolations, a subsample of needles was removed from each collected tissue sample and lyophilized for 2-7 days. Genomic DNA was extracted from between 10 mg and 20 mg of lyophilized needle tissue using a Qiagen DNeasy Plant Mini Kit and a DNeasy 96 Plant Kit (Qiagen, Chatsworth, California, USA). Manufacturer instructions were followed for DNA isolation except the incubation of the samples on ice during the protein and polysaccharide precipitation step was increased from 5 min to 30 min to increase yield and purity. Estimates of DNA concentrations and purity ratios were determined using a Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific,

Wilmington, DE, USA). Genomic DNA was diluted to 5 ng/µl, suspended in PCR grade H_20, and stored at -20°C.

SSR primers were selected from 21 previously described eastern hemlock and Carolina hemlock primers (Shamblin et al. 2008; Josserand et al. 2008). First, all 21 primers were tested against a panel of 3 genotypes from 3 Minnesota sites representing native trees and trees of unknown provenance. PCR conditions were as follows: 2.5 min at 95°C; 21 cycles of 20 s at 95°C (denaturation), 20 s at 65°C for the first cycle with a decrease of 0.5°C each subsequent cycle (annealing), and 30 s at 72°C (extension); and 24 cycles of 20 s at 95°C, 30 s at 55°C, and 30 s at 72°C; followed by a final 15 min extension at 72°C and an indefinite hold at 4°C. Various primer and DNA template concentrations were tested with these 3 genotypes.

Amplified fragments were evaluated on an agarose gel for consistent amplification in single and multiplexed reactions. Based on these initial results, 12 forward primers were fluorescently direct labeled on the 5' end and tested across a panel of 8 genotypes from 8 Minnesota sites across a range of provenances. The markers were optimized for primer and DNA template concentrations and multiplexing and pooling capacities using an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA) at the University of Minnesota Genomics Center.

Based on our assessments, 8 primers from Shamblin et al. (2008) were selected based on reliable amplification and high polymorphism rates in Minnesota trees: *Tcn10A12*, *Tcn10B01*, *Tcn12C01*, *Tcn2C08*, *Tcn3E02*, *Tcn3H04*, *Tcn2B04*, *and Tcn10A07* (Table 2-4). These 8 primers were subsequently used for genotyping Minnesota trees. A subset of 4 of these primers (*Tcn10B01*, Tcn12C01, *Tcn3E02* and *Tcn3H04*) were also used to genotype seedlings of Minnesota trees based on high

polymorphic information content (PIC), low null allele frequency, and ease of scoring over a panel of 225 – 259 individuals. In each case, forward primers were fluorescently direct labeled on the 5' end (Table 2-4). PCR was performed in 10 µL volumes, each containing 5 ng genomic DNA, 0.4 mM dNTPs, 0.3 µM forward primers and reverse primers, 1X Taq ThermalPol® Buffer, and 0.125 µl Taq DNA polymerase. Final PCR conditions for primers *Tcn10A12*, *Tcn10B01*, *Tcn12C01*, *Tcn2C08*, *Tcn3H04*, *Tcn2B04*, *Tcn10A07* were as follows: 2.5 min at 95°C; 30 cycles of 20 s at 95°C (denaturation), 20 s at 56°C (annealing), and 30 s at 72°C (extension); followed by a 15 min extension at 72°C and an indefinite hold at 4°C. PCR conditions for primer *Tcn3E02* were 2.5 min at 95°C; 30 cycles of 20 s at 95°C (denaturation), 20 s at 56°C (annealing), and 30 s at 72°C (extension); followed by a 15 min extension at 72°C and an indefinite hold at 4°C. Reactions were run on two machines; an Eppendorf Mastercycler Nexus Gradient Thermal Cycler (Eppendorf, Hamburg, Germany) and a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

PCR products were separated on an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Peaks were sized and binned, and then alleles were called using Geneious R10 (Biomatters Ltd., Auckland, New Zealand) with Liz®-500 as an internal size standard for each sample. Visual checks were performed on all peaks and hand-sized if necessary.

Statistical analysis

Allele calls were used to calculate diversity statistics and measures of differentiation for eastern hemlock trees and their seedlings. We assessed observed and expected heterozygosity, null allele frequencies and polymorphic information content (PIC) of loci across a panel of between 225 – 259 individuals using the parentage

program, Cervus 3.0.7 (Table 2-4) (Kalinowski, Taper, & Marshall, 2007; Marshall, Slate, Kruuk, & Pemberton, 1998). We calculated general F-statistics such as the observed and expected heterozygosity, and the fixation index over all loci and individuals from each collection site (Table 2-5) using GeneAlEx 6.5 (Peakall & Smouse, 2006). Marker *Tcn10A12* was excluded from the analysis due to >10% missing data and 13 individuals were excluded from the analysis because they had 2 or more loci with missing data. The following sites were excluded from analyses due to small population size: Private Property (PP), Carleton Cowling Arboretum (CCA), and Jay Cooke State Park (JC). Data from one known native individual from Jay Cooke (JC) was combined with data from Hemlock Ravine (HR) due to geographic proximity to create one single site for analysis. Additionally, the Minnesota Landscape Arboretum trees were split into two sites for data analysis: cultivated (MLA) and native (MLA-ML), based on the native origin of the trees originating from the extirpated Mille Lacs Lake population. We calculated F-statistics by maternal accession for Minnesota Landscape Arboretum seedlings and by site for native seedlings (Table 2-6). We conducted an AMOVA (Slatkin, 1995; Weir & Cockerham, 1984) with interpolated data to account for missing data and calculated R_{st} and R_{is} to measure admixture and inbreeding within and among populations and individuals for all sites (Table 2-7) and native sites (Table 2-8).

To better understand and visualize the similarities between collection sites, we created a dendrogram (Figure 2-2) of all sites using Nei's genetic distance matrix output from GeneAlEx in the statistical program R version 3.1.2 (R Core Team, 2013) and RStudio (RStudio Team, 2012). We also conducted a Principal Coordinate Analysis (PCoA) (Figure 2-3) based on pairwise population F_{st} values in GeneAlEx to visualize similarities across collection sites.

We used a Bayesian based software program, STRUCTURE (Pritchard, Stephens, & Donnelly, 2000), to identify subgroups that have distinct allele frequencies and group these into genetically distinct clusters to visualize admixture and trends. We did this across all known native populations, including the trees from the Minnesota Landscape Arboretum that originated from Mille Lacs Lake. We tested the number of genetic clusters (K) for K=1 through K=10 with 10 iterations with a burn-in length of 20,000 and 70,000 Markov Chain Monte Carlo (MCMC) runs. The correct K was selected by using the Evanno ΔK method of the change in log likelihood values using STRUCTURE HARVESTER (Table 2-9) (Earl & VonHoldt, 2012; Evanno, Regnaut, & Goudet, 2005). Results were visualized using CLUMPAK and DISTRUCT (Figure 2-4) (Kopelman, Mayzel, Jakobsson, & Rosenberg, 2015).

Allele calls were also used to assign paternity of seedlings and measure genetic contributions of Minnesota native and the Minnesota Landscape Arboretum trees to the next generation seedlings. Paternity assignment was measured using Cervus, which takes a maximum-likelihood approach and estimates error rate and accounts for mutation rates. We ran Cervus simulations assuming a 5% genotyping error rate and based on the assumption that 90% of pollen donors (fathers) had been sampled. We narrowed down candidate fathers if there were positive LOD scores (log of likelihood ratio). LOD scores were calculated with the following simulation parameters for all runs: 10,000 offspring simulated, 90% candidate fathers sampled, with a minimum of 2 loci typed. Two separate simulations and parentage analyses were run, one for native seedlings and one for seedlings from the Minnesota Landscape Arboretum. The number of potential candidate fathers varied from 59 for native trees (including all native genotyped trees within a 30 kilometer radius) and 100 for the Minnesota Landscape

Arboretum trees (including all genotyped trees within a 30 kilometer radius). The most likely parents were determined by taking the highest trio LOD scores for matched mother-father pairs.

RESULTS

Field Work and Herbarium Specimens

Overall, we discovered 36 mature native eastern hemlock trees over 1-meter tall growing in three disjunct Minnesota sites: Hemlock Ravine, West Duluth, and Jay Cooke State Park (Appendix B). Hemlock Ravine had a substantial number of seedlings growing on site, often within deer exclosures introduced by the Minnesota Department of Natural Resources. One sapling was growing within the isolated deer exclosure at Jay Cooke State Park. There was no regeneration found at the West Duluth sites, although trees were of reproductive age. Trees at Hemlock Ravine and West Duluth were found on slopes along drainages or creeks and the Jay Cooke State Park tree was near the St. Louis River.

Unknown provenance trees were found growing in a variety of site conditions. Notably, there were a variety of age classes at Tischer Creek and Glensheen Mansion, as well as in the Eloise Butler Wildflower Garden, indicating regeneration among these sites. The largest trees encountered during this study within Minnesota were growing in the Eloise Butler Wildflower Garden. The widest tree had a DBH of 69.2 cm (EB027) and the tallest tree was 25.3 meters (EB017) (Appendix B). Two trees growing at the Minnesota Landscape Arboretum that were planted in 1960 were less than 3.7 cm DBH (Appendix B).

The oldest herbarium specimen we extracted DNA from was collected in Carlton County, MN in 1893. The amount of DNA extracted from herbarium specimens ranged

from 1.1 ng/µl to 47.9 ng/µl. In contrast, fresh samples rarely yielded less than 10 ng/µl and up to 198.8 ng/µl. Although DNA was successfully extracted from herbarium specimens, PCR amplification was only successful with one specimen, HERB007 from the Minnesota Landscape Arboretum Herbarium, which was collected from Mille Lacs County, MN in 1971 (Table 2-3).

Genetic Diversity and Differentiation

Of the 8 tested SSR markers, marker *Tcn10A12* was the most polymorphic (PIC = 0.825) and had a comparatively low incidence of null alleles (19.39%; Table 2-4). This marker, however, yielded >15% total missing data due to failed PCR amplification and was eventually dropped from the analysis to reduce bias. The next most polymorphic marker was Tcn10B01 (PIC = 0.756; null alleles = 22.35%; Table 2-4). The lowest PIC was 0.170 in marker *Tcn2B04*, which had only 3 alleles observed in our collection sites. Marker *Tcn2C08* had a high frequency of null alleles as calculated by Cervus (40.50%). For all markers, the observed heterozygosity (H_o) was lower than the expected heterozygosity (H_e). All but two markers (*Tcn2B04*, *Tcn10A07*) revealed significantly fewer heterozygotes than expected under Hardy-Weinberg Equilibrium.

Across ten collection sites, native Minnesota sites had the highest fixation indices [Hemlock Ravine (HR), F = 0.463; West Duluth, (WD) F = 0.365], while North Carolina (NC) had the lowest fixation index (F = -0.007), followed by the Minnesota Landscape Arboretum native trees (MLA-ML, F = 0.060). Across cultivated trees and trees of unknown provenance, there was also lower incidence of heterozygotes than expected, besides McCarthy Beach State Park (MB) which had comparably more heterozygotes (Table 2-5).

Among seedlings, those from seed collected from Hemlock Ravine (HR) and West Duluth (WD) populations also had higher fixation indices than trees collected from the native origin Minnesota Landscape Arboretum trees (Table 2-6). Seedlings originating from maternal accession MLA17 had a higher observed than expected heterozygosity (H_o = 0.327, H_e=0.282).

Population differentiation as measured by Slatkin's R_{st} across all collection sites was relatively low (R_{st} = 0.059), with moderate inbreeding (R_{is} = 0.183) (Table 2-7). Population differentiation and inbreeding increased (R_{st} = 0.123; R_{is} = 0.219) when cultivated trees and trees of unknown provenance were removed from the analysis and only known native-source sites were included (Table 2-8).

Population Clustering

From the Bayesian clustering test, STRUCTURE, the most likely number of genetic clusters was K=4. There was little admixture for these clusters in North Carolina (NC), Hemlock Ravine (HR), and Minnesota Landscape Arboretum native (MLA-ML) trees (Figure 2-2). These same sites also had higher proportions of individuals from the same genetic cluster (Figure 2-2) and distinctly different genetic clusters. Hemlock Ravine (HR) and Minnesota Landscape Arboretum native trees (MLA-ML) were also different from West Duluth (WD) trees, which were more similar to trees in Wisconsin (LM).

The dendrogram of Nei's genetic distances revealed in its hierarchical structuring that Hemlock Ravine (HR) and Mille Lacs Lake native-sourced trees at the Minnesota Landscape Arboretum (MLA-ML) were different from all other sites, with North Carolina (NC) being the next most differentiated (Figure 2-3). Cultivated and naturalized trees in Theodore Wirth Park (TWP) and the Eloise Butler Wildflower Garden (EB) clustered

together, as did trees from Tischer Creek and Glensheen Mansion (TC) and McCarthy Beach State Park (MB). The native Minnesota West Duluth (WD) site was more genetically similar to Lake Minnesuing, Wisconsin (LM), Baraga State Forest, Michigan (MI), and North Carolina (NC), than Hemlock Ravine (HR) in Minnesota (Figure 2-2). There were similar trends in the Principal Coordinates Analysis (PCoA) based on the F_{st} distance matrix (Figure 2-4).

Paternity Analysis

For Minnesota native trees, 58 out of 77 seedlings (75%) had positive LOD scores for mother-father matches. One candidate mother-father match was assigned at a strict (95%) confidence level and 5 mother-father matches were assigned at a relaxed (80%) confidence level (Table 2-10). There was a high average observed error rate (12.4%) in the analysis over all four markers, assuming all known parent-offspring pairs were equally independent. Of 77 known offspring-mother matches, there was a total of 20 mother-offspring mismatches in genotyping, 18 of which were due to null alleles and the remaining due to genotyping error. Of the 18 with null alleles, 16 were mismatched at marker *Tcn3H04* (Table 2-11). For seedlings originating from accession WD011 (West Duluth, 2015 collection), an accession from the Tischer Creek and Glensheen Mansion site (TC018) had the most positive LOD scores. The individuals HR017 and HR007 from Hemlock Ravine were assigned positive LOD scores for Hemlock Ravine derived seedlings, although not at a significant confidence level (Table 2-10).

For Minnesota Landscape Arboretum seedlings, 111 out of 131 had positive LOD scores for mother-father matches with 9 mother-father matches assigned at relaxed (80%) confidence level for paternity assignment (Table 2-12). The average error rate in analysis over all markers was 7.77%. Out of 131 known offspring-mother matches, there

were a total of 16 mother-offspring mismatches in genotyping, 11 of which were due to null alleles and the remaining due to genotyping error (Table 2-11). Of the 111 assignments, 7 most likely candidate fathers came from outside of the Minnesota Landscape Arboretum. These candidate fathers were from the Eloise Butler Wildflower Garden (EB032, EB034), located approximately 25 km away (Table 2-12). The most frequent most likely fathers were MLA017 (n=23), MLA020 (n=23) and MLA018 (n=22). Additionally, 8 of 9 statistically significant mother-father pairs (at 80% confidence level) had an assigned father from a single accession, MLA018 (Table 2-12).

DISCUSSION

Field Work and Herbarium Specimens

The number of native trees in Minnesota has noticeably declined from recent estimations (Smith, 2008) to 36 mature trees greater than 1 meter tall. In 2012, floods in the Duluth area caused mortality in a number of trees at the Hemlock Ravine Scientific and Natural Area and Sanctuary (M. Cleveland, personal communication) and the remaining trees are growing along steep slopes in the ravine, often in perilous conditions. Jay Cooke State Park also has limited regeneration within a substantial deer exclosure. We expect that deer exclosures added by the Minnesota DNR have allowed significant regeneration of eastern hemlock. Eastern hemlock provides wind and snow protection during harsh winter months and a winter food source for white-tailed deer (*Odocoileus virginianus* Zimmerman), porcupines (*Erthizon dorsatum* Linnaeus), and other small mammals. It is likely that the exclusion of these animals through fences has helped with regeneration. There is currently no protection for trees in West Duluth, which are growing off a pedestrian trail. Trees in West Duluth produce viable seed (see Chapter 3), but conditions on site are not conducive to seedling survival. This could be

due to deer and small rodent browse. It has been shown that browsing by white-tailed deer causes decline in eastern hemlock forests and affects the ability for regeneration, even more than a changing climate and poor seedbed conditions (Anderson & Loucks 1979; Frelich & Lorimer 1985; Rooney 2001; Faison et al. 2016).

It is interesting that the largest trees in Minnesota are growing in the cultivated site at the Eloise Butler Wildflower Garden and Theodore Wirth Park. It is possible that the protection and regular maintenance of these sites could lead to more growth. Age could also be a factor to their size, as many of the trees at Eloise Butler Wildflower Garden were recorded as being planted between 1907 and 1914. Trees at Tischer Creek were likely planted along the creek by the Congdon family who built and owned Glensheen Mansion in the early 1900s (Leavitt, 1907). These trees, growing closely along the creek, seem to be regenerating, with seedlings and saplings appearing among the rocky ridges along the creek.

Herbarium specimens proved to be difficult to use for marker analyses. Although DNA quantification estimates showed a minimum of 1.1 ng/µl and a maximum of 47.9 ng/µl, only one of 31 herbarium accessions reliably yielded PCR product. Interestingly, the successful specimen, HERB007, had an estimated DNA concentration of only 1.9 ng/µl. This small amount of DNA suggests that the problem with downstream analysis lies not with the amount of DNA, but rather the purity. Eastern hemlock needles contain tannins, which can negatively affect purity and in turn, downstream analysis (Hiesinger, Ritt, & Phenol, 2001). Herbarium specimen DNA has been shown to frequently be degraded, often due to specimen preparation (Staats et al., 2011; Wandeler, Hoeck, & Keller, 2007). It degrades quickly after extraction, and is ill-suited for long-term storage. Drabkova et al. (2002) found that mixer mill grinding and extraction with a Qiagen

DNeasy Plant Kit, but with longer than recommended incubation times, more AP1 buffer, less final elution buffer, and a longer elution time, yielded more DNA and gave better PCR results. Higher numbers of PCR cycles and shorter DNA storage time in TE (10 mm Tris, 1 mm EDTA) buffer, as opposed to H_2O, may also be required for higher quality PCR results (Drabkova, Kirschner, & Vlcek, 2002). The age of specimens and the type of Taq polymerase used could also affect PCR amplification (Telle & Thines, 2008). We extended the incubation time for our DNeasy Plant Kit DNA extractions, but other recommendations of Drabkova et al. (2002) warrant consideration. Additionally, although DNA was stored for a relatively short amount of time (< 3 months) it was stored in double deionized H_2O. Storing in TE (10mm Tris, 1mm EDTA) buffer may be more effective.

Genetic Diversity and Differentiation

We measured F-statistics for all loci that amplified in our collection sites to inform primer pair choice for seedling analysis. All markers yielded 3 or more alleles across our collection sites and were considered polymorphic. Marker *Tcn10A12*, although the most polymorphic in our populations, was difficult to score and had much missing data due to failure in PCR product amplification. Missing data and scoring inconsistences could have been due to PCR conditions, such as insufficient annealing temperatures or number of cycles. Marker *Tcn2C08* produced a high frequency of null alleles, which are defined as non-amplified alleles that result in either scoring a heterozygote as a homozygote or PCR reaction failure (Guichoux et al., 2011). These may be due to mutations in primer binding sites. This marker also had a much lower observed than expected heterozygosity (Table 2-4) and was ultimately not employed for seedling analyses. Overall, 6 of 8 SSR markers yielded fewer than expected heterozygotes. While the presence of null alleles can contribute to this observation, our data are consistent with a

lack of heterozygotes across our collection sites. Potter et al. (2012) found smaller null allele frequencies in many of the same markers (*Tcn3E02*: 0.04; *Tcn3H04*: 0.08; *Tcn10A07*: 0.073), but also found significantly reduced observed than expected heterozygosity.

We found generally high fixation indices, or inbreeding coefficients (F) in native Minnesota sites, especially Hemlock Ravine (F = 0.463). This fixation index measures inbreeding within sites and indicates that there is a higher level of inbreeding in Minnesota native sites than sites in Michigan (F=0.164) and North Carolina (F = -0.007). This pattern of inbreeding in disjunct Minnesota sites is corroborated by a study conducted in 1987 that also found a decrease in heterozygotes within disjunct Minnesota sites (Zabinski, 1992). However, when we separated the Minnesota Landscape Arboretum trees from garden or nursery origin (MLA) and those collected as seed from the extirpated Mille Lacs Lake trees (MLA-ML), we observed little inbreeding in the MLA-ML trees. This discrepancy could be due in part to the smaller population size of MLA-ML (n = 15) or that the trees from Mille Lacs Lake may have originally been less inbred than the current native trees in Minnesota. A negative fixation index in the North Carolina population indicates that we observed more heterozygotes than expected under Hardy-Weinberg equilibrium, suggesting a panmictic population. This is not surprising, as the North Carolina population is not disjunct or on the edge of the range, but rather is the center of species diversity (Potter et al., 2012).

A similar pattern of inbreeding was found among seedlings. Seedlings from Hemlock Ravine and West Duluth had higher fixation indices than seedlings from native Minnesota Landscape Arboretum trees, with Hemlock Ravine being the most inbred (Table 2-6). This could be due to there being more possible pollen donors in closer

geographic proximity to Minnesota Landscape Arboretum trees (at least 32 trees within a 3 km radius) compared with Hemlock Ravine and West Duluth (between 22 and 13 trees within a 3 km radius, respectively). Also, Minnesota Landscape Arboretum trees are growing in artificial sympatry with cultivated forms of eastern hemlock from a variety of sources, which could introduce different genetics from sources outside of Minnesota or the general Great Lakes region (Havens et al., 2006).

Observed levels of inbreeding in cultivated and unknown provenance trees could be due to the provenance of the trees planted. For example, trees planted from nursery stock could be more genetically similar, especially if some were propagated from vegetative cuttings, collected from a single maternal accession, or grown as seed from full-sib crosses. Additionally, the high frequency of null alleles observed in this study suggests the possibility that some genotypes scored as homozygous (+/+) for a particular marker could in fact be heterozygous (+/null). In this instance, our calculated inbreeding coefficient would be inflated. If trees are meant to be maintained as a conservation collection, these genetic consequences need to be taken into consideration. A study by Enßlin et al. (2011) found that the genetic diversity of populations of a short-lived perennial plant, *Cynoglossum officinale* L., decreased with the time spent in cultivation. Eastern hemlock has much longer generation times in comparison, so capturing genetic variation with targeted collections and management will be essential (Cibrian-Jaramillo et al., 2013). While the original goal of cultivated sites in Minnesota may not have been to conserve genetic diversity, knowing which trees are less inbred and what is their likely provenance will help in developing conservation plans.

We found low levels of genetic differentiation among all collections sites (R_{st} = 0.059) and moderate levels of inbreeding (R_{is} = 0.183). These numbers were increased when cultivated and unknown provenance trees were removed from the AMOVA analysis (R_{st} = 0.123, R_{is} = 0.219). The low levels of population differentiation when measured across all sites could again be in part due to the unknown provenance trees. Since trees in cultivation are not natural populations, it makes little sense to treat them as such. However, it is interesting to note that the populations show more genetic structure when only native populations are included in the analysis.

Population Clustering

In our analysis of population structure, we found K=4 genetic clusters across known native collection sites. Sites such as Hemlock Ravine (HR), Minnesota Landscape Arboretum native trees (MLA-ML) and North Carolina (NC) overwhelmingly belonged to different genetic clusters than all other sites (Table 2-2). This was also true for both the dendrogram based on Nei's genetic distance matrix (Table 2-3) and the Principal Coordinate Analysis (PCoA) based on Wright's F_{st} values (Table 2-4). It is especially interesting that Hemlock Ravine, which contains disjunct Minnesota native trees, was dissimilar in all analyses from West Duluth (WD), which is another disjunct Minnesota site located less than 30 kilometers from Hemlock Ravine. Palynological evidence shows that eastern hemlock has existed at Hemlock Ravine for at least 1,200 years and that the number of trees at this site was probably never greater than 12 (Calcote, 1987). The age of the stand in West Duluth is unknown, but is more genetically similar to Wisconsin (LM) and Michigan (MI) trees.

Although North Carolina (NC) is expected to be near the center of species diversity, we see that it is most admixed with Michigan (MI), but not other population

clusters. This could be because it is geographically removed from the other collection sites. It is important to remember that this is not a range-wide genetic diversity study, but rather a study of the disjunct native trees in Minnesota and their relation to a select native populations and cultivated sites. It would be helpful in the future to collect from and compare among additional sites across the range, especially disjunct Wisconsin sites. Collections from more disjunct sites could reveal more genetic similarities or differences from Minnesota disjunct populations.

From the dendrogram and PCoA, the clustering of trees from Eloise Butler Wildflower Garden (EB) with those from Theodore Wirth Park (TWP) suggests that they are more genetically similar. These trees are in the same park system in Minneapolis, MN and although we could not find planting records for Theodore Wirth Park, the close statistical clustering suggests trees from both sites could have originated from a common source. In general, trees from cultivated sources seem to be mixed with native Michigan (MI) and Wisconsin (LM) trees. Some of these sites, including Tischer Creek (TC), Eloise Butler Wildflower Garden (EB) and McCarthy Beach State Park (MB) have records of being planted in the early 1900s and this clustering is evidence that they may have come from sources in the Great Lakes area. Again, sampling and genotyping trees from more populations across Wisconsin and Michigan could provide additional insights.

Paternity Analyses

Few mother-father pairs were assigned at a statistically significant confidence level for native seedlings or seedlings from the Minnesota Landscape Arboretum. Despite having few statistically significant results, we see some patterns in the most likely parent pairs. For example, tree TC018 is overwhelmingly the most likely candidate father for seedlings grown from seed collected from West Duluth in 2015. This tree from

Tischer Creek (TC) is growing on the Glensheen Mansion formal grounds, although along Tischer Creek, close to where the creek meets Lake Superior. However, it is unlikely that long-distance pollen dispersal could bring pollen from Tischer Creek to West Duluth, a distance of over 20 kilometers. A paternity and pollen dispersal study on *Quercus robur* L. and *Q. petraea* (Matt.) Liebl. mixed stands observed that 65% of pollen donors were from distances over 100 meters from maternal trees (Streiff et al., 1999) and another by O'Connell et al. (2007) found long distance dispersal in pines to be 3,000 meters. Seedlings collected from WD011 each had one locus where the seedlings' genotype was a potential match with the mother and father separately, but were not matches with the mother-father pair, which could account for candidate father error (Table 2-10: Trio Mismatched Loci). Seedlings originating from trees collected in West Duluth in 2014 did not match with TC018 as a candidate father.

At the Minnesota Landscape Arboretum, accession MLA018 was assigned as the most likely candidate father for 8 seedlings and is a non-native-sourced accession. It is also in very close proximity (within 10 meters) to its most common seedling match, MLA017. The other accession assigned as the most likely paternal candidate, MLA005, was grown from seed from a Mille Lacs Lake native tree. There was also potential for trees from Eloise Butler Wildflower Garden to pollinate trees at the Minnesota Landscape Arboretum. In general, we find that both native origin and non-native trees are likely pollinating native-sourced trees at the Minnesota Landscape Arboretum.

Evidence of mismatched genotypes between mothers and offspring means there are some genotyping errors, which include the presence of null alleles and mistyped alleles. Although a range of 4-8 loci has been reported as enough for parentage analyses (Koch et al., 2010), using only four loci in the current study may not have

provided enough information given the frequency of null alleles, especially in marker *Tcn3H04*. Including more loci with lower null allele frequencies would increase the statistical power of the parentage assignment.

CONCLUSIONS

Our data indicate that, although Minnesota disjunct trees are indeed inbred, they are also genetically distinct from trees from Wisconsin, Michigan, and the center of species diversity in North Carolina. With eastern hemlock considered near threatened and the looming prospect of hemlock woolly adelgid and climate change approaching the upper Midwest, it will be important to conserve the studied populations both as in situ land management sites, and as ex situ resources in seed banks and parks and gardens. The native origin Minnesota Landscape Arboretum trees could be especially important for conservation due to their provenance and they are distinctly dissimilar from other Minnesota Landscape Arboretum accessions and all other collection sites.

TABLES

Table 2-1: Collection sites of eastern hemlock and number of individuals sampled for tissue and seed.

ID	Site Name	Location, County/State	Total No. Maternal Trees	Trees (Tissue)	Trees (Seed)
CCA	Carleton Cowling Arboretum	Rice County, MN	14	13	-
EB	Eloise Butler Wildflower Garden	Hennepin County, MN	42	35	3
HR	Hemlock Ravine SNA†	Carlton County, MN	34	22	3
JC	Jay Cooke State Park†	Carlton County, MN	3	3	1
LM	Lake Minnesuing	Douglas County, WI	>100	20	-
MB	McCarthy Beach State Park	St. Louis County, MN	65	30	-
MI	Baraga State Forest	Baraga County, MI	>100	21	-
MLA	Minnesota Landscape Arboretum†	Carver County, MN	43	32	5
NC	DuPont National Forest	Transylvania County, NC	>100	20	-
PP	Private Property, Duluth	St. Louis County, MN	3	3	2
TC	Tischer Creek and Glensheen Mansion	St. Louis County, MN	19	19	6
TWP	Theodore Wirth Park	Hennepin County, MN	79	29	-
WD	West Duluth†	St. Louis County, MN	13	13	2

†Denotes sites containing Minnesota native trees

Table 2-2: Descriptions and provenance information for Minnesota eastern hemlock trees used in this study.

Site ID*	Native[a]	Cultivated[b]	Naturalized[c]	Planted[d]	Unknown Provenance[e]	Putatively Native[f]
CCA		x	x	x		
EB		x		x	x	x
HR	x					
JC	x			x		
MB			x	x	x	x
MLA	x	x		x		x
PP			x		x	
TC		x	x	x	x	x
TWP			x	x	x	x
WD	x					

A) Native: there is no evidence of planting or human disturbance other than research and conservation practices. B) Cultivated: trees that exist in an area with regular maintenance by an institution. C) Naturalized: trees are producing seed and seedlings and are growing outside of cultivated with trees of various size and age classes. D) Planted: trees with historic records of being planted. E) Unknown Provenance: trees with unknown origin in cultivated, planted, or naturalized settings. F) Putatively Native: trees that could be native based on early planting dates. NOTE: Tischer Creek (TC) trees occur along a naturalized creek setting, but also within formal, maintained garden grounds. Jay Cooke (JC) contained one tree that was planted. More information on site history available in Appendix A.

*see table 2-1 for site ID names

Table 2-3: Eastern hemlock herbarium specimen collections, the quantified amount of DNA extracted, and PCR amplification success.

ID	Source	Ref No.	County/State	Year Collected	DNA (ng/µl)	PCR[z]
HERB001	MLA	02249	Carver County, MN	1981	41.3	No
HERB002	MLA	01850	Mille Lacs County, MN	1960	3.9	No
HERB003	MLA	01851	Mille Lacs County, MN	1960	2.5	No
HERB004	MLA	01852	St. Louis County, MN	1960	7.8	No
HERB005	MLA	01853	Carlton County, MN	1960	7.2	No
HERB006	MLA	01867	Mille Lacs County, MN	1971	4.2	No
HERB007	MLA	01868	Mille Lacs County, MN	1971	1.9	YES
HERB008	MLA	01869	Mille Lacs County, MN	1957	2.1	No
HERB009	MLA	01746	Pulaski County	1978	16.6	No
HERB010	MLA	02181	Goodhue County, MN	1981	2.7	No
HERB011	MLA	02526	Carver County, MN	1984	5.2	No
HERB012	BELL	589358	Swain County, NC	1927	1.5	No
HERB013	BELL	56817	Carlton County, MN	1893	3	No
HERB014	BELL	680202	Ashland, WI	1976	34.9	No
HERB015	BELL	777571	Carlton County, MN	1981	16.8	No
HERB016	BELL	676306	Carlton County, MN	1942	16.5	No
HERB017	BELL	332181	Pine County, MN	1936	1.1	No
HERB018	BELL	298013	St. Louis County, MN	1935	9.5	No
HERB019	BELL	353594	St. Louis County, MN	1939	14.9	No
HERB020	BELL	900032	St. Louis County, MN	2000	6.9	No
HERB021	BELL	738061	Carlton County, MN	1981	36.9	No
HERB022	BELL	738059	Carlton County, MN	1981	38.3	No
HERB023	BELL	738060	Carlton County, MN	1981	30.5	No
HERB024	BELL	568970	St. Louis County, MN	1960	8.7	No
HERB025	BELL	441907	Carlton County, MN	1945	13.3	No
HERB026	BELL	334311	Mille Lacs County, MN	1935	5.7	No
HERB027	BELL	360776	Mille Lacs County, MN	1937	1.4	No
HERB028	BELL	573748	St. Louis County, MN	1961	21.4	No
HERB029	BELL	573758	Carlton County, MN	1961	3.8	No
HERB031	BELL	490494	Carlton County, MN	1995	4.8	No
HERB032	BELL	351998	Door County, WI	1938	37.5	No
HERB033	BELL	422586	St. Louis County, MN	1948	47.9	No

Sources for herbarium specimens include the Minnesota Landscape Arboretum Herbarium (MLA) and the Bell Museum of Natural History Herbarium (BELL)

[z]PCR was attempted on all samples, only HERB007 successfully amplified

Table 2-4: Microsatellite (SSR) marker details for eastern hemlock markers used in this study

Name	Motif	Size Range	A	Primer Sequences 5' – 3'	Dye	Ho	He	PIC	HWE	Null
Tcn10A12	(AG)19	139 - 187	23	F: CTCAGACCAGCACTCCAG R: AGTCATGGGGCCTCTTTGC	NED	0.560	0.835	0.825	***	0.1939
Tcn10B01†	(GT)26	187 - 209	10	F: GTCAGTCTTGCTTTCGTTTGG R: CACCTCGATCATACATCGGTC	VIC	0.498	0.785	0.756	***	0.2235
Tcn12C01†	(AAG)7…(AAG)12	354 - 369	7	F: GAACAACAGAAGGACCCATC R: AGCCCACCGTCTCTCTAAG	NED	0.486	0.609	0.559	**	0.1108
Tcn2C08	(AC)9…(AC)7	237 - 349	7	F: GGTGGGTGGTTTCTTGAAGTC R: ACTCCACCCCTTTTAGCCC	PET	0.292	0.696	0.633	***	0.4053
Tcn3E02†	(AG)16	361 - 382	9	F: GCCACCATAGAGCTGAGG R: GTGCAAGGTTAAGGCCACG	6-FAM	0.295	0.571	0.541	***	0.3212
Tcn3H04†	(GT)16…(AG)11	298 - 310	7	F: GGAACCAACTTCGTGCGAG R: GTGGTTGGTCTCTTTCACTGG	VIC	0.494	0.679	0.634	***	0.1558
Tcn2B04	(TG)12	170 - 174	3	F: CATGTACCGGTCCTCCTG R: AGAGGCCCTTCTTGAACCC	PET	0.144	0.186	0.170	ND	0.1330
Tcn10A07	(TACA)7	411 - 427	4	F: TGGGGAGTTGATCACTGGG R: GGTGAAGAAACCGGGGAATG	VIC	0.440	0.525	0.417	NS	0.0862

All primer pairs were derived from Shamblin et al. (2009). Dyes were labeled on 5' end of forward primers for all primers.

All statistics were observed from a panel of between 225 to 259 distinct individuals across collection sites

A = No. of observed alleles, Ho = Observed heterozygosity, He = Expected heterozygosity, PIC = Polymorphic Information Content, HWE = Hardy-Weinberg exact test of heterozygote deficiency with a Bonferroni adjusted false discovery rate where NS = not significant, ND = not done, and *** = significant at p=0.01, ** significant at p=0.05, Null = Null allele frequency

†denotes primers also used in seedling analysis

Table 2-5: Genetic diversity statistics for each collection site at 7 nuclear microsatellite (SSR) markers

ID		N	Na	Ne	I	Ho	He	uHe	F
Hemlock Ravine	**Mean**	22.571	3.714	2.119	0.864	0.242	0.473	0.484	0.463
	SE	0.297	0.565	0.236	0.160	0.051	0.085	0.087	0.085
West Duluth	**Mean**	12.857	3.429	2.191	0.880	0.277	0.496	0.516	0.365
	SE	0.143	0.369	0.241	0.134	0.050	0.073	0.076	0.114
Tischer Creek	**Mean**	18.429	4.286	2.404	0.990	0.284	0.515	0.529	0.384
	SE	0.369	0.747	0.365	0.173	0.039	0.080	0.082	0.091
McCarthy Beach	**Mean**	28.429	4.000	2.499	0.958	0.464	0.518	0.527	0.093
	SE	0.429	0.655	0.416	0.185	0.086	0.092	0.093	0.031
Eloise Butler	**Mean**	34.143	4.571	2.412	1.042	0.386	0.552	0.560	0.301
	SE	0.261	0.685	0.274	0.130	0.067	0.053	0.053	0.100
Theodore Wirth Park	**Mean**	30.429	4.714	2.378	1.030	0.383	0.554	0.563	0.321
	SE	1.307	0.808	0.215	0.121	0.066	0.049	0.050	0.100
MN Landscape Arboretum (MLA)	**Mean**	14.857	3.286	2.213	0.871	0.346	0.495	0.513	0.256
	SE	0.143	0.474	0.273	0.148	0.062	0.078	0.081	0.081
MLA-Mille Lacs	**Mean**	14.286	2.286	1.716	0.590	0.342	0.369	0.382	0.060
	SE	0.286	0.286	0.183	0.123	0.077	0.078	0.081	0.071
Michigan	**Mean**	18.857	4.143	2.383	0.985	0.449	0.534	0.549	0.164
	SE	0.459	0.459	0.335	0.117	0.061	0.056	0.058	0.083
Wisconsin	**Mean**	19.714	3.571	2.205	0.876	0.290	0.489	0.502	0.327
	SE	0.286	0.429	0.283	0.152	0.047	0.081	0.083	0.094
North Carolina	**Mean**	17.286	4.143	2.537	1.026	0.553	0.565	0.581	-0.007
	SE	0.421	0.508	0.365	0.126	0.039	0.051	0.052	0.078

N: Number of individuals
Na: No. of Different Alleles
Ne: No. of Effective Alleles = 1/(Sum pi2)
I: Shannon's Information Index = 1*Sum(pi*Ln(pi))
Ho: Observed Heterozygosity = No. heterozygotes/ N
He: Expected Heterozygosity = 1 = Sum pi2
uHe: Unbiased Expected Heterozygosity = (2N / (2N – 1))*He
F: Fixation Index = (He = Ho) / He = 1-(Ho / He)

Table 2-6: Genetic diversity statistics for seedlings grouped by maternal accession and Minnesota native site at 4 nuclear microsatellite (SSR) markers

		N	Na	Ne	I	Ho	He	uHe	F
Hemlock Ravine	**Mean**	45	2.750	1.464	0.472	0.156	0.264	0.267	0.497
	SE	0	0.854	0.241	0.189	0.090	0.111	0.112	0.227
West Duluth	**Mean**	33	2.500	1.528	0.494	0.205	0.303	0.308	0.168
	SE	0	0.289	0.226	0.131	0.053	0.096	0.098	0.189
MLA-Mille Lacs-12	**Mean**	38	2.250	1.322	0.315	0.158	0.187	0.189	0.151
	SE	0	0.629	0.229	0.150	0.099	0.110	0.112	0.110
MLA-Mille Lacs-17	**Mean**	39	2.500	1.462	0.484	0.327	0.282	0.286	0.017
	SE	0	0.289	0.182	0.141	0.128	0.091	0.093	0.214
MLA-Mille Lacs-19	**Mean**	42	2.500	1.632	0.547	0.274	0.321	0.325	0.111
	SE	0	0.289	0.326	0.183	0.098	0.116	0.117	0.046
MLA-Mille Lacs-20	**Mean**	15	2.500	1.457	0.467	0.200	0.258	0.267	0.062
	SE	0	0.289	0.262	0.167	0.047	0.104	0.108	0.153

N: Number of individuals genotyped for genetic diversity analysis
Na: No. of Different Alleles
Ne: No. of Effective Alleles = 1/(Sum pi2)
I: Shannon's Information Index = 1*Sum(pi*Ln(pi))
Ho: Observed Heterozygosity = No. heterozygotes/N
He: Expected Heterozygosity = 1 = Sum pi2
uHe: Unbiased Expected Heterozygosity = (2N / (2N – 1))*He
F: Fixation Index = (He = Ho) / He = 1-(Ho / He)

Table 2-7: AMOVA across all collection sites using Slatkin's R_{st}

Source	df	Sum of Squares	Mean Sum of Squares	Estimated Variation	% of Variation
Among Populations	10	5555.135	555.514	8.931	6%
Among Individuals	229	38909.994	169.913	26.283	17%
Within Individuals	240	28163.079	117.346	117.346	77%
Total	479	72628.208		152.560	100%
R_{st}:	**0.059**				
R_{is}:	**0.183**				

Table 2-8: AMOVA across native sites (HR, WD, MLA-ML, NC, LM, MI) using Slatkin's R_{st}

Source	df	Sum of Squares	Mean Sum of Squares	Estimated Variation	% of Variation
Among Populations	4	3448.297	862.074	18.751	12%
Among Individuals	89	14462.042	162.495	29.173	19%
Within Individuals	94	9789.980	104.149	104.149	68%
Total	187	27700.319		152.073	100%
R_{st}:	**0.123**				
R_{is}:	**0.219**				

Table 2-9: Delta K results from STRUCTURE analysis for parental trees

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(k)	| Ln'(k) |	Delta K
1	10	-1735.20	0.34	---	---	---
2	10	-1623.54	1.69	111.66	18.58	10.95
3	10	-1530.46	1.14	93.08	18.37	16.12
4	10	-1455.75	1.08	74.71	48.26	44.67
5	10	-1429.30	8.63	26.45	4.57	0.53
6	10	-1407.42	36.63	21.88	18.82	0.53
7	10	-1404.36	22.99	3.06	18.17	0.79
8	10	-1383.13	18.52	21.23	19.85	1.07
9	10	-1381.75	16.91	1.38	9.35	0.55
10	10	-1389.72	5.74	-7.97	---	---

Note: The grey bar indicates the number of genetic clusters, K, which STRUCTURE HARVESTER found to be most likely for these collection sites.

Table 2-10: Paternity analysis assignments for native seedlings that had a most likely mother-father match with a positive LOD score. (-) Indicates positive LOD score, (+) Indicates significance at relaxed (80%) level, and (*) indicates significance at a strict (95%) level.

Offspring ID	Mother ID	Candidate father ID	Trio loci compared	Trio loci mismatching	Trio top LOD	Trio confidence
SEEDHR4001	HR004	HR006	4	0	2.59	-
SEEDHR4004	HR004	HR006	4	0	2.66	-
SEEDHR4003	HR004	HR020	4	0	2.52	-
SEEDHR8002	HR008	HR007	4	0	4.11	-
SEEDHR8012	HR008	HR007	4	0	4.99	+
SEEDHR8028	HR008	HR007	4	0	4.99	+
SEEDHR8034	HR008	HR007	4	1	4.40	-
SEEDHR8003	HR008	HR017	4	1	0.61	-
SEEDHR8008	HR008	HR017	4	0	3.51	-
SEEDHR8010	HR008	HR017	4	0	3.51	-
SEEDHR8013	HR008	HR017	4	0	3.51	-
SEEDHR8015	HR008	HR017	4	0	3.51	-
SEEDHR8016	HR008	HR017	4	1	0.61	-
SEEDHR8021	HR008	HR017	4	1	0.61	-
SEEDHR8023	HR008	HR017	4	0	3.51	-
SEEDHR8024	HR008	HR017	4	0	3.51	-
SEEDHR8025	HR008	HR017	4	0	3.51	-
SEEDHR8026	HR008	HR017	4	0	3.51	-
SEEDHR8031	HR008	HR017	4	0	3.51	-
SEEDHR8033	HR008	HR017	4	0	3.51	-
SEEDHR8036	HR008	HR017	4	1	0.61	-
SEEDHR8038	HR008	HR017	4	0	3.51	-
SEEDHR8041	HR008	HR017	4	0	3.51	-
SEEDHR8043	HR008	HR017	4	0	3.51	-
SEEDHR8042	HR008	HR020	4	1	2.90	-
SEEDHR8011	HR008	WD009	4	1	2.44	-
SEEDWD1014	WD001	TC004	4	1	1.92	-
SEEDWD1012	WD001	WD002	4	0	4.89	+
SEEDWD1001	WD001	WD005	4	0	3.90	-
SEEDWD1002	WD001	WD005	4	0	4.72	-
SEEDWD1005	WD001	WD005	4	0	3.33	-
SEEDWD1011	WD001	WD005	4	0	3.33	-
SEEDWD1015	WD001	WD005	4	0	3.33	-
SEEDWD1016	WD001	WD005	4	0	3.90	-
SEEDWD1017	WD001	WD005	4	0	3.33	-
SEEDWD1003	WD001	WD007	4	1	0.83	-
SEEDWD1013	WD001	WD007	4	1	0.83	-
SEEDWD1006	WD001	WD010	3	0	2.79	-
SEEDWD1009	WD001	WD010	3	0	2.79	-
SEEDWD1004	WD001	WD012	4	0	4.17	-
SEEDWD1008	WD001	WD012	4	0	4.17	-
SEEDWD1010	WD001	WD012	4	0	7.18	*
SEEDWD11007	WD011	TC018	3	1	1.11	-

Table 2-10 cont.

Offspring ID	Mother ID	Candidate father ID	Trio loci compared	Trio loci mismatching	Trio top LOD	Trio confidence
SEEDWD11015	WD011	TC018	3	1	1.11	-
SEEDWD11018	WD011	TC018	3	1	1.11	-
SEEDWD11022	WD011	TC018	3	1	1.11	-
SEEDWD11027	WD011	TC018	3	1	1.11	-
SEEDWD11033	WD011	TC018	3	1	1.11	-
SEEDWD11035	WD011	TC018	3	1	1.11	-
SEEDWD11037	WD011	TC018	3	1	1.11	-
SEEDWD11038	WD011	TC018	3	1	1.11	-
SEEDWD11043	WD011	TC018	3	1	1.11	-
SEEDWD11045	WD011	TC018	3	1	1.11	-
SEEDWD11047	WD011	TC018	3	1	1.11	-
SEEDWD11048	WD011	TC018	3	1	1.11	-
SEEDWD11049	WD011	TC018	3	1	1.11	-
SEEDWD11050	WD011	WD001	4	1	3.65	-
SEEDWD11026	WD011	WD004	4	1	5.16	+

Table 2-11: Marker information for probability of detecting the correct candidate parent and estimated error rates.

Locus Name	No. compared	No. mismatching	No. null	Detection probability	Est. error rate
Native MN					
Tcn10B01	77	1	0	40.62%	15.99%
Tcn12C01	77	0	0	8.35%	0.00%
Tcn3E02	77	2	2	20.51%	6.33%
Tcn3H04	77	17	16	26.49%	41.68%
			mean observed error rate:		12.40%
Minnesota Landscape Arboretum					
Tcn10B01	131	7	5	17.77%	15.04%
Tcn12C01	131	3	1	23.86%	4.80%
Tcn3E02	131	6	5	20.35%	11.25%
Tcn3H04	89	0	0	4.67%	0.00%
			mean observed error rate:		7.77%

Table 2-12: Paternity analysis assignments for Minnesota Landscape Arboretum seedlings that had a most likely mother-father match with a positive LOD score. (-) Indicates positive LOD score, (+) Indicates significance at relaxed (80%) level, and (*) indicates significance at a strict (95%) level.

Offspring ID	Mother ID	Candidate father ID	Trio loci compared	Trio loci mismatching	Trio top LOD	Trio confidence
SEEDMLA12035	MLA012	EB032	4	0	2.98	-
SEEDMLA12003	MLA012	MLA009†	4	0	1.55	-
SEEDMLA12014	MLA012	MLA009†	4	0	1.55	-
SEEDMLA12015	MLA012	MLA009†	4	0	1.55	-
SEEDMLA12023	MLA012	MLA009†	4	0	1.55	-
SEEDMLA12029	MLA012	MLA009†	4	0	1.55	-
SEEDMLA12034	MLA012	MLA009†	4	0	1.55	-
SEEDMLA12038	MLA012	MLA009†	4	0	1.55	-
SEEDMLA12041	MLA012	MLA009†	4	0	1.55	-
SEEDMLA12046	MLA012	MLA009†	4	0	1.55	-
SEEDMLA12008	MLA012	MLA014	4	0	2.05	-
SEEDMLA12009	MLA012	MLA014	4	0	2.05	-
SEEDMLA12011	MLA012	MLA014	4	0	2.05	-
SEEDMLA12021	MLA012	MLA014	4	0	2.05	-
SEEDMLA12024	MLA012	MLA014	4	0	2.05	-
SEEDMLA12025	MLA012	MLA014	4	0	2.05	-
SEEDMLA12027	MLA012	MLA014	4	0	2.05	-
SEEDMLA12028	MLA012	MLA014	4	0	2.05	-
SEEDMLA12042	MLA012	MLA014	4	0	2.05	-
SEEDMLA12002	MLA012	MLA017†	4	1	2.23	-
SEEDMLA12001	MLA012	MLA018	4	1	3.33	-
SEEDMLA12016	MLA012	MLA018	4	1	1.75	-
SEEDMLA12030	MLA012	MLA018	4	0	5.04	+
SEEDMLA17001	MLA017	MLA018	4	0	5.05	+
SEEDMLA17004	MLA017	MLA018	4	0	2.87	-
SEEDMLA17005	MLA017	MLA018	4	0	2.87	-
SEEDMLA17008	MLA017	MLA018	4	0	5.05	+
SEEDMLA17009	MLA017	MLA018	4	0	5.05	+
SEEDMLA17013	MLA017	MLA018	4	0	2.87	-
SEEDMLA17017	MLA017	MLA018	4	0	2.87	-
SEEDMLA17018	MLA017	MLA018	4	0	2.87	-
SEEDMLA17021	MLA017	MLA018	4	0	5.05	+
SEEDMLA17022	MLA017	MLA018	4	0	5.05	+
SEEDMLA17023	MLA017	MLA018	4	0	2.87	-
SEEDMLA17025	MLA017	MLA018	4	0	2.87	-
SEEDMLA17026	MLA017	MLA018	4	0	2.87	-
SEEDMLA17029	MLA017	MLA018	4	0	2.87	-
SEEDMLA17031	MLA017	MLA018	4	0	5.05	+
SEEDMLA17032	MLA017	MLA018	4	0	2.87	-
SEEDMLA17037	MLA017	MLA018	4	0	5.05	+

Table 2-12 cont.

Offspring ID	Mother ID	Candidate father ID	Trio loci compared	Trio loci mismatching	Trio top LOD	Trio confidence
SEEDMLA17042	MLA017	MLA018	4	0	2.87	-
SEEDMLA17044	MLA017	MLA018	4	0	2.87	-
SEEDMLA17033	MLA017	MLA020†	4	0	4.64	-
SEEDMLA17047	MLA017	MLA020†	4	3	3.37	-
SEEDMLA17002	MLA017	MLA028	4	0	1.97	-
SEEDMLA17007	MLA017	MLA028	4	0	1.97	-
SEEDMLA17010	MLA017	MLA028	4	0	1.97	-
SEEDMLA17011	MLA017	MLA028	4	0	1.97	-
SEEDMLA17012	MLA017	MLA028	4	0	1.97	-
SEEDMLA17014	MLA017	MLA028	4	0	1.97	-
SEEDMLA17015	MLA017	MLA028	4	0	1.97	-
SEEDMLA17016	MLA017	MLA028	4	0	1.97	-
SEEDMLA17019	MLA017	MLA028	4	0	1.97	-
SEEDMLA17020	MLA017	MLA028	4	0	1.97	-
SEEDMLA17027	MLA017	MLA028	4	0	1.97	-
SEEDMLA17035	MLA017	MLA028	4	0	1.97	-
SEEDMLA17036	MLA017	MLA028	4	0	1.97	-
SEEDMLA17038	MLA017	MLA028	4	0	1.97	-
SEEDMLA17041	MLA017	MLA028	4	0	1.84	-
SEEDMLA17043	MLA017	MLA028	4	0	1.97	-
SEEDMLA17045	MLA017	MLA028	4	0	1.97	-
SEEDMLA17003	MLA017	MLA029	4	0	2.73	-
SEEDMLA19042	MLA019	MLA005◊	4	0	5.24	+
SEEDMLA19027	MLA019	MLA009†	4	0	3.60	-
SEEDMLA19015	MLA019	MLA013	3	0	2.73	-
SEEDMLA19048	MLA019	MLA014	4	1	1.78	-
SEEDMLA19001	MLA019	MLA017†	4	0	1.90	-
SEEDMLA19003	MLA019	MLA017†	4	0	2.63	-
SEEDMLA19005	MLA019	MLA017†	4	0	1.90	-
SEEDMLA19010	MLA019	MLA017†	4	0	2.63	-
SEEDMLA19011	MLA019	MLA017†	4	0	1.90	-
SEEDMLA19016	MLA019	MLA017†	4	0	2.63	-
SEEDMLA19017	MLA019	MLA017†	4	0	1.90	-
SEEDMLA19023	MLA019	MLA017†	4	0	2.63	-
SEEDMLA19024	MLA019	MLA017†	4	0	2.63	-
SEEDMLA19025	MLA019	MLA017†	4	0	1.90	-
SEEDMLA19028	MLA019	MLA017†	4	0	2.63	-
SEEDMLA19035	MLA019	MLA017†	4	0	1.90	-
SEEDMLA19036	MLA019	MLA017†	4	0	1.90	-
SEEDMLA19037	MLA019	MLA017†	4	0	1.90	-
SEEDMLA19038	MLA019	MLA017†	4	0	1.90	-
SEEDMLA19039	MLA019	MLA017†	4	0	1.90	-
SEEDMLA19041	MLA019	MLA017†	4	0	1.90	-

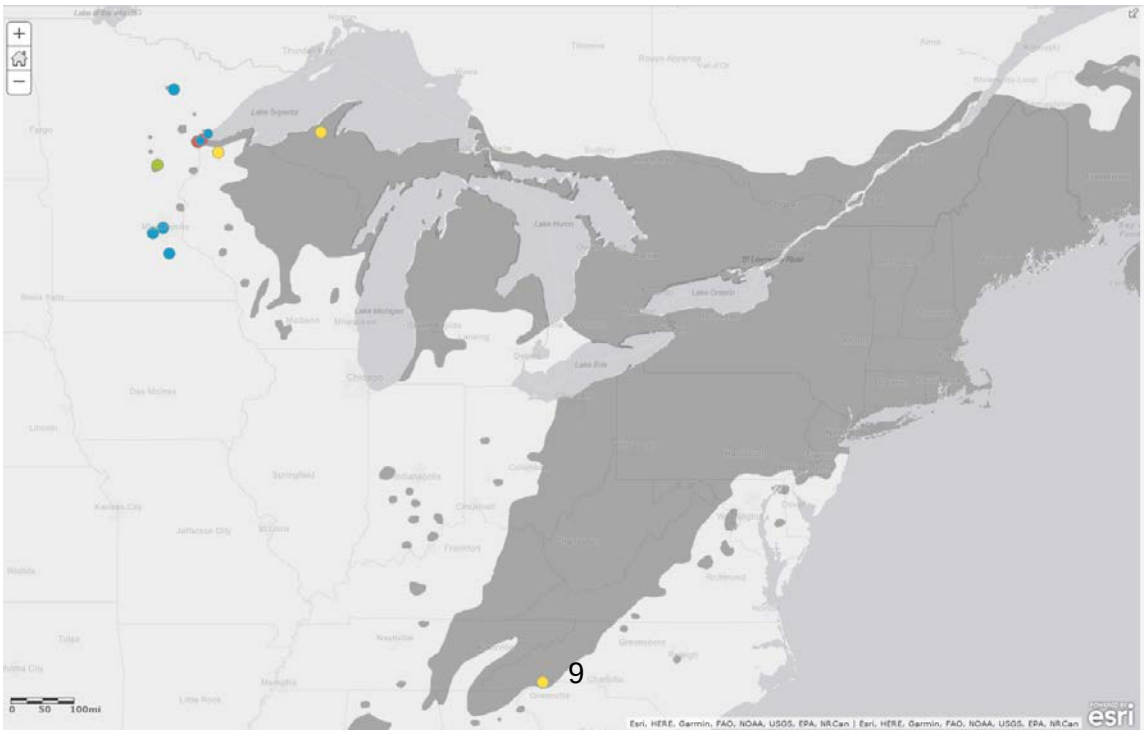
Table 2-12 cont.

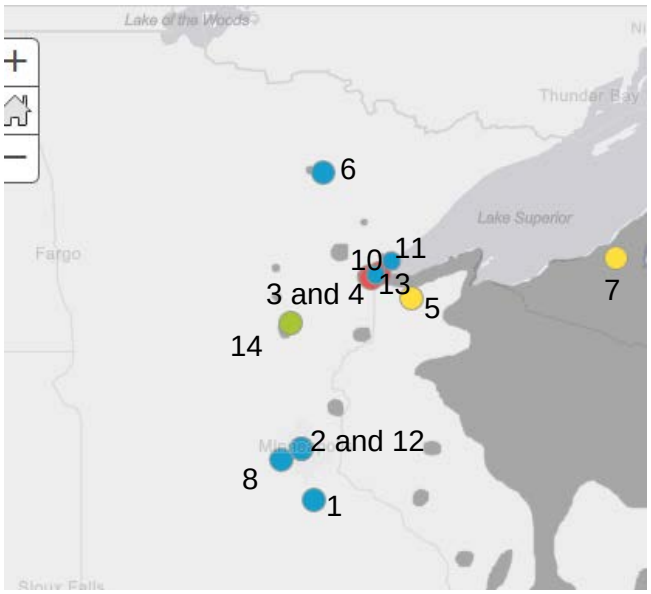
Offspring ID	Mother ID	Candidate father ID	Trio loci compared	Trio loci mismatching	Trio top LOD	Trio confidence
SEEDMLA19044	MLA019	MLA017†	4	0	1.90	-
SEEDMLA19045	MLA019	MLA017†	4	0	2.63	-
SEEDMLA19046	MLA019	MLA017†	4	0	1.90	-
SEEDMLA19047	MLA019	MLA017†	4	0	2.63	-
SEEDMLA19008	MLA019	MLA020†	4	3	3.37	-
SEEDMLA19012	MLA019	MLA020†	4	0	4.59	-
SEEDMLA19018	MLA019	MLA020†	4	0	4.59	-
SEEDMLA19022	MLA019	MLA020†	4	0	4.58	-
SEEDMLA19026	MLA019	MLA020†	4	0	4.59	-
SEEDMLA19032	MLA019	MLA020†	4	0	4.58	-
SEEDMLA19033	MLA019	MLA020†	4	0	4.59	-
SEEDMLA19040	MLA019	MLA020†	4	3	3.37	-
SEEDMLA19002	MLA019	MLA028	4	0	1.83	-
SEEDMLA19004	MLA019	MLA028	4	0	1.83	-
SEEDMLA19007	MLA019	MLA028	4	0	1.83	-
SEEDMLA19019	MLA019	MLA028	4	0	1.83	-
SEEDMLA19021	MLA019	MLA028	4	0	1.83	-
SEEDMLA19031	MLA019	MLA028	4	0	1.83	-
SEEDMLA20015	MLA020	EB034	4	0	3.41	-
SEEDMLA20016	MLA020	EB034	4	0	3.41	-
SEEDMLA20024	MLA020	EB034	4	0	3.41	-
SEEDMLA20028	MLA020	EB034	4	0	3.41	-
SEEDMLA20037	MLA020	EB034	4	0	3.41	-
SEEDMLA20048	MLA020	EB034	4	0	3.41	-
SEEDMLA20013	MLA020	MLA002◊	4	0	3.80	-
SEEDMLA20003	MLA020	MLA005◊	4	1	4.71	-
SEEDMLA20004	MLA020	MLA017†	4	0	2.59	-
SEEDMLA20032	MLA020	MLA027	4	1	1.60	-

◊ denotes trees grown from seed collected from native-sourced trees within the Arboretum
† denotes native sourced-trees collected from Mille Lacs Lake

FIGURES

Figure 2-1: Eastern hemlock North American range (grey highlight) and study collection sites. Red indicates collections from Minnesota native trees, blue indicates collections from cultivated and unknown provenance trees, green indicates collection from herbarium specimens included in analysis, and yellow indicates collections from out-of-state native populations. Map from ArcGIS online and range from Clemson Center for Geospatial Technologies (2016).

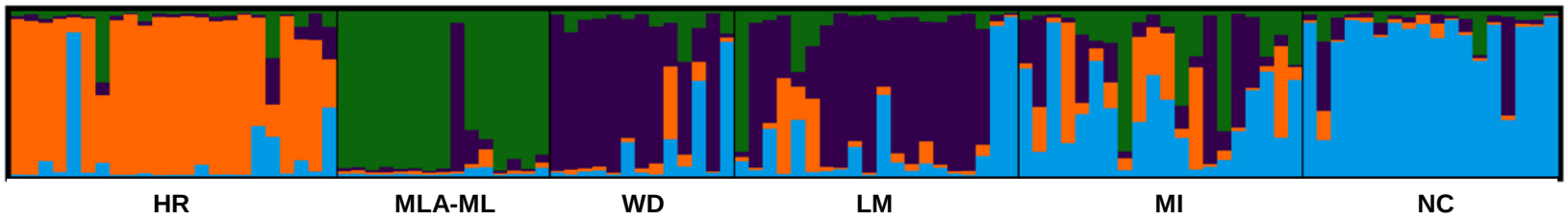




LEGEND

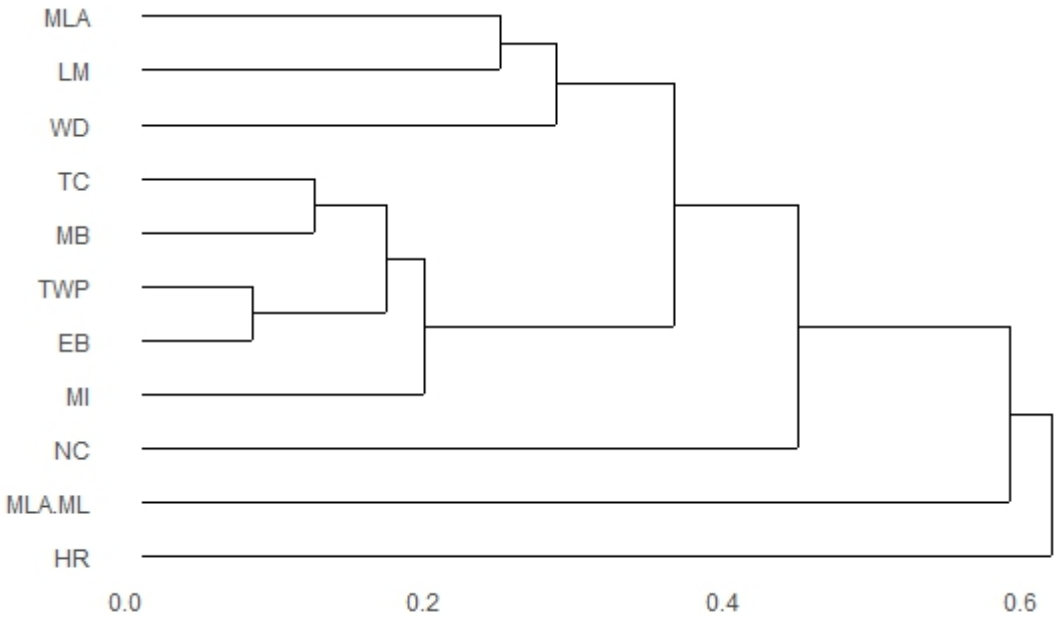
1 = CCA
2 = EB
3 = HR
4 = JC
5 = LM
6 = MB
7 = MI
8 = MLA
9 = NC
10 = PP
11 = TC
12 = TWP
13 = WD
14 = HERBARIUM

Figure 2-2: STRUCTURE plot across known native collection sites with K=4 genetic clusters



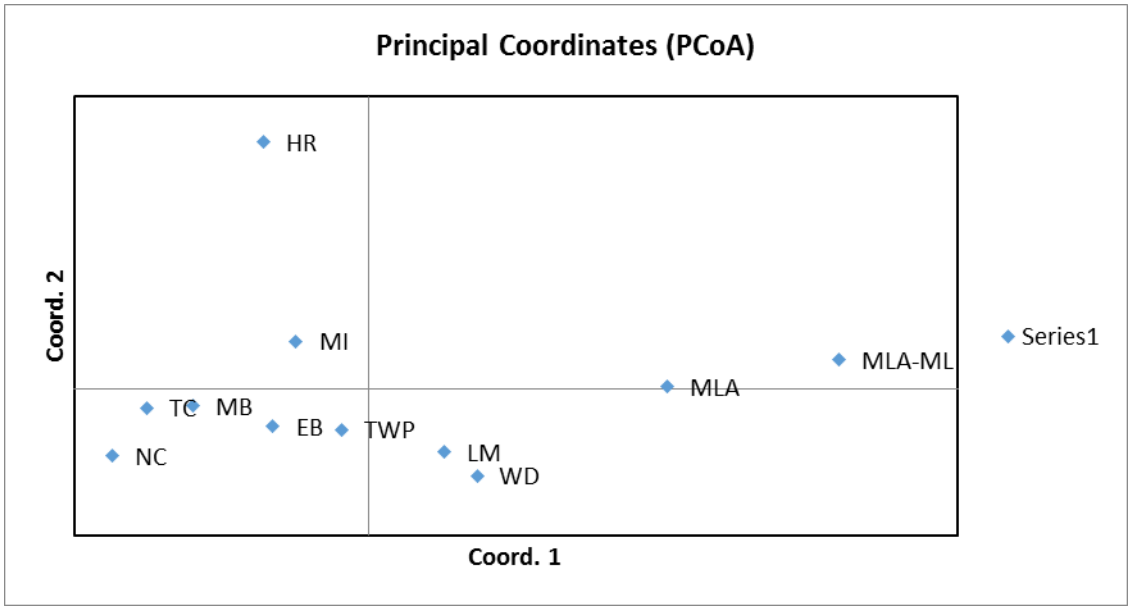
Collection Sites: Hemlock Ravine, MN (HR), Minnesota Landscape Arboretum-Mille Lacs Lake origin, MN (MLA-ML), West Duluth, MN (WD), Lake Minnesuing, WI (LM), Baraga State Forest, MI (MI), and DuPont National Forest, NC (NC)

Figure 2-3: Dendrogram calculated from Nei's Genetic Distance of genetic relationships across collection sites using 7 microsatellite (SSR) markers.



Collection Sites: Minnesota Landscape Arboretum, MN (MLA), Lake Minnesuing, WI (LM), West Duluth, MN (WD), Tischer Creek and Glensheen Mansion, MN (TC), McCarthy Beach State Park, MN (MB), Theodore Wirth Park, MN (TWP), Eloise Butler Wildflower Garden, MN (EB), Baraga State Forest, MI (MI), DuPont National Forest, NC (NC), Minnesota Landscape Arboretum-Mille Lacs Lake origin, MN (MLA-ML), and Hemlock Ravine, MN (HR)

Figure 2-4: Principal Coordinates Analysis (PCoA) based on F_{st} distance matrix across collection sites. Coord.1 explains 37.18% of the variation, Coord. 2 explains an additional 26.2% of the variation.



Collection Sites: Minnesota Landscape Arboretum, MN (MLA), Lake Minnesuing, WI (LM), West Duluth, MN (WD), Tischer Creek and Glensheen Mansion, MN (TC), McCarthy Beach State Park, MN (MB), Theodore Wirth Park, MN (TWP), Eloise Butler Wildflower Garden, MN (EB), Baraga State Forest, MI (MI), DuPont National Forest, NC (NC), Minnesota Landscape Arboretum-Mille Lacs Lake origin, MN (MLA-ML), and Hemlock Ravine, MN (HR)

CHAPTER 3:

Propagation Methods and Landscape Management Recommendations for Eastern Hemlock (*Tsuga canadensis* (L.) Carrière) in Minnesota

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INTRODUCTION

Eastern hemlock (*Tsuga canadensis* (L.) Carrière) is a foundation species across its range of occurrence. It is facing range-wide threats due to climate change (Saladyga & Maxwell, 2015) and an aphid-like invasive insect, the hemlock woolly adelgid (*Adelges tsugae* Anaand.) (McClure, 1991). Efforts to conserve eastern hemlock range from research into their ecological effects (Ellison, Barker-Plotkin, Foster, & Orwig, 2010), paleoecology (Calcote, 1987; Davis, Calcote, Sugita, & Takahara, 1998), and genetics (Lemieux et al., 2011; Potter et al., 2012). Important to the current research, some conservation researchers have also emphasized study of propagation techniques (Jetton et al., 2005, 2014) and seed-banking prioritization (Hastings, Potter, Koch, Megalos, & Jetton, 2017).

Eastern hemlock is considered near-threatened across its range by the International Union for the Conservation of Nature (Farjon, 2013). It is clear that the

threat of hemlock woolly adelgid requires conservation of eastern hemlock not only through in situ preservation of wild and native stands, but also through ex situ conservation via seed-banking, informed propagation, and the preservation of genetic resources in cultivated landscapes and seed orchards (Dosmann, 2006). Around 90% of all genetic resources saved for future conservation use are held in seed banks (Pritchard 2004). It has been shown that seed source is especially important in restoring ecosystems, with practitioners seeking regionally adapted, but genetically diverse seed sources (Johnson et al., 2010). It is important for seed banks and plant collections to capture this genetic diversity (Griffith et al., 2015).

Eastern hemlock is a wind-pollinated species and its seed is wind and gravity dispersed (Godman & Lancaster, 1990). Seeds have reportedly low germination rates between 15% and 50% (Barbour et al., 1980), but germination success increases with stratification (Jetton et al., 2014; Olson et al., 1959). Eastern hemlock is also commonly propagated via vegetative cuttings, especially in the landscape nursery industry where characteristics of cultivars must be retained (Hartmann, 2011).

Eastern hemlock is endangered in Minnesota and faces range-wide threats. There are fewer than 40 mature known native trees in Minnesota, as well as a number of scattered saplings and seedlings. At least eight of Minnesota's mature eastern hemlock trees are found growing on the steep banks of a ravine. Unfortunately, several trees were lost when the banks eroded due to severe flooding in 2012 (M. Cleveland personal communication). Efforts to eliminate deer-browse in this area have been successful, but the ravine faces challenges with erosion control. The existing trees, along with other native eastern hemlock, are being preserved through in situ land conservation, but there is an opportunity to preserve this genetic material through ex situ conservation via

propagation, seed-banking, and the preservation of genetic resources in parks and gardens. Specifically, the Minnesota Landscape Arboretum in Chanhassen, MN contains trees that were collected as seed from a now extirpated disjunct population that was located near Mille Lacs Lake, MN.

In order to conserve trees, we need to understand the propagation potential of native and non-native trees in Minnesota. It is important to determine which mature trees produce viable seed and how to vegetatively propagate old and not always vigorous native trees in order to effectively focus conservation efforts. The goal of our research was to not only understand the genetic diversity of trees per se, but to use this information in conjunction with vegetative and seed propagation techniques to create land management recommendations for eastern hemlock trees in Minnesota.

Our specific objectives were to grow Minnesota native and non-native eastern hemlock from seed in a greenhouse setting to observe germination trends. We also examined best methods for vegetative propagation for Minnesota native-sourced trees by investigating the effect of seasonal differences in rooting success and by measuring the effect of different concentrations of rooting hormones indole-3-butyric acid (IBA) and 1-napthaleneacetic acid (NAA). Lastly, armed with the information from genetic diversity studies and propagation studies, we provide recommendations to land managers on the conservation practices and restoration potential of eastern hemlock in Minnesota.

MATERIALS AND METHODS

Seed Propagation

Seed Collection and Establishment

Female cones were collected from a total of 22 trees across 7 sites in Minnesota (Figure 3-1). Of these trees, 6 were native, 4 were of known native provenance cultivated at the Minnesota Landscape Arboretum, and 12 were of unknown provenance in cultivated settings in parks and gardens and in naturalized areas. Since there are few native trees and not all trees were reproductive, trees were selected for seed collection based on presence of ripe cones and their accessibility. Permits from the Minnesota Department of Natural Resources (DNR) allowed collection of up to 20% of the cones from each native tree greater than about 25 cm (10 inches) diameter at breast height (DBH) with a maximum of 200 cones from any particular tree.

Due to permitting constraints, in 2014 cones were collected during the last half of October after seed dispersal had already begun. In 2015, trees were assessed for their cone set in early September and cones were collected when they were ripe and turning from purple to brown, prior to seed dispersal. Cones were dried at room temperature in paper bags and seed was extracted by shaking cones in a closed container. A soil sieve was used to eliminate excess chaff, needles, and cone scales. Extracted seeds were organized by maternal accession and imbibed in deionized water for 21-24 hours. Seeds were dried on paper towels for 2-3 hours at room temperature and cold moist-stratified between 4°C and 7°C in a 1:1 or 1:2 sand/peat mixture for 12-14 weeks (Table 3-1).

Seeds collected in 2014 were sown after cold-stratification, whereas seeds collected in 2015 were pre-sown prior to cold-stratification. Seeds were divided evenly among flats for representative viewing. Temperatures were recorded using HOBO Pro v2 U23-003 dataloggers (Onset Computer Corporation, Bourne, MA, USA). Seeds collected in 2014 were germinated in trays with dome lids in February and March, 2015. These were placed under fluorescent lights for a 16-hour light cycle for 7 days and

misted twice daily. Trays were removed after 7 days due to excessive heat (27°C) from fluorescent lighting and placed in greenhouses at the Horticultural Research Center in Chanhassen, MN, where germination was assessed weekly once the first seed germinated. Seeds collected in 2015 were germinated in seedling trays with dome lids in March, 2016. These were placed directly in the greenhouse at the Horticultural Research Center under a greenhouse light for 12 hours a day and misted twice daily. Dome lids were removed after 6-8 weeks to allow for better air circulation around seedlings.

Seedlings were transplanted 11-14 weeks after removal from stratification and grown in controlled greenhouses at the Horticultural Research Center. Seedlings were grown in various media (Table 3-1) and fertilized at rates between 100 ppm N and 200 ppm N with Peters Excel 21-5-20 fertilizer (The Scotts Company LLC, Marysville, OH, USA) once weekly between March and October.

Germination Estimates and Analysis

To obtain estimated germination rates, we first estimated the number of seeds collected from each maternal accession. In 2014, seed number estimates were taken post-stratification. The stratification mix with seeds for each maternal accession were thoroughly mixed and the number of seed were counted in a subsample of each mix. In 2015, seed number estimates were taken pre-stratification by weighing 3 independent subsamples of 25 seeds per maternal accession, and averaging the weights of these three subsamples. In both years, the estimate of seed number per maternal accession was calculated by multiplying the seed number per sample or average seed number per subsample by the weight of the total seed or stratification mix divided by the weight of the subsample. This resulted in an estimate of the total number of seeds. Estimated

germination rates were calculated by dividing the number germinated by the estimated total number of seeds sown per accession.

Germination was determined after the first seed germinated by visual counts of seedling flats every week for 7-8 weeks. Seedlings were defined as germinated when the hypocotyl emerged from the germination medium. Germination of seedlings ceased beyond 8 weeks after removal from stratification.

Vegetative Propagation

Experimental Design

We investigated vegetative propagation in winter and summer in 2015. Both winter and summer cuttings studies tested 6 eastern hemlock accessions from the Minnesota Landscape Arboretum; four native-sourced cultivated trees, MLA12, MLA17, MLA19, MLA20, one tree grown from seed from a native-sourced arboretum tree, MLA13, and one non-native tree, MLA18. Treatments included four hormone concentrations in a 50% ethanol and 50% water solution: control; 5,000 ppm IBA; 10,000 ppm IBA, or 5,000 ppm IBA + 5,000 ppm NAA. The experiment comprised a randomized design with 12 replications, 24 treatment combinations, and 1 cutting per treatment for a total of 288 cuttings.

Cuttings establishment

Cuttings were taken from terminal and lateral branch tips on the lower half of the crown on two-year wood. They were cut at an angle on the basal end of second year growth and stripped of the lower 1/3 of their needles. The last 2 cm of the basal end were dipped in rooting hormone for 5 seconds. The combination IBA + NAA treatment required that cuttings were first dipped in IBA and then NAA, each for 5 seconds.

Winter cuttings were taken on January 27, 2015, and stuck in a 2:1 perlite/vermiculite mix on January 28, 2015. Cuttings were placed in a polyethylene humidity tent (Figure 3-2) with bottom heat between 21°C and 25°C. Air temperature and soil temperature was recorded using HOBO Pro v2 U23-003 dataloggers. Winter cuttings were harvested for data collection on July 15, 2015.

Summer cuttings were taken on July 20, 2015, placed in plastic bags with wet paper towels and refrigerated between 4°C and 7°C, and stuck on July 21, 2015 using the treatment protocol outlined above. Cuttings were stuck in 5:0.75 perlite/peat media and misted in the propagation house at the Horticultural Research Center, without a polyethylene humidity tent. Cuttings were harvested for data collection on January 5, 2016.

Data Collection and Analysis

Mortality, presence of callus formation, presence and number of adventitious roots, and length of longest root were recorded for all cuttings at harvest time. Cuttings were considered alive if approximately 75% of needles were still attached, as noted from a visual check. Callus formation was judged as any amount of callus growth around the needle-stripping wound or basal tip (Figure 3-3a, b). Root length was measured if longer than 2 mm (Figure 3-3c). Cuttings were saved if they had callus formation and/or roots and were still alive.

Statistical analyses were performed in R version 3.1.2 (R Core Team, 2013) and RStudio (RStudio Team, 2012). Two-way ANOVA tests were performed for the variables number of roots and length of longest root and significant differences ($p < 0.05$) were further analyzed using Tukey's honest significance difference test (HSD) as a post hoc

test to examine pairwise comparisons. We used two-way binomial ANOVA tests to test for significant effects on survivability, callus formation, and root formation. The analyzed variables included percent alive, percent with callus formation, percent with root formation, the number of roots, and the length of the longest root. Plots were made with ggplot2 (Wickham, 2009).

RESULTS

Seed Propagation

Eastern hemlock in Minnesota was successfully propagated from seed collected from all sites except Jay Cooke State Park in Esko, MN. In total, two accessions had a final estimated germination rate of 0%: HR34 (Hemlock Ravine) and JC2 (Jay Cooke State Park) (Table 3-2). No seed germinated from HR34 and one seed germinated from JC2, however it died within three weeks of germinating. The average total estimated germination rate among known native trees was lower (4.18%) than both trees of known provenance (26.5%) and unknown provenance (9.02%; Table 3-3). The MLA17 seed collected in 2015 was excluded from this calculation because they were germinated from previous years' (2014) cones and were not representative of average viable seed.

Vegetative Propagation

There was little overall rooting and widespread mortality among both winter and summer cuttings (Table 3-4). Due to high mortality (89.24%), low percentage callus formation (6.25%), and no root formation (0%), summer cuttings were not included in the analysis (Table 3-4). For winter cuttings, binomial ANOVAs revealed both accession and treatment had significant effect on survivability (p = 9.082e-09; p = 0.004), callus formation (p = <2e-16; p = 0.03187), and root formation (p = 0.00019; p = 0.017) (Tables 3-5, 6, 7). The 10,000 ppm IBA treatment had higher average survival rates than other

treatments (Figure 3-4). One accession, MLA20, had no cuttings classified as alive at time of harvest and MLA12 only survived with one treatment, 5,000 IBA ppm + 5,000 NAA ppm (Figure 3-4). In addition to low survival, accession MLA20 had low callus formation, while accession MLA12 had low survivability but high callus formation (Figure 3-5). Among winter cuttings, 9 of 12 rooted cuttings were from a singular accession, MLA19 (Table 3-10).

Of cuttings that rooted, accession had a significant effect on both the number of roots (p = 5.08e-07) and the length of the longest root (p = 2.55e-05) (Tables 3-8, -9). MLA19 was observed to be significantly different than all other accessions in regards to the number of roots and length of longest root. There was also a significant interaction effect for number of roots (p = 0.0079) and the length of longest root (p = 0.0108) (Tables 3-8, -9). Tukey's pairwise comparisons revealed that the accession MLA19 differed significantly from all other accessions and the 10,000 ppm IBA treatment, although not statistically significant, had the lowest p-values for both root number and length of longest root (Table 3-11). For MLA19, the 10,000 ppm IBA treatment was the most successful (Figures 3-6, -7). The effect of rooting hormone treatment on the number of roots and length of the longest root was not significant across accessions.

DISCUSSION

Seed Propagation

Seed propagation of eastern hemlock will be integral for conservation efforts in Minnesota. We found that eastern hemlock in Minnesota can be propagated via seed originating from both native trees and trees of unknown provenance. There were differences in estimated germination rates between sites and genotypes which could be attributed to seed handling and genetics. In 2014, cones from the Minnesota Landscape

Arboretum trees were collected on October 15, two weeks earlier than cones collected from Hemlock Ravine and West Duluth. In the later instances, cones were collected after they had turned brown and started to open, an indication that seed dispersal had begun. This likely contributed to lower estimated germination rates in native trees in 2014, as seeds that are dispersed from the cones later are more likely to be sterile (Olson et al., 1959). It is interesting to note that MLA17 did not produce seed in 2015, thus seed collected from MLA17 in 2015 (Table 3-2) was collected from previous years' (2014) cones. Since these cones had been open for an entire year, the low germination rate of MLA17 from 2015 collections (0.4%) is also likely attributable to non-viable seed.

We observed total average estimated germination rates for Minnesota native and unknown provenance trees (~4 - 26%) that were generally lower than previously reported for eastern hemlock (15-50%) (Barbour et al., 1980). The reported rates, which are low, may be due to the difficulty of differentiating poor from viable seed or difficulty in replicating natural emergence conditions in nursery and greenhouse settings (Dirr, 2006). Two trees from Hemlock Ravine and Jay Cooke State Park, HR34 and JC2, failed to successfully germinate in our study. Both trees are native and growing in the Duluth area, within 2 km of each other. Cones from HR34 were closed when collected, but notably smaller in size than cones from other Hemlock Ravine trees. Additionally, fewer seeds could be extracted from HR34 and JC2 than other accessions (Table 3-2).

The timing of cold-moist stratification has been shown to increase germination speed and rates of eastern hemlock (Jetton et al., 2014; Safadi, 2011). Stratification time in 2014-15 was twelve weeks and in 2015-16, it was thirteen weeks, however more northern sourced seeds may need a longer cold-stratification period. It may be beneficial to increase stratification time for seed collected from more northern locales (Olson et al.,

1959). Other seed handling parameters that could affect germination and growth are medium type (Coffman, 1978), photoperiod, and temperature (Olson et al., 1959).

Seed from the Minnesota Landscape Arboretum was observed to have higher estimated germination rates than those from native trees and trees that are in other naturalized and cultivated sites across Minnesota. These trees are healthy; when they were planted, they were sited for optimal growth and have been maintained for over 50 years. However, methods for estimated seed counts in 2014 were not ideal and actual seed counts and germination percentages could have been lower or higher than reported. Additional replication with optimized methods may be warranted.

Vegetative Propagation

The Minnesota Landscape Arboretum has a unique resource in eastern hemlock trees of native provenance growing on the arboretum property. These trees were grown from seed collected from trees at a now extirpated population of disjunct eastern hemlock near Mille Lacs Lake, MN. The objective of this study was to test the effects of seasonality, different rooting hormones, and accessions on the general success of eastern hemlock cuttings.

In general, survivability of cuttings was low, regardless of when the cuttings were collected. However, summer cuttings faired worse than winter cuttings. Summer cuttings failed to root, formed little callus, and displayed a >85% mortality rate. Although summer cuttings may put on new growth earlier than winter cuttings, several studies indicate that winter cuttings of eastern hemlock are used preferentially to summer cuttings. Jetton et al. (2005) found that summer cuttings resulted in a lower rooting rate than was reported for dormant or semi-dormant winter cuttings.

Both accession and treatment had significant effects on survivability, callus formation, and root formation. A surprising trend arose in accession MLA12. This genotype produced callus but had very low survival. Callus often formed along the wounds where needles were stripped from the stem, but often did not produce roots. We also found that one treatment, 10,000 ppm IBA, produced more roots and longer roots when compared with other treatments with accession MLA19 (Figure 3-6, -7). Also, controls often faired better than the IBA + NAA treatment in survivability (Figure 3-4). These results are in contradiction to a study by Fordham *et al.* (1971) that demonstrated the use of IBA and NAA together was most successful in rooting eastern hemlock winter cuttings. However, Doran (1952), found success in cuttings with several IBA treatments.

Research has been done on the positive effects of different soil microbial communities on adventitious rooting in conifers. Eastern hemlock has recorded associations with ectomycorrhizal fungi and the introduction of these fungi to soil may be beneficial for germination and growth of seed/seedlings and possibly the formation of callus and roots on cuttings. Chanway et al. (1995) reported that *Tsuga heterophylla* (Raf.) Sarg. seedlings had a 30% biomass increase when seeds were inoculated with the plant growth-promoting bacterium *Baccillus polymyxa* (Prazmowski) Macé. A study on *Pinus pinaster* Ait. found that the fertilization of mother plants and cuttings with nitrogen positively effects rooting (Martinez-Alonso et al., 2012). Although some winter cuttings were successful, it is possible that changing the fertilization regime (cuttings were not regularly fertilized), adding a soil inoculum, and even changing the rooting medium may improve callus formation and rooting. It should be noted we made cuttings from second year wood due to the small size of first year wood. Although second year

growth may produce longer and more abundant roots, cuttings from first year growth have been shown to survive and root.

We found evidence for genotypic specificity for all rooting responses measured, most notably in accession MLA19, which displayed more success in traits measured. In general, the high mortality noted in these experiments could be attributed to genotypic specificity. Genotypes can vary in their success rates (Del Tredici, 1985; Ky-dembele et al., 2016) and it has been shown that younger, 5-year old Eastern hemlock trees have had greater success in rooting than older, 12-year old trees (Waxman, 1985). However, 120-year old Eastern hemlocks have been rooted successfully (Del Tredici, 1985). Given the treatments and timeframes evaluated, we found that Minnesota Landscape Arboretum trees are not particularly amenable to vegetative propagation. However, it appears that future efforts should be focused on rooting winter collected cuttings. Using first year growth cuttings, experimenting with inoculations of ectomycorrhizal fungi in the rooting media, and different nitrogen fertilizer regimes should be investigated.

RECOMMENDATIONS

Our data yield tangible recommendations for management of native and non-native eastern hemlock stands in Minnesota. The following recommendations are informed by our understanding of the genetics (Chapter 2) and propagation potential of trees in Minnesota, as well as field data collected from 2014 – 2017. We specify sites and trees that should be prioritized for use in conservation and, in turn, outline recommendations. Our recommendations directly address trees managed by the Minnesota DNR, the Minneapolis Park Board, and the University of Minnesota Landscape Arboretum.

1. Continue to Preserve Native Sites In Situ

Hemlock Ravine Scientific and Natural Area and Sanctuary

The Minnesota DNR lists Hemlock Ravine Scientific and Natural Area and Sanctuary under the strictest form of preservation in the state. We recommend this status continues. Trees in Hemlock Ravine are more inbred compared with other trees in Minnesota (Table 2-5), but are also genetically distinct (Figure 2-2, -3, -4). These trees should be targeted for seed-banking, despite being inbred.

West Duluth, MN

The native site in West Duluth has 13 mature eastern hemlock trees. Based on analysis of SSR marker data (Chapter 2), these trees are genetically similar to trees from Lake Minnesuing, Wisconsin (LM) and Baraga State Forest, Michigan (MI), but distinct from those at Hemlock Ravine (Figure 2-2, -3). These trees are reproductive but there is no evidence of regeneration in situ. Therefore, it is recommended that deer exclosures be tested at this site. Despite being distinct from the Hemlock Ravine trees, similarity to upper Midwestern populations in Wisconsin and Michigan makes these trees a secondary consideration for seed-banking.

2. Focus Propagation and Seed-Banking Efforts on Native Trees

Seed Propagation Methods

Although our investigations for seed propagation were not exhaustive, anecdotal evidence from two years of growing eastern hemlock provided insights into successful seedling germination and care. Methods described above were moderately successful for growing eastern hemlock. We found it is easier to pre-sow seedlings in germination

flats, as opposed to broadcast sowing upon removal from stratification, although care should be taken to obtain sterile propagation medium. Seed should be collected before cones are open, as they turn from purple to brown. Also, seed source should be taken into account when stratifying seed and seed from more northerly Minnesota sources should be cold-moist stratified between 40-45°F for 14 weeks.

Trees for Seed Propagation and Seed-Banking

Genetic diversity analyses (Chapter 2) revealed that seedlings grown from native trees in Hemlock Ravine and West Duluth sites were inbred. However, trees at Hemlock Ravine were also genetically distinct from those at all other sites and could be useful for conservation efforts. Thus, we recommend trees from Hemlock Ravine be a focus for future seed propagation. Trees from West Duluth should be a secondary priority, as they are related to other trees from the upper Midwest. When collecting seed, it is important to collect cones at the right time, as the color and size of the cone may be indicators that seed is viable.

Although no seeds were seed-banked in this study, eastern hemlock seeds are orthodox and thus able to be seed-banked. The USDA-ARS Germplasm Resources Information Network (GRIN) of the National Plant Germplasm System (NPGS) has several holdings of eastern hemlock (GRIN-Global, 2017). However, this database does not report Minnesota sourced seed other than seed from the Minnesota Landscape Arboretum. The additions of Minnesota-sourced eastern hemlock seed to NPGS is recommended – especially seed from Hemlock Ravine. Trees from West Duluth also germinated successfully, are more accessible, and would be useful additions to the NPGS.

Seedlings from the Minnesota Landscape Arboretum that were from Mille Lacs Lake-sourced arboretum trees experienced only moderate inbreeding and germinated successfully within normal germination ranges for eastern hemlock. They were also genetically distinct from all other collection sites. However, from paternity analyses, we saw that the most likely candidate father for many seedlings grown from these trees was a non-native accession (Table 2-12). Although this may be the reason these seedlings are not inbred, more importantly, it also indicates that these seedlings are outcrossing with non-Minnesota native eastern hemlock trees. For seed from Minnesota Landscape Arboretum trees to be useful in restorations, either non-native sources should be removed or controlled crosses on the native trees with native sourced pollen could be attempted. Controlled crosses would be time and labor intensive and would likely result in relatively low seed yields.

Vegetative Propagation

We had low success rates with vegetative propagation from Minnesota Landscape Arboretum trees. Possible causes for this were discussed. However, the native-origin accession MLA19 was the tree most amenable to propagation. Seeing as this tree is most likely from Mille Lacs Lake, vegetative propagation may be a good option for propagation to avoid outcrossing with non-native trees. We recommend creating clonal replicates of this tree by taking winter hardwood cuttings, using 10,000 ppm IBA, and using cuttings from first year growth to possibly increase the rate of rooting. Additional experiments along the lines discussed should be undertaken to determine methodologies to vegetatively propagate other native trees.

3. Continue Maintenance of Ex Situ Trees

Our studies on genetic diversity revealed that cultivated trees cluster with other cultivated trees and trees sampled from Wisconsin and Michigan populations. Trees in cultivated spaces, i.e. at the Eloise Butler Wildflower Garden and Theodore Wirth Park, are some of the largest in the state. Additionally, the cultivated trees at the Minnesota Landscape Arboretum that were collected from Mille Lacs Lake seed are important genetic resources for conservation. We found that these trees, in particular, had low levels of inbreeding compared with other cultivated and native trees in Minnesota (Table 2-5). The goal of conservation collections is generally to maximize genetic diversity and reduce inbreeding (Guerrant, Havens, & Vitt, 2014). For eastern hemlock in Minnesota, any institution or agency that is introducing eastern hemlock to its grounds should consider provenance. We recommend that parks and gardens that have naturalized eastern hemlock to plant trees native to Minnesota or the Great Lakes region. In addition, if clonal propagation of native sourced trees proves successful, establishment of isolated seed orchards consisting of said native clones should be considered.

CONCLUSIONS

Eastern hemlock is an important tree in ecosystems throughout its range. It acts as a foundation species, fundamentally regulating its surroundings, and is important to the horticulture industry. The introduction of hemlock woolly adelgid to the east coast has turned eastern hemlock into a species under threat in both native and cultivated surroundings. Although eastern hemlock is not yet under threat from hemlock woolly adelgid in Minnesota, pressures from climate change put Minnesota's most endangered tree species at further risk and jeopardize a tree that is important to the natural history of the state. Genetic resources in Minnesota can also be useful for conservation across the

range of eastern hemlock, especially considering the unique genetics of trees growing in Hemlock Ravine and at that Minnesota Landscape Arboretum. The propagation methods and recommendations put forth in this study can be utilized to preserve the genetic resources of eastern hemlock and safeguard the species for the future of our ecosystems.

TABLES

Table 3-1: Eastern hemlock seed propagation details by year.

Year	Stratification Mix	Germination Mix	Growing Mix	Date Start Cold-Stratification	Date End Cold-Stratification
2014	1:1 sand/peat	1:2 sand/peat	1:1:1/2 peat/pine bark/perlite	Nov. 25, 2014	Feb. 20, 2015
2015	1:2 sand/peat	1:2 sand/peat	1:1 pine bark/Gertens mix*	Nov. 29, 2015	March 1, 2015

*Gertens Mix is peat, composted bark, perlite, and slow release fertilizer (14-14-14)

Table 3-2: Eastern hemlock seed collection and estimated germination rates for all Minnesota trees.

Accession	No. Cones Collected	Estimated Seed Extracted	Estimated Germination Rate (%)	Collection Date
EB17	<100	90	3.3%	Oct. 23, 2015
EB23	<100	1452	3.5%	Oct. 23, 2015
EB24	<200	587	7.2%	Oct. 23, 2015
HR4*	57	272	1.8%	Oct. 30. 2014
HR8*	<200	1388	7.9%	Oct. 14, 2015
HR34*	<150	40	0%	Oct. 14, 2015
JC2*	<30	13	0%	Oct. 21, 2015
MLA12*	>200	5642	32.9%	Oct. 15, 2014
MLA17*	>200	4657	21.0%	Oct. 15, 2014
MLA17*	<100	463	0.4%	Nov. 2, 2015
MLA18	<100	25	8.0%	Nov. 2, 2015
MLA19*	>200	4143	14.7%	Oct. 15, 2014
MLA20*	>200	4686	37.4%	Oct. 15, 2014
PP2	97	854	3.7%	Oct. 9, 2015
PP3	134	2053	9.5%	Oct. 9, 2015
TC1	223	4102	11.5%	Oct. 9, 2015
TC2	<200	2192	31.4%	Oct. 9 and Oct. 21, 2015
TC6	<200	1814	3.3%	Oct. 21, 2015
TC8	239	2059	4.2%	Oct. 9 2015
TC9	94	989	9.2%	Oct. 9, 2015
TC16	<50	89	12.4%	Oct. 21, 2015
WD1*	228	1087	3.5%	Oct. 30, 2014
WD11*	212	492	11.6%	Oct. 14, 2015

*indicates native provenance maternal individual

Table 3-3: Averages of estimated eastern hemlock germination rates, grouped by provenance

	Average Estimated Germination Rate
Known native trees	4.13%
Native-sourced cultivated trees	26.5%
Unknown provenance trees	9.02%

Table 3-4: Basic summary statistics for 2015 summer and winter cuttings

Season of Cuttings	% Alive	% Callus Formation	% Rooted
Winter	20.14%	68.75%	4.17%
Summer	10.76%	6.25%	0%

Table 3-5: Binomial ANOVA table for survivability of 2015 winter cuttings

	Df	Deviance	Residual Df	Residual Deviance	Pr(>chi)
NULL			287	289.34	
Accession	5	46.00	282	243.34	9.082e-09 ***
Treatment	3	12.993	279	230.34	0.004652 **
Accession:Treatment	15	23.471	264	206.87	0.074648

Significance Codes: 0.001 = ***, 0.01 = **, 0.05 = *

Table 3-6: Binomial ANOVA table for callus formation of 2015 winter cuttings

	Df	Deviance	Residual Df	Residual Deviance	Pr(>chi)
NULL			287	357.75	
Accession	5	89.592	282	268.15	< 2e-16 ***
Treatment	3	8.814	279	259.34	0.03187 *
Accession:Treatment	15	17.760	264	241.58	0.27547

Significance Codes: 0.001 = ***, 0.01 = **, 0.05 = *

Table 3-7: Binomial ANOVA table for root formation of 2015 winter cuttings

	Df	Deviance	Residual Df	Residual Deviance	Pr(>chi)
NULL			287	99.766	
Accession	5	24.2745	282	75.492	0.0001923 ***
Treatment	3	10.1687	279	65.323	0.0171855 *
Accession:Treatment	15	7.9901	264	57.333	0.9241787

Significance Codes: 0.001 = ***, 0.01 = **, 0.05 = *

Table 3-8: ANOVA table representing number of roots for 2015 winter cuttings

	Df	Sum Sq	Mean Sq	F value	Pr(>f)
Accession	5	25.85	5.17	7.978	5.08e-07***
Treatment	3	4.70	1.568	2.420	0.06651
Accession:Treatment	15	21.02	1.402	2.163	0.00791**
Residuals	264	171.08	0.648		

Significance Codes: 0.001 = ***, 0.01 = **, 0.05 = *

Table 3-9: ANOVA table representing the length of the longest root for 2015 winter cuttings

	Df	Sum Sq	Mean Sq	F value	Pr(>f)
Accession	5	262.7	52.53	6.047	2.55e-05***
Treatment	3	40.3	13.43	1.546	0.2030
Accession:Treatment	15	272.1	18.14	2.008	0.0108*
Residuals	264	2293.4	8.69		

Significance Codes: 0.001 = ***, 0.01 = **, 0.05 = *

Table 3-10: The number of cuttings per accession that survived, formed callus, and formed roots in the 2015 winter cuttings study

Accession	No. Alive (out of 72 per acccession)	No. with Callus Formation	No. Rooted
MLA12	2	47	0
MLA13	10	32	1
MLA17	11	35	1
MLA18	17	39	1
MLA19	18	37	9
MLA20	0	8	0

Table 3-11: Tukey's Honest Significance Difference table for the number of roots and length of longest root for 2015 winter cuttings

Accession Combination	Number of Roots P Value	Length of Longest Root P Value
MLA13-MLA12	0.973744	0.999605
MLA13-MLA17	0.98838	0.999885
MLA13-MLA18	0.995876	0.991728
MLA13-MLA20	0.973744	0.999605
MLA17-MLA12	0.999995	1.000000
MLA17-MLA20	0.999995	1.000000
MLA18-MLA12	0.999856	0.94616
MLA18-MLA17	0.999995	0.961530
MLA18-MLA20	0.999856	0.94616
MLA19-MLA12	0.000011***	0.0000197***
MLA19-MLA13	0.000329***	0.006406**
MLA19-MLA17	0.000020***	0.000264***
MLA19-MLA18	0.000036***	0.000714***
MLA19-MLA20	0.000011***	0.0000197***
MLA20-MLA12	1.000000	1.000000
Treatment Combination		
5000 IBA + 5000 NAA-CONTROL	0.887198	0.755515
5000 IBA-CONTROL	0.203252	0.312911
10000 IBA-CONTROL	0.083264	0.20956
5000 IBA-5000 IBA + 5000 NAA	0.600564	0.882882
10000 IBA-5000 IBA + 5000 NAA	0.349083	0.771523
10000 IBA-5000 IBA	0.976027	0.995912

Significance Codes: less than 0.001 = ***, 0.01 = **, 0.05 = *

FIGURES

Figure 3-1: Eastern hemlock North American range and seed collection sites. Each color represents a different site. Figures from Esri, ArcGIS online (Clemson Center for Geospatial Technologies, 2016).

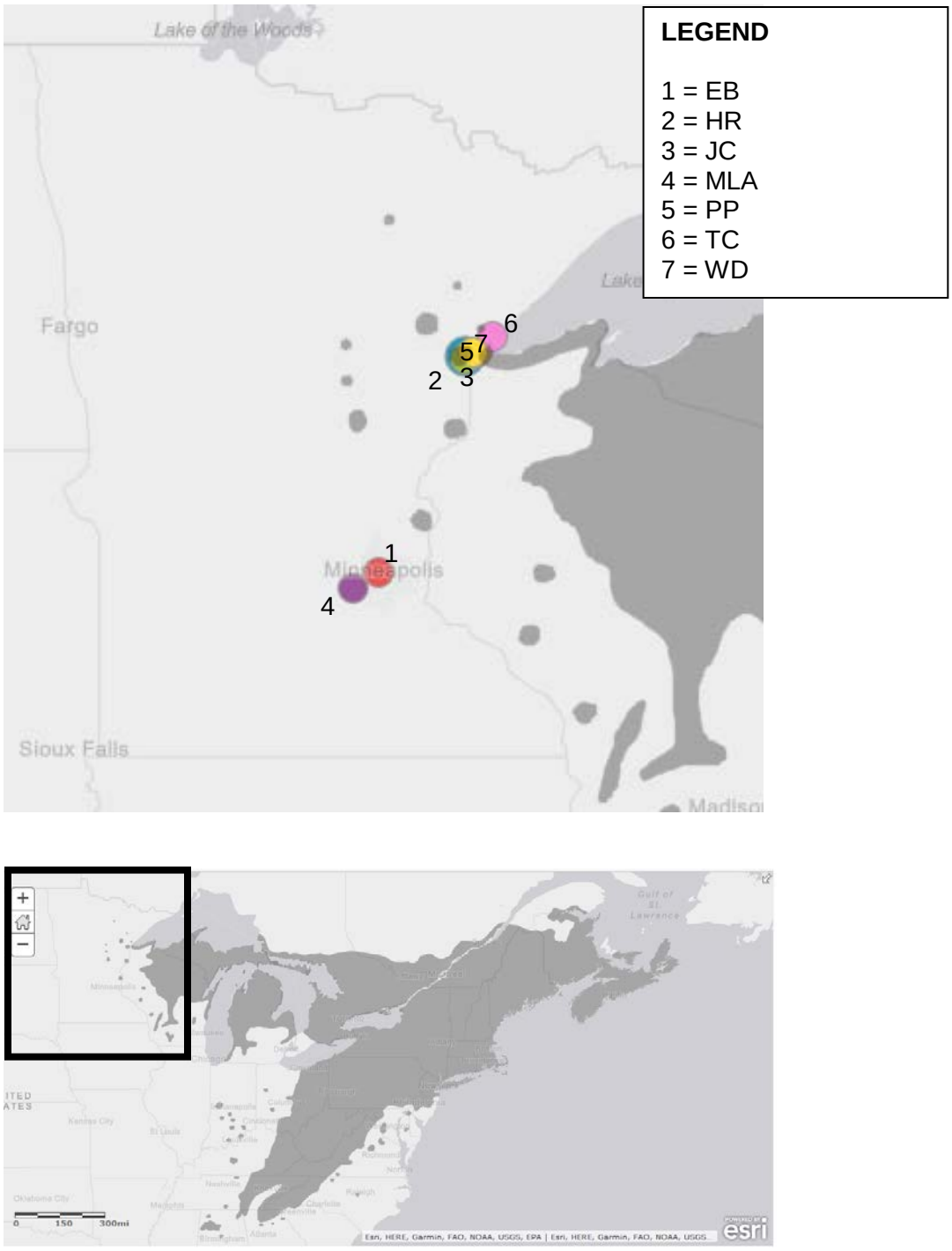
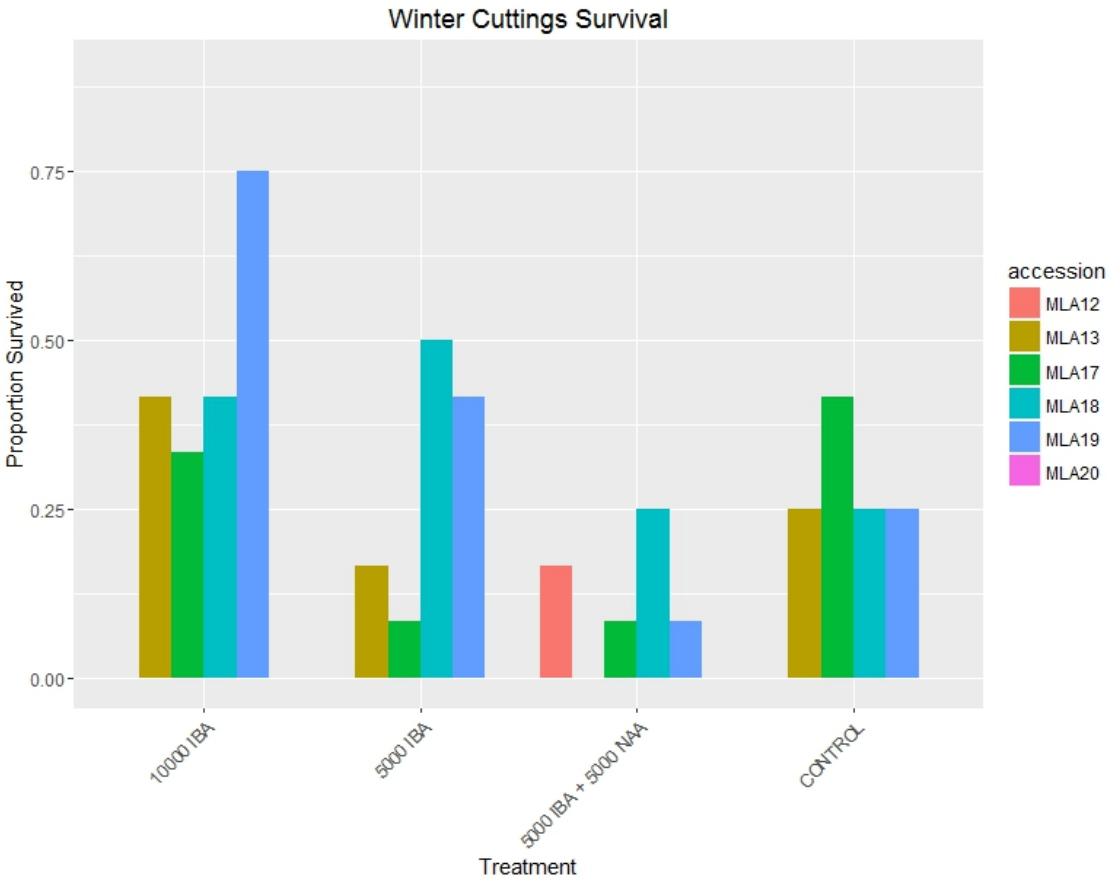


Figure 3-2: Polyethylene humidity tent built to house 2015 winter cuttings of eastern hemlock.

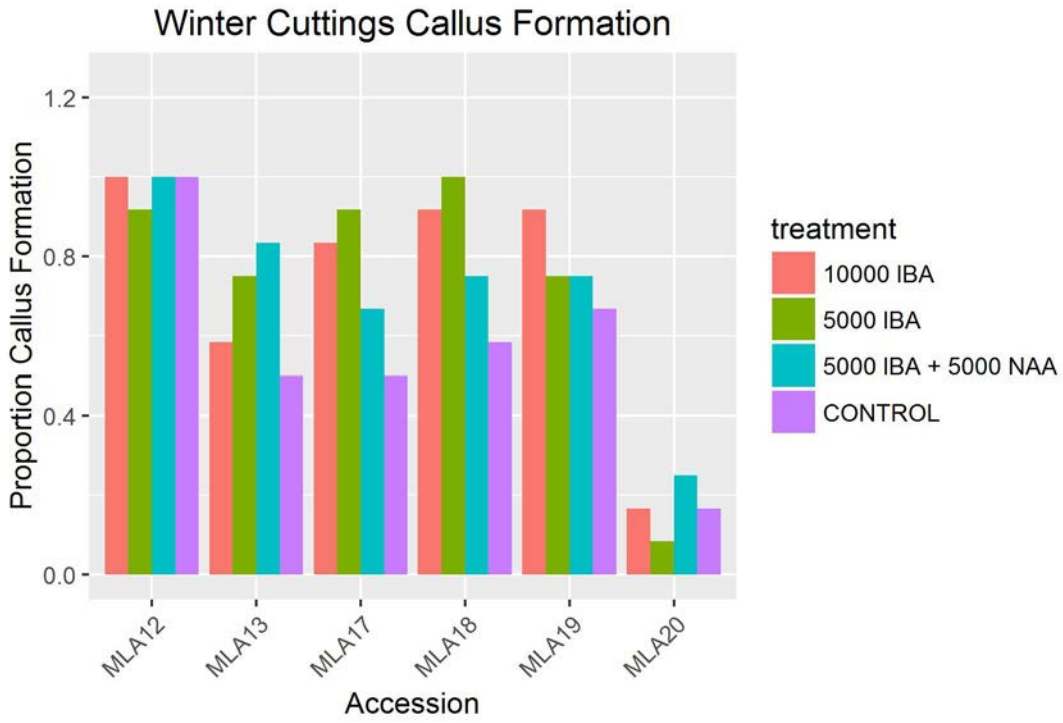
Figure 3-3: Examples of eastern hemlock vegetative cutting variables. A) Example of an eastern hemlock cutting with no callus formation B) Example of an eastern hemlock cutting with callus formation and new growth C) Example of an eastern hemlock cutting with measurable adventitious roots and new growth.

Figure 3-4: Graph of the survivability of winter cuttings in 2015 at time of harvest.



Note: no cuttings from accession MLA20 survived and only cuttings from the combination 5000 ppm IBA + 5000 ppm NAA treatment survived for accession MLA12.

Figure 3-5: Callus formation by accession of winter cuttings in 2015 at time of harvest.



Note that MLA12 has significant callus formation, despite high mortality rates.

Figure 3-6: Graph of the average number of roots for accession MLA19 for 2015 winter cuttings

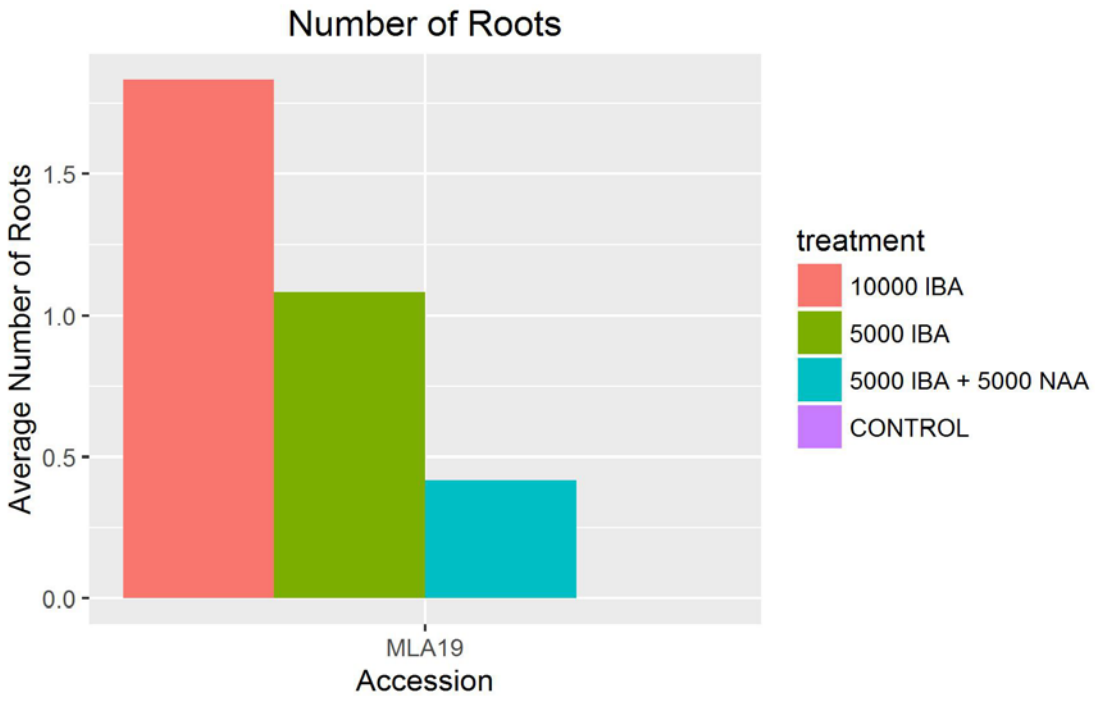
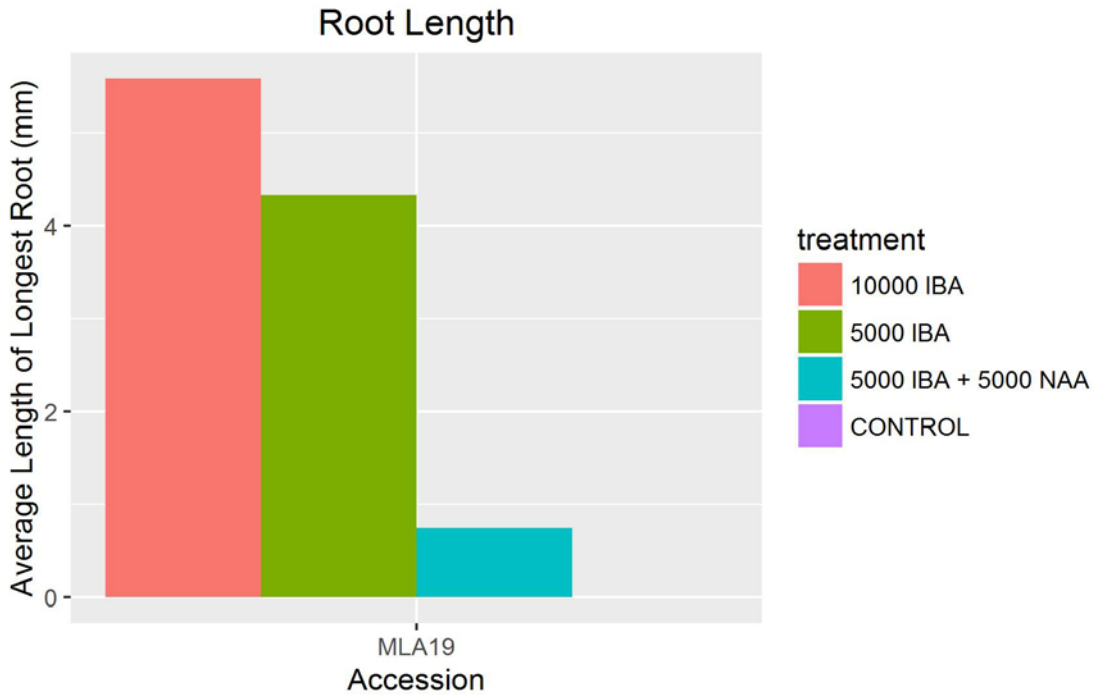


Figure 3-7: Graph of average length of the longest root for accession MLA19 for 2015 winter cuttings



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APPENDIX A

Historic Cultivated Tree Records

The cultivated and planted trees of Minnesota have varying degrees of provenance information. Appendix A synthesizes the history of Eastern hemlock plantings in select cultivated sites in Minnesota, with specific regards to the Minnesota Landscape Arboretum, McCarthy Beach State Park, Tischer Creek and Glensheen Mansion, and the Eloise Butler Wildflower Garden and Theodore Wirth Park.

The Minnesota Landscape Arboretum (Chanhassen, MN)

The Minnesota Landscape Arboretum contains trees that were grown from seed collected from a now extirpated population at Mille Lacs Lake, MN. One accession growing on the arboretum property was grown from seed from these cultivated wild-sourced seeds (Table A-1).

McCarthy Beach State Park (Side Lake, MN)

There were massive planting efforts in the 1930s by the Civilian Conservation Corps (CCC) and between 1933 and 1942, at least 43,736,547 trees were planted on state and public owned lands in Minnesota (Bachman, 1969). Between May 8 and May 16, 1935, 21,980 conifers including White pine, White Spruce, and Eastern hemlock were planted over 31.5 acres in the McCarthy Beach State Park area, possibly by the CCC (T. Westbrook, personal communication).

Glensheen Mansion and Tischer Creek (Duluth, MN)

The Congdon Family built the Glensheen Mansion Estate in Duluth, MN between 1905 and 1908. Within this time period, the grounds underwent significant change under the direction of Charles Wellford Leavitt, Jr., a civil and landscape engineer from New

York City. Records indicate that Eastern hemlock were initially described as “stock not quite hardy in Duluth, but can be tried on small scale for experiment” (Figure A-1) (Leavitt, 1906). The Congdons planted Eastern hemlock in 1907 under the title “evergreen trees for protected places” (Figures A-2, -3) (D. Hartman personal communication; Leavitt 1907a, Leavitt 1907b). The Congdon estate owned and developed the land along Tischer Creek and it is possible that Eastern hemlock were planted along the creek, past what is now London Road (E. Ford, personal communication).

Eloise Butler Wildflower Garden and Theodore Wirth Park (Minneapolis, MN)

Records indicate that 36 trees were planted between 1907 and 1914 at The Eloise Butler Wildflower Garden, which is within Theodore Wirth Park (Table A-2). There were additional trees planted in the 1980s (S. Wilkins, personal communication). Plantings were recorded in a log that Eloise Butler kept, as well as an index filing catalog. We did not find records for Theodore Wirth Park trees.

The exact nursery provenance of the Eloise Butler Wildflower Garden and Theodore Wirth Park trees is unknown. The first state tree nursery was established in 1905, although there was no record of Eastern hemlock being grown in the nursery (Bachman, 1969). There were also a number of nurseries in Minnesota in the early 1900s, some of which grew Eastern hemlock between 1900 and 1915, the time period the Eloise Butler Wildflower Garden received trees. These nurseries include The Jewell Nursery, Holm & Olson Park Nurseries, and L.L. May & Co (The Jewell Nursery Co., 1914; Holm & Olson, 1915; L.L. May & Co., 1914). There was a conifer nursery in Anoka called Hanson Evergreen Nurseries which was operating in the early 1900s, as well as the Anoka Nursery, which grew and sold evergreen trees in 1872 (Martin, 1872). We

were unable to locate records referencing the Minnesota Park Board Nurseries in the early 1900s. Interestingly, Theodore Wirth II, the son of the park's namesake, married the daughter of O. J. Olson, who was a co-owner of Holm & Olson Park Nurseries (Widmer, 1997). This nursery sold Eastern hemlock in the early 1900s, but records do not indicate that this was the source of trees in Theodore Wirth Park or the Eloise Butler Wildflower Garden and any assumption along these lines would be pure conjecture.

TABLES

Table A-1: Native-sourced accession data from the Minnesota Landscape Arboretum

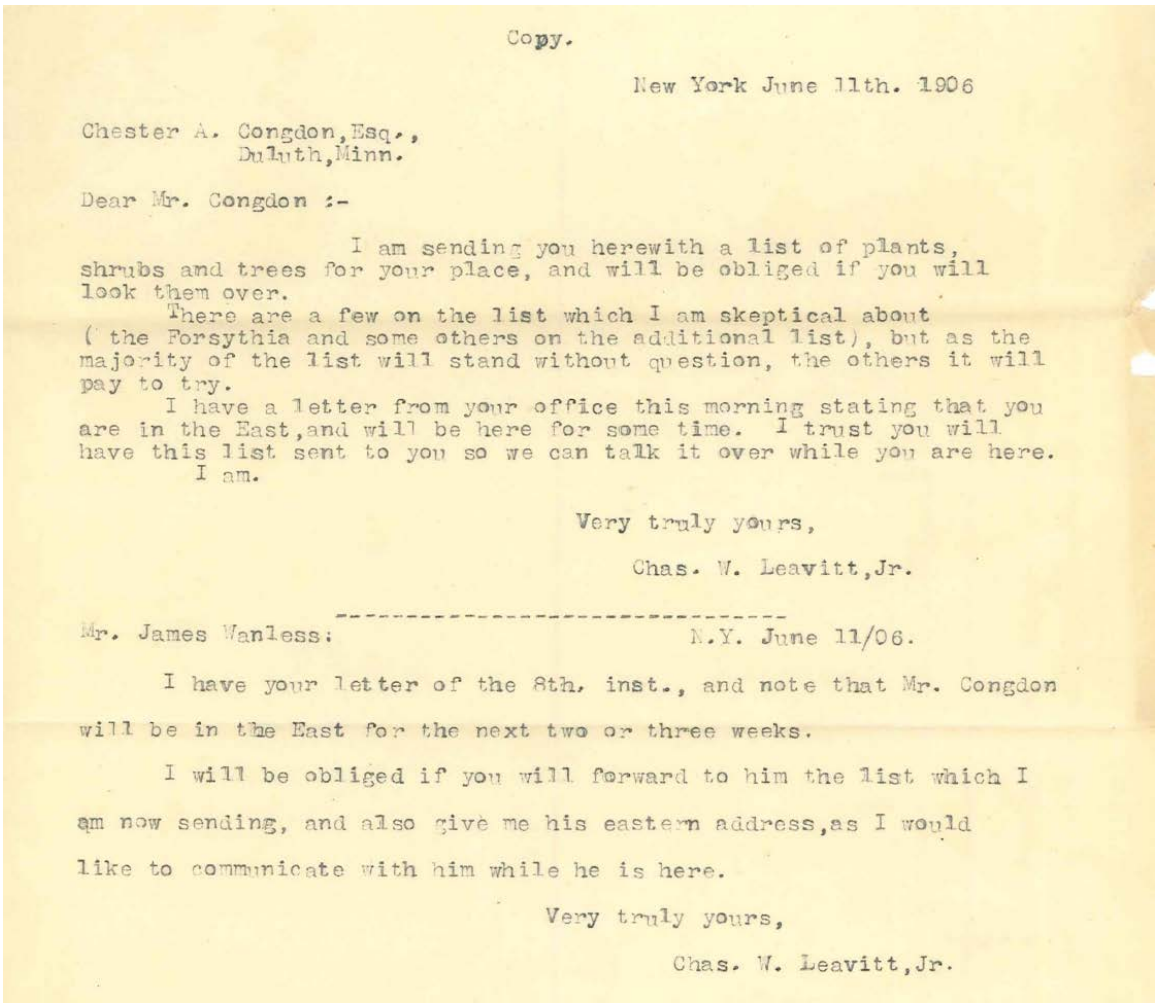
Accession No.	Recorded Provenance	No. Individuals
19570432	HWY 27 – Mille Lacs Lake, MN	3
19601118	HWY 27 – Mille Lacs Lake, MN	14
19820893	Seedling of 19570423	3

Table A-2: Available accession data for Eloise Butler Wildflower Garden Eastern hemlock plantings

No. Trees	Recorded Provenance	Date Received
2	Anoka, MN	May 26, 1907
6	Park Board Nursery	September 11, 1911
28	Park Board Nursery	May 28, 1914

FIGURES

Figure A-1: Note from Charles W. Leavitt, Jr. to Mr. Congdon, preceding the proposed 1906 plantings for Glensheen Mansion grounds (Leavitt, 1906).



Copy.

New York June 11th. 1906

Chester A. Congdon, Esq.,
Duluth, Minn.

Dear Mr. Congdon :-

I am sending you herewith a list of plants, shrubs amd trees for your place, and will be obliged if you will look them over.

There are a few on the list which I am skeptical about (the Forsythia and some others on the additional list), but as the majority of the list will stand without question, the others it will pay to try.

I have a letter from your office this morning stating that you are in the East, and will be here for some time. I trust you will have this list sent to you so we can talk it over while you are here.

I am.

Very truly yours,

Chas. W. Leavitt, Jr.

Mr. James Wanless: N.Y. June 11/06.

I have your letter of the 8th. inst., and note that Mr. Congdon will be in the East for the next two or three weeks.

I will be obliged if you will forward to him the list which I am now sending, and also give me his eastern address, as I would like to communicate with him while he is here.

Very truly yours,

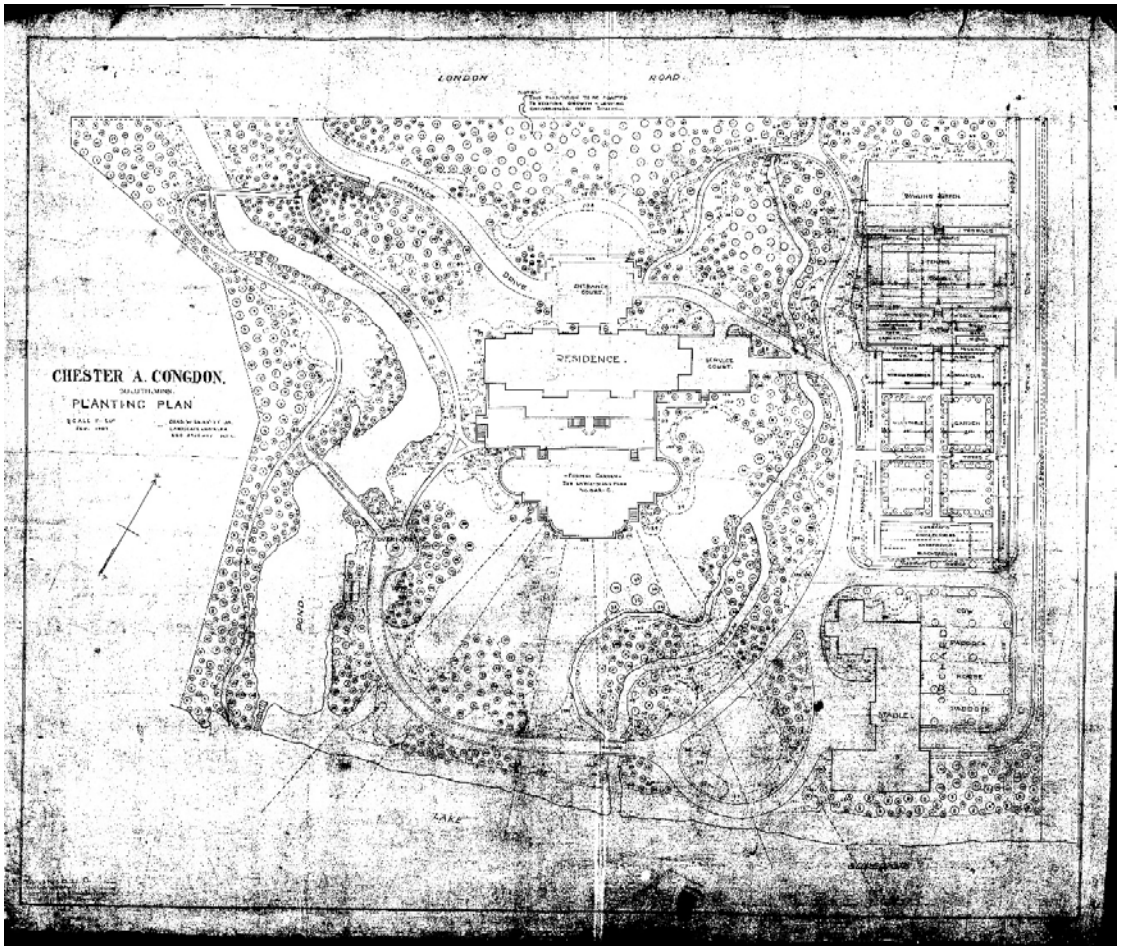
Chas. W. Leavitt, Jr.

Figure A-2: A list of evergreen trees for protected places, including Eastern hemlock, on the 1907 Glensheen Mansion finalized planting list (Leavitt, 1907a).

Evergreen Trees for Protected Places.

57	Abies concolor- - - - - - - - - - - -	Colorado Silver Fir
58	Juniperus sabina- - - - - - - - - - -	Savin Juniper
59	Picea excelsa inverta- - - - - - - - -	Weeping Spruce
60	Pseudo-tsuga Douglassi- - - - - - - -	Douglas spruce
61	Pinus Cembra- - - - - - - - - - - -	Swiss Stone Pine
62	Tsuga Canadensis- - - - - - - - - -	Hemlock

Figure A-3: The Glensheen Mansion planting plan map from 1907 (Leavitt, 1907b).



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