5-2018

Conservation and collection of Castanea dentata germplasm in the South

Trent Deason
University of Tennessee at Chattanooga, hvj617@mocs.utc.edu

Conservation and collection of *Castanea dentata* germplasm in the South

Trent Deason

Departmental Thesis

The University of Tennessee at Chattanooga

Department of Biology, Geology, and Environmental Sciences

Examination Date: April 5, 2018

_____	_____
Dr. J. Hill Craddock	Dr. David Aborn
Professor	Assistant Professor
Biology, Geology, and Environ. Sci.	Biology, Geology, and Environ. Sci.
Thesis Director	Department Examiner

_____

Dr. Jennifer Boyd
Assistant Professor
Biology, Geology and Environmental Science
Department Examiner

Table of Contents

i. Tables and Figures

ii. Abstract

Conservation and Collection of *Castanea dentata* germplasm in the South

Trent Deason

The American chestnut, *Castanea dentata*, has been devastated by the exotic invasive pathogens *Cryphonectria parasitica* and *Phytophthora cinnamomi* to which it has no resistance. The American Chestnut Foundation (TACF) has developed an interspecific backcross breeding program to introgress disease resistance from Asian chestnut species, primarily *Castanea mollissima*, into *C. dentata* hybrid populations. The genetic base of this program can be expanded by utilizing vegetative propagation through grafting in order to collect and conserve American chestnut individuals not amenable to traditional breeding. As the majority of the surviving American chestnuts are confined to the understory, they are shaded out by the forest canopy and unable to reach sexual maturity. Additionally, southern populations of chestnut harbor greater genetic diversity and more frequent occurrence of rare alleles. Conservation of these diverse populations would widen the genetic base of TACF breeding program and strengthen restoration of the species. This study has located and collected scionwood from 33 American chestnuts, 19 (~58%) of which have not been collected prior, across 9 sites in Tennessee and Alabama which will be conserved through grafting. Four types of rootstocks (*C. dentata*, *C. mollissima,* and F1 and BC3F2 hybrids) were chosen to account for possible graft incompatibility, although compatibility was not measured in this study. The whip-and-tongue and bark-flap grafting techniques were used depending on scion-rootstock diameter. These container-grown grafted plants will be conserved *ex situ* in a nursery where, released from competition for light, they should produce flowers. Pollen collected from these grafts will be used by TACF breeders to capture cytoplasmic genes and potentially develop new line of resistance when crossed with novel Asian *Castanea* sources.

1. Introduction

The American chestnut, *Castanea dentata* (Marshall) Brokh., is susceptible to a fungal blight (*Cryphonectria parasitica* [Murrill] Barr) as well as to Phytophthora root rot (PRR) caused by *Phytophthora cinnamomi* Rands. Both of these pathogens are considered nonindigenous, having been introduced to the United States with the earliest reported symptoms on American Chestnut dating back to 1904 and 1825, respectively (Anagnostakis, 2001). There is evidence that suggests that these pathogens originated in Asia as the Asiatic *Castanea* species, such as the Chinese (*Castanea mollissima*) and Japanese (*Castanea crenata*) chestnuts, have shown resistance to both (Anagnostakis, 2001; Burnham, 1988).

The American Chestnut Foundation (TACF) has been focused on the introgression of Asian sources of resistance into the American chestnut by interspecific-backcross breeding. These efforts have produced viable Chinese-American chestnut hybrids that capture the disease resistance from their Asian parent, while maintaining the morphological characters of the American (Burnham, 1988; Diskin, 2006). However, the primary breeding method requires locating flowering American chestnuts, which is difficult, effectively limiting the gene pool and reducing the effective population required for successful restoration (Fei, 2007). Most individuals in surviving *C. dentata* populations never bloom because they are in the understory. Shaded out by the canopy, this creates conditions unfavorable for competitive growth and sexual maturation (Paillet, 2002). Even when competitive release occurs by wind throw, timber harvest, or other means, breeding wild trees is time consuming and involves repeated visits to the field in order to place and retrieve pollination bags for

the collection of pollen or seeds. This method is complicated when field conditions, such as terrain and distance from roads, make it difficult to collect pollen and nuts efficiently.

These problematic circumstances create the conditions for repeated visits to known flowering trees in relatively more convenient locations. As a result, less accessible or nonflowering American chestnut trees may be excluded. The consequence of this may be the over-representation of some American individuals, the loss of potentially rare alleles, and regional variation from the breeding program.

I evaluated a graft-based method that focuses on collecting scionwood, rather than pollen or seeds, from naturally occurring American chestnut trees. Collection of scionwood is not dependent on the sexual maturity of the plant; even trees that are not blooming can be thus brought into the breeding program. American scions grafted to rootstocks can be planted in the field, in a germplasm conservation orchard (GCO), or maintained in containers. Container grown trees can be manipulated in ways that may accelerate the development of flowers. The increased temperature and photoperiod conditions in a lighted greenhouse, or growth chamber may expedite flowering and shorten the time to pollen collection (Baier et al., 2012; Sanz-Pérez, 2008). If these southern American clones produce seeds, we also capture their cytoplasmic genes, and possibly rare alleles, that will further enhance the genetic diversity of the breeding program and conservation efforts.

2. Literature Review

2.1 *Castanea dentata*: Description of American Chestnut

The American chestnut, *Castanea dentata,* is one member of a genus with a distribution throughout the northern hemisphere in eastern North America, eastern Asia, and Europe. Four species occur in Asia (*C. mollissima, C. henryi, C. seguinii,* and *C. crenata*), one in Europe (*C. sativa*), and two in North America (*C. dentata* and *C. pumila*), though the taxonomy is under debate (Perkins, 2017). The American chestnut has a long latitudinal range, encompassing much of the Appalachian Mountains and foothills, from Alabama to Maine and southern Ontario and spreads longitudinally from western Kentucky to the central Carolina's (Anagnostakis, 2001). The largest of all other *Castanea* species, the American chestnut was a dominant figure in the canopy throughout the eastern hardwood forest. Unlike many other forest trees such as oak (*Quercus*), *Castanea* species produce a reliable annual mast, and thus are of ecological importance in ecosystems where they occur. (Fei, 2012). American chestnut typically produces three nuts per burr, a character that distinguishes it from the other native North American species, *C. pumila* (Anagnostakis, 1987; Nixon, 1997).

In the eastern hardwood forests of the United States, and particularly in the Appalachian Mountains of the Southeast, the American chestnut was important economically and culturally, possibly more than any other one tree in its range (Ashe, 1911). Its annual production of choice-edible nuts was a reliable staple food and as it often grew to heights of over 30 m., American chestnut was a valuable timber product for a multitude of uses (Roane et al., 1987). Following the introduction and spread of

chestnut blight, caused by *Cryphonectria parasitica*, this dominant canopy tree has been reduced to the understory as a small tree and coppice resprouts, unable to reach the canopy before succumbing to blight (Paillet, 2002).

2.1.1 Biogeography: Migration of American Chestnut

Forests in eastern North America have been repeatedly compressed and displaced as a result climate change, driven primarily by some 18 to 20 glacial events that occurred over the last 2 million years of the Pleistocene epoch (Davis, 1983). Periods of glacial advancement, or maxima, were sustained throughout much of the Pleistocene. The most recent glaciation, the Wisconsinan, occurred between 18,000 and 20,000 years ago. These events caused the compression of species ranges into unglaciated regions further south and some species were relegated to a few pockets which severed as refugia until glacial ice retreated (Davis, 1983). During interglacial periods, deciduous species began to disperse northward, as evident by the pollen records studied by Davis (1983) and Delcourt et al. (1980).

Davis (1983) and Delcourt et al.'s (1980) palynology studies suggest that migration of deciduous species occurred in a south to north fashion from their glacial refugia. This migration, as Davis (1983) explains, occurred at varying rates for each species. These rates were influenced largely on dispersal methods and fertilization restriction, but also due to other factors such as herbivory. Palynology of *Castanea dentata* has placed it in western Tennessee and central Alabama about 15,000 years before present (Davis, 1983). Huang (1998) suggests that American chestnut likely

occurred in multiple refugia, primarily in south-central Alabama and on the continental shelf in North Carolina and Virginia.

Though the exact migration mechanism requires more study, one explanation offered by Davis (1983) proposes that because *Castanea dentata* are monecious obligate out-crossers, the need for two individuals to produce fertile offspring likely slowed its progression northward (Davis, 1983). From its refugium in the south, American chestnut migrated north along the axis of the Appalachian Mountains, reaching the northeast only as recently as 2,000 years before present (Davis, 1983). This time represents the establishment of American chestnut throughout its modern range.

2.1.2. Genetic Diversity: Southern Hotspots

The American chestnut has a high genetic variability across its range, though considered narrower when compared to other species within the genus *Castanea* (Kubisiak & Roberd, 2006). Much of this diversity exists within populations (95% of diversity can be sample within a population), however given the expanse of its range, between population diversity is measureable (Huang, 1998, Kubisiak and Roberds, 2006).

Through a study of 12 populations across its present range, Huang (1998) has indicated that the center of diversity of American chestnut occurs in south-central Alabama. This finding is supported by its occurrence here as a refugium during the last glacial maximum, serving as the founder population for interglacial migration northward (Davis, 1983; Gailing & Nelson, 2017). Huang also discovered that genetic diversity of American chestnut has a negative correlation between genetic and

geographic distance, where diversity decreases from south to north; though he noted there is intermediate levels of heterozygosity in the central Appalachian population compared to the southernmost and northern populations. This finding contradicts other works regarding Pleistocene interglacial migration, which have found lower diversity at the extremes of ranges of some conifer species (Critchfield, 1984). Additionally, he suggests that distinct populations across the range can be identified, segregating as southern (AL), southern Appalachian (GA, NC, VA, including OH and MI), north-central Appalachian (PA) and northern Appalachian (CT, NY), where the southernmost populations in Alabama represents the highest genetic diversity. This segregation has been disputed by Kubisiak and Roberds (2006) as being insufficiently quantified, but they do acknowledge that between population variation does exist. In light of this evidence, the population genetics make up of American chestnut is consistent with that of a single metapopulation influenced by genetic drift, rather than disjunction events (Kubisiak & Roberds, 2006; Gailing & Nelson, 2017).

Genetic diversity and distribution in American chestnut was examined further by Kubisiak and Roberds (2006). They expanded RAPD markers to include chloroplast (cp) DNA in addition to noncoding regions of the nuclear genome. The inclusion of cpDNA in this study is useful in that chloroplast genomes are slow to evolve and serve as reliable phylogenetic markers (Palmer et al., 1988). Further, as cpDNA is inherited from the mother parent, it commonly segregates into distinct haplotypes. The American chestnut segregates into a number of haplotypes, which have been found to be distributed along a latitudinal gradient. Northern populations are typically fixed at a more recently mutated haplotype D1 (Li & Dane, 2013) and/or D2 (Shaw et al., 2012)

and southern populations (particularly in Alabama) show higher frequencies of more ancient and unique types that are not found anywhere else. These haplotypes include rare “D-types” and non-D-types (D2, D11, D12, D13, R2; Li & Dane, 2013). Shaw et al. (2012) report additional haplotypes in southern populations (P1, M4, M6, M7, and M10), and showed that these unique haplotypes are reflected in the morphology. Further, they demonstrated that morphology can be used to predict which haplotype an individual has (Perkins, 2016; Shaw et al., 2012), thus an important factor in targeting areas for conservation.

In contrast to Huang (1998), Kubisiak and Roberds (2006) report findings of clear longitudinal and latitudinal variation in allele frequency, where the highest frequency of genetic diversity and rare alleles occurs in southwestern populations. This result is echoed by Shaw et al. (2012) and Li and Dane (2013), as both studies discuss the occurrence of unique haplotypes of south-central Alabama (Ruffner Mountain Nature Preserve, Birmingham, AL). This region is of particular interest as it represents the location of glacial refugium for many species in addition to the American chestnut (Soltis et al., 2006). The high genetic diversity and frequency of rare alleles in the southern range of the American chestnut highlights the need for conservation, capture, and introduction of these genes into the TACF breeding program.

2.2 Introduced Pathogens

2.2.1 Chestnut Blight

The preblight range of the American chestnut extended throughout much of the North American eastern hardwood forest and held a prominent place in the culture of both Native Americans and settlers of European descent (Burnham, 1988). However,

this important canopy tree was destroyed by the introduction of the fungal plant pathogen *Cryphonectria parasitica*. The first evidence of chestnut blight was recorded in 1904 in the Bronx Zoological Park in New York City and mortality was reported as quickly as 1905 (Merkel, 1905; Roane et al., 1986).

Initially described as *Endothia parasitica*, *Cryphonectria parasitica* is an ascomycete fungus that causes canker development in its infected host. American chestnut has little to no natural resistance, which lead to rapid devastation of nearly every individual throughout its native range (Anagnostakis, 1987). Chestnut blight can be easily spotted on young trees as cankers are more pronounced on the smoother bark of juvenile trees. Additionally, *C. parasitica* produces an orange pycnidial fruiting body that may be found near the canker or along other portions of an infected tree. Mortality results when chestnut blight kills the cambium layer, preventing development of new vascular tissue. The portion of the tree above the canker is effectively severed from nutrients supplied by the roots; wilting above the canker occurs as the fungus spreads and kills all above ground tissue (Anagnostakis, 1987; Anderson, 1914). Unable to spread to the roots, blight killed American chestnuts will continue to coppice from the root collar of the original trunk (Graves, 1926). These sprouts linger in the understory were they once stood, repeating a cycle of sprout, infection, death, and re-spout for decades (Paillet, 2002).

Drastic efforts were taken to prevent the spread of chestnut blight, particularly in Pennsylvania where large “fire-breaks” were cut in forests between 1912 and 1914 (Gravatt, 1949). This was method was not successful as chestnut blight, though producing animal-vectored conidia under certain conditions, is primarily spread via

airborne ascospores (Anderson, 1914; Gravatt, 1949). By 1926 chestnut blight had been reported across the entirety of its range (Gravatt & Marshall, 1926) and mortality of nearly all mature individuals by 1950 (Anagnostakis, 2001). The devastation of such an economic and culturally significant tree prompted the U.S. Congress to pass the Plant Quarantine Act of 1912: the United States' first regulation to control the import and distribution of exotic plants in order to prevent the introduction of other catastrophic plant pathogens.

Though the majority of trees have been effectively eradicated, there are several rare cases of large surviving American chestnuts (LSA) reaching heights of 12 to 18 meters (Day et al., 1977; Diller & Clapper, 1965). These trees represent an interesting phenomenon, though not the original tree, these individuals have survived for some time after infection with blight (Day et al., 1977). In these cases, the fungus has shown to be less virulent, or hypovirulent. Evidence of this condition was first discovered in Italy, where chestnut blight also occurred on *Castanea sativa* (Grente & Sauret, 1969). Further investigation showed that *C. parasitica* had been infected by a dsRNA virus, reducing its virulence on the host plant. Cultures of hypovirulent strains were used to inoculate trees showing symptoms of blight and after a few years, cankers healed and blight symptoms subsided. Not long after manual treatment began, hypovirulent strains spread and slowly restoration of *C. sativa* occurred (Grente & Berthelay-Sauret, 1978).

Samples taken from LSA's were matched to isolates from Europe indicating that some hypovirulence does in fact occur in the range of *C. dentata* (Day et al., 1977). However, due to vegetative compatibility restrictions, hypovirulent strains must match at every gene for successful mating (Anagnostakis, 1977). It is still unclear as to why

hypovirulence spreads more rapidly in Europe than the United States. Continued research is ongoing which may uncover a more readily transmissible strain that will reverse the damage of chestnut blight in North America (Zhang & Nuss, 2016)

2.2.2 Phytophthora Root Rot: *Phytophthora cinnamomi*

The genus *Phytophthora* is host to many pathogens capable of widespread destruction. Among other diseases caused by this genus, the infamous Irish potato famine was caused by *Phytophthora infestans*, which wreaked havoc on the Irish food supply during the 1840s resulting in historic migration of people out of western Europe (Yoshida et al., 2013). This genus of oomycetes includes *Phytophthora cinnamomi*, the subject of concern in American chestnut.

Predating chestnut blight, Phytophthora root rot (PRR) caused by *P. cinnamomi*, was first recorded in 1825 on native *Castanea* species in Riceboro, Georgia (Anagnostakis, 2001); though it is believed to have been present some 100 years before (Crandall et al., 1945). While this disease is credited with widespread chestnut mortality in areas of the Carolinas, its seriousness was likely overlooked due to the unprecedented destruction by *Cryphonectria parasitica* occurring in the northeast around the same time (Crandall & Gavatt, 1967).

PRR produces necrotic lesions on root tissue causing them to turn black, thus also called ink disease (Anagnostakis, 2001), killing root tissue and reducing nutrient uptake from the soil (Maurel et al., 2001). Prior to the inspection of roots, PRR symptoms can manifest above ground as leaf yellowing and wilt, branch die-back, and reduced vigor (Maurel et al., 2001). Different from chestnut blight, PRR resides in the

soil, spreading via spores in moist soil and rain events though erosion and runoff. Further, this pathogen kills the tree from the roots eliminating its ability to resprout as in the case of chestnut blight. In this way, PRR may pose a more serious threat to restoration efforts if trees die before germplasm collection takes place.

Although relatively recent, the TACF expanded its breeding program to combat PRR and progress has been made (Jeffers et al., 2008). In the same way that Asiatic *Castanea* species have resistance to blight, they are also resistance to *Phytophthora cinnamomi*, and the backcross breeding program has since been selecting for resistance to PRR (Jeffers et al., 2008; Robinson, 2016)

2.3. Restoration Efforts

Soon after chestnut blight began to spread through the northeastern U.S., the Department of Agriculture developed a multipronged program designed to save and restore the American chestnut. This program focused on three areas: finding American chestnuts with some level of genetic resistance, investigate whether an Asian *Castanea* species might replace the devastated American, and begin breeding hybrids for resistance (Diller & Clapper, 1965).

Early on, researchers were optimistic about finding a resistant American. Diller and Clapper (1965) describe that the American chestnut's ability to resprout after succumbing to blight, and the occurrence of large surviving American chestnuts (LSA) were indicators of possible resistance. Though, as they explain, these hopes were not met with positive results. Even as several state and federal research organizations, as

well as chestnut hobbyists, were avidly breeding American chestnuts, resistance to blight was not discovered (Diller & Clapper, 1965).

Commissioned by the Department of Agriculture in 1927, Dr. R. Kent Beattie went to Asia in search of a suitable blight-resistant *Castanea* species (Diller &Clapper, 1965). He returned to the United States with seed from several species and began to study the growth and habit of these trees under direction of the Dept. of Agriculture. Later, large areas of forest land were converted into Asian chestnut orchards for the purposes of studying blight resistance. One tree, a *C. mollissima* from Nanking, China (now referred to as "Nanking") showed adequate blight resistance, growth and form, and quality of nuts (Diller & Clapper, 1965). Though these Asiatic species were found unsuitable ecosystem replacements for the American chestnut, they do represent a valuable source of resistance for interspecific breeding (Burnham et al., 1986; Diller & Clapper, 1965).

The hopes for discovery of American blight resistance faded and efforts shifted towards breeding resistance through interspecific crosses of American and Asian species. The most successful of which was a backcross breeding method where an American (*Castanea dentata*) was hybridized with a Chinese (*C. mollissima*), then bred back to an American (*C. dentata*; Diller & Clapper, 1965). Developed by Russel Clapper, this "Clapper method" and others like it (Arthur Graves: *C. dentata* X *C. henryi*) would go on to be the foundation of the interspecific backcross breeding program embraced by TACF (Burnham, 1981; Diskin et al., 2005).

2.3.1 Role of The American Chestnut Foundation

Founded in 1983, The American Chestnut Foundation (TACF) works to breed resistance for chestnut blight and PRR in order to restore the American chestnut to the eastern hardwood forest. This organization seeks to accomplish restoration through breeding, biotechnology, and bio-control (3BUR Proposal, 2016; Anagnostakis, 2001).

Designed by Charles Burnham (1988), the backcross breeding program involves the introgression of resistance from the Asian *Castanea* species into American populations. Built on the early success of plant breeders such as Arthur Graves and Russel Clapper, this program is designed to incorporate blight-resistance from Asian *Castanea* species (primarily *C. mollissima*) into the American, while selecting for American phenotype (Burnham et al., 1986; Diskin et al., 2005). Maintaining American morphology is essential for restoration of the species as a hybrid tree must be able to fill the same ecological niche as the pure American (Diskin et al., 2005). The third generation of the third backcross (BC3-F3) is the generation hypothesized to capture genetic resistance to chestnut blight and PRR while recovering every phenotypic character of the American (Diskin et al., 2005). It is at this level where hybrids are expected to be 93.75% American.

Biotechnology, on the other hand, focuses on the development and approved implementation of a transgenic gene, oxalate oxidase (OxO). This genetically engineered solution has shown to effectively render American chestnut immune to chestnut blight (Steiner et al., 2016). This technology, while additional research and approval pending, may prove to be an effective means of restoring the American chestnut.

Another goal of TACF is to establish germplasm conservation orchards (GCO) where wild type American chestnuts are sourced from its native range and concentrated into orchards by means of grafting, transplanting, and/or seed. GCOs are an important area in regard to the potential use of the transgenic OxO gene (Steiner et al., 2016). Once its use is approved these GCOs will serve as common locations where pollen from an OxO treated founder tree can be used to breed resistance to a wide range of regional genetic diversity (Steiner et al., 2016).

Finally, the bio-control aspect of TACF approach involves developing an efficient means of introducing hypovirulence into American chestnut populations. Research is ongoing for development and implementation of hypovirulent donor strains that allow successful transmission of this viral fungus pathogen (Anagnostakis, 2001). The combined efforts of the TACF across its range of state and national research organizations are making progress at achieving its goal.

2.4. *Ex situ* Conservation by Vegetative Propagation

Techniques utilized in the conservation of at-risk or special interest species can be simplified into two broad categories: *in situ* and *ex situ*. *In situ* conservation focuses on protecting and managing the physical environment in which the species is found. Ensuring populations of species-of-interest are conserved, as well as the surrounding ecosystem is the preferred method of action in forestry and affiliated conservation organizations (McIlwrick et al., 2000). However, this approach is not always feasible when habitat loss and/or lack of control over land management negatively impacts *in situ* conservation. Additionally, as pathogen pressure from chestnut blight and PRR

increase on American chestnut, *ex situ* methods are important for conserving genetic diversity before wild populations decline further.

In these circumstances, *ex situ* methods are required to capture and conserve genetic material in locations outside the natural range or environment it occupied. These methods include grafting, the subject of the present research, transplanting, and planting seed harvested from individuals within their natural range. The material collected and conserved *ex situ* can be stored, grown in a greenhouse, nursery, and orchards offering the ability to manage growing conditions conducive to plant health and propagation (Alexander et al., 2003; McIlwrick et al., 2000; Wang et al., 1993).

Chestnut breeding and conservation could be advanced by including *ex situ* vegetative propagation methods in order to collect individuals previously excluded due to reproductive immaturity and/or geographic inaccessibility. Expanding the current breeding program to include regionally sourced, grafted American chestnuts will provide potential development of additional resistant lineages as well as conserve regional variation of germplasm and cytoplasm diversity.

2.4.1. Graft Propagation in American Chestnut

The mostly widely employed method of vegetative propagation in the genus *Castanea* is grafting (Keys, 1978; McKay & Jaynes, 1969). Other methods such as rooting and budding have been employed with limited success due in large part to *Castanea* being difficult to root (Wright, 1976). The advantage of grafting is that it requires no elaborate or complex equipment and can be done relatively quickly.

Although the technique is a skill that requires practice, it can be learned and executed with relative ease (Craddock & Bassi, 1993).

2.4.2. Graft Compatibility: Implications of Rootstock Selection

As in many hardwood species, proper rootstock selection is important for the success of grafted chestnuts. In these cases, scion-rootstock compatibility is fundamental to short- and long-term success. Compatibility among *Castanea* species has been studied and some evidence, though limited, has supported incompatibility on grafts of interspecific combinations (Huang et al., 1994; Santamour et al., 1986). Santamour et al. (1986) examined 10 *Castanea* species and found three variable anodal isoperoxidase bands in the cambial zones. They reported that graft incompatibility exists where cambial bands differ, including even intraspecific scion and rootstock. Although these findings where disputed by Huang et al. (1994), graft failure may be more pronounced in interspecific combinations.

Determining graft incompatibility is a difficult task because of the number of factors involved in graft success. As outlined by Jaynes (1979) four conditions that commonly influence graft success are (1) winter hardiness, (2) graft union infection by chestnut blight, (3) improper grafting technique, and (4) scion-rootstock incompatibility. Additionally, due to the unique stem morphology of *Castanea* species (often fluted or grooved), alignment of phloem bundles is difficult, contributing to increased graft failure (Huang et al., 1994). The age of the rootstock may also contribute to graft failure, as rootstocks of 2 to 3 years have more distinguishable phloem bundles which can be identified and aligned more easily. Additionally, scion

diameter plays a role in graft success. Depending on the grafting technique, ensuring the scion and rootstock are of equal diameter will increase the amount of vascular tissue contact. Grafted plants with similar diameter scion and rootstock develop smoother graft unions with less swelling, increasing the continuity of vascular bundles and improve graft success (Craddock & Bassi, 1993)

Another common phenomenon involves early graft success, which may yield some growth of the scion, but fails after a few months. This can be attributed to an interruption of phloem bundles by a mass of nonvascular tissue at the graft union, eventually cutting off vascular connectivity between rootstock and scion (Huang et al., 1994). Initially categorized as graft incompatibility, Huang et al. (1994) suggests this is simply a delayed graft failure due to growth of nonvascular tissue. These numerous factors make it difficult to discern whether graft success is a result of rootstock-scion incompatibility or other factors. Long-term studies on chestnut grafting are needed to better diagnose the exact cause of graft failure (Craddock & Bassi, 1999; Huang et al., 1994).

In light of these conditions, it is common practice to use a rootstock of the same species as the scion (Weber & MacDaniels, 1969). However, because *C. dentata* is susceptible to PRR, it may prove to more advantageous to graft susceptible American scion to resistant Chinese rootstocks where *P. cinnamomi* is a concern. Given the limited evidence of graft incompatibility between *C. dentata* and *C. mollissima*, conservation may be better served by utilizing this interspecific combination, especially in the southeastern U.S where *P. cinnamomi* is a concern. This practice would allow

successfully grafted plants to be transplanted into orchards that test positive for or are predicted to have *P. cinnamomi*.

2.5. Plant Growth Manipulation

When *ex situ* methods are employed, container grown plants can be subjected to a number of experiments in order to test their response to a given environment. Manipulating light and temperature conditions are of increasing interest given the possible implication of climate change on plant-animal interaction (Chmielewski & Rotzer, 2002; Sanz-Perez et al., 2007). In *C. dentata*, research on increased photoperiod and high light intensity has yielded more vigorous growth, increase biomass production, and reduced the time to bud burst and flower induction (Baier et al., 2012; Wang et al., 2006). Additionally, these studies may offer best practices on how to accelerate the currently long generation cycle *C. dentata* in the TACF breeding program.

Wang et al. (2006) studied American chestnut's response to light limitations and exposure in order to better understand how the species will respond to future forest plantings. Through a review of previous literature, Wang et al. (2006) found contradicting data on the shade tolerance of chestnut, where some authors describe it as shade intolerant and others report it as tolerant. In this study, Wang et al. (2006) designed a light exposure experiment to measure the photosynthetic rate, biomass allocation, and growth at four levels of irradiance (4%, 12%, 32%, 100%). Their findings show that American chestnut is a shade tolerant species, evident by its ability to persist in the understory and alteration of vertical to lateral growth ratio in high shade conditions. While this study was designed to measure shade tolerance in relation to

future restoration efforts, Wang et al. (2006) also found that in high light conditions, *C. dentata* exhibits rapid growth. These findings, similar to other eastern deciduous species, suggest that American chestnut can be treated with high light environments in order to accelerate growth.

Additional light manipulation studies, such as Baier et al. (2012), show that phenotypic plasticity in American chestnut can produce more vigorous growth and acceleration of flower induction. In an initial study of the transgenic American chestnut cultivar 'Hinchee 1', Baier et al. (2012) found that under the high light environment of a growth chamber (16hr photoperiod of 700-900 microEinsteins) 14 (43%) of the seedlings developed catkins between 9 and 11 months after planting. Importantly, pollen collected from these male flowers were tested and found viable.

This test was then performed on nontransgenic American (*C. dentata*) and Chinese (*C. mollissima*) chestnut and similar results were found. The trial consisted of six Chinese and six American chestnuts grown under the same conditions as the transgenic 'Hinchee 1'. Baier et al. (2012) discovered that four (67%) of Chinese and 1 (17%) American seedling produced catkins as early as six months after planting. Pollen collected from these catkins were tested and also found viable. Further, one (17%) Chinese chestnut produced female flowers. This study demonstrates the ability of high light exposure to induce early flowering in chestnut species. Additionally, accelerated flowering may speed breeding efforts by shortening the generation through earlier pollen collection and crossing (Baier et al., 2012). Using light to speed flower induction relies on plasticity within the species rather than through genetic modification, as other research has explored.

This research (Baier et al., 2012) provides evidence that supports the application of high light environments to advance TACF breeding program and conservation, however, it is outside the scope of the current study. The focus of my study was to collect potentially rare alleles and individuals outside the current TACF breeding program. As this work could only be performed during winter dormancy, the short duration of this study did not allow for the procurement of specialized lighting equipment and the time to collect, graft, and grow plants under the conditions laid out by Baier et al. (2012).

3. Methods

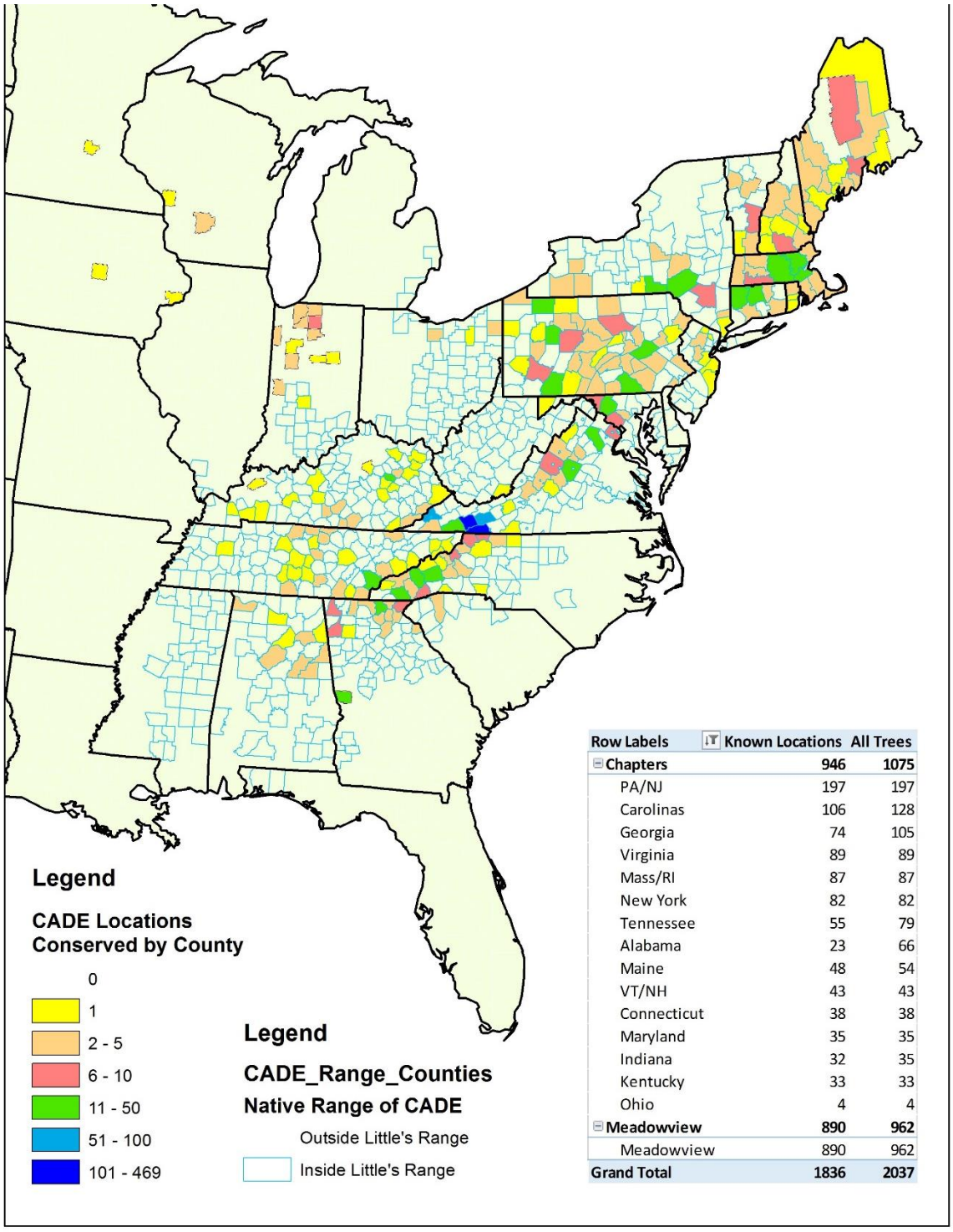
3.1. Study Area: Targeted Scion Collection

This study was designed to capture new and/or under-sampled American chestnut germplasm through scionwood collection from locations not represented in the TACF breeding program. Further, locations in the southernmost extent of the range of American chestnut targeted due to high genetic diversity and more frequent occurrence of rare alleles (Kubisiak & Roberds, 2006; Li & Dane, 2013; Perkins, 2016; Shaw et al. 2012). I divided areas for collection into four regions: (1) southeast Tennessee/northwest Georgia, (2) south-central Tennessee/northern Alabama, (3) north-central Tennessee/southwestern Kentucky, and (4) western Tennessee/northern Mississippi.

Although an artificial boundary, counties were used as a convenient marker for scion collection due to the common use of county-level occurrence reporting. A county-

by-county map of flowering American chestnuts conserved in the TACF breeding program (Figure 1) was obtained from Ben Jarret, produced by TACF, to guide collection efforts toward locations not well represented on the map. Additionally, we drafted an announcement requesting information on known locations of naturally occurring American chestnut. This announcement was sent out by TACF to all members of the Alabama, Georgia, Kentucky, and Tennessee chapters. Thanks to the network of dedicated TACF members and volunteers, we received a number of locations to trees within each region and an additional offer to help locate those trees upon our visit. This resource proved invaluable as it represented the majority of the material collected.

Locations obtained from TACF members were to be visited once in the fall of 2017 to confirm location and identification, though due to poor logistical planning, only one site was visited (Cannon County, TN). The remaining sites were visited in the winter of 2017-2018 when trees were dormant in order to properly collect scionwood. In total, we received information on 11 sites from TACF landowners and volunteers: 4 in Tennessee, 6 in Alabama, and 1 in Kentucky. Two additional Tennessee sites were identified through Southeastern Regional Network of Expertise and Collection (SERNEC) herbarium records.



Row Labels	Known Locations	All Trees
Chapters	**946**	**1075**
PA/NJ	197	197
Carolinas	106	128
Georgia	74	105
Virginia	89	89
Mass/RI	87	87
New York	82	82
Tennessee	55	79
Alabama	23	66
Maine	48	54
VT/NH	43	43
Connecticut	38	38
Maryland	35	35
Indiana	32	35
Kentucky	33	33
Ohio	4	4
Meadowview	**890**	**962**
Meadowview	890	962
Grand Total	**1836**	**2037**

Figure 1. TACF Breeding Program & Conservation of *Castanea dentata* by County

3.2 Scion Collection and Storage

Quality scionwood is material with dormant, unopened buds of the previous year's growth, thus collection cannot occur prior to winter dormancy. Collecting trips began in the middle of December 2017 and continued through the beginning of February 2018. Scions were collected by hand pruners and a pole pruner for trees exceeding 8-10ft. In order to optimize graft success, scionwood of pencil to index finger diameter was collected when possible. This diameter should be sufficient to perform the whip-and-tongue graft, which requires matching diameter of rootstock and scion (Craddock & Bassi, 1993). However, not all material collected was of desired diameter. These smaller samples will be used with other grafting techniques such as the bark-flap graft, commonly used for smaller diameter scionwood (Garner, 1947).

At each field site, scionwood collected from dormant trees were cut to the width of a standard gallon size freezer storage bag. Scions were placed in freezer storage bags labeled according to the name of the tree (tree code or common tree name, i.e., TNCAN01 or Fern Trail 01) and dated then rolled and pressed to remove excess air. Pressed bags were then double-bagged to reduce the likelihood of desiccation, then stored in an iced cooler (0°- 4° C) to maintain dormant conditions for the duration of the collecting trip. At the completion of the collecting trip, scions were removed from the cooler and resealed using a straw to remove a much air as possible, creating a semi-vacuum seal, then placed in a refrigerator (0°-1° C) Initially, scions were stored in a personal refrigerator, then moved to a dedicated refrigerator located in the STEM Annex at the University of Tennessee at Chattanooga.

3.3 Rootstock Selection

Grafting *Castanea* species, as well as other hardwoods, can be difficult given the number of variables involved in the process. Interspecific scion-rootstock compatibility may have some influence on graft success (Huang et al., 1994; Santamour et al., 1986), however, pathogen pressure on *C. dentata* rootstocks is a major concern within our study area. As PRR is prevalent in our greenhouse and nursery (Fortwood Street Greenhouse, University of Tennessee at Chattanooga, Chattanooga, TN), consideration of rootstock survival is as important as graft compatibility.

For this reason, a variety of rootstock species and hybrids were chosen aimed at achieving resistance to PRR and chestnut blight, while also accounting for possible graft incompatibility. Rootstock variation include pure *C. mollissima*, pure *C.* dentata, F1 hybrids of *C. mollissima X C. dentata,* and BC3F2 hybrids of *C. mollissima X C. dentata*. Rootstocks were sourced from researchers at TACF (Sara Fitzsimons, Penn State University), U.S. Forest Service (James McKenna, Hardwood Improvement) as well as from a commercial nursery (Greg Miller, Route 9 Cooperative) and stock grown on site at the Fortwood Greenhouse.

It should be noted that this study is not designed to test graft compatibility. Although a variety of rootstocks have been selected, it is only to account for the potential for graft incompatibility, not to test for it. Each individual will be grafted to all rootstock types for as many replications as allowed be the number of rootstocks in each type.

All rootstocks will be potted into 7.19 L or 14.76 L Rootmaker® pots from

Stuewe and Sons, Inc., depending on size. Currently, all *C. dentata* and the locally grown *C. mollissima* rootstocks have been potted in a medium of Sun Gro Metro-Mix 852, fertilized with Osomocote Plus 15-9-12 slow release (8-9 months) and Peters Professional water soluble 21-7-7 Acid Special fertilizer, and treated with the systemic fungicide Allude to prevent infection by *C. paracitica* and *P. cinnamomi*. The same combination of potting medium, fertilizer, and fungicide will be used for all rootstocks. Each rootstock will be treated every two weeks with a combination of soluble fertilizer and fungicide to promote vigorous growth and prevent fungal infection.

4. Results

4.1 Scionwood Collection

Throughout the range of *Castanea dentata* genetic diversity is highest in the southern-most populations found in and around central Alabama, decreasing northward (Huang, 1998; Kubisiak & Roberds, 2006; Li and Dane, 2013). Likely the refugium location during the last glacial maximum, these diverse southern populations harbor more unique or rare alleles than any other population throughout its range (Davis, 1983; Gailing & Nelson, 2017; Li & Dane, 2013).

This study targeted these highly diverse, undersampled populations for conservation by vegetative propagation. Scionwood, totaling 375 scions, was collected from 33 individuals in nine sites (Table 1) corresponding to three out of the four regions designated: (1) southeast Tennessee/northwest Georgia, (2) south-central

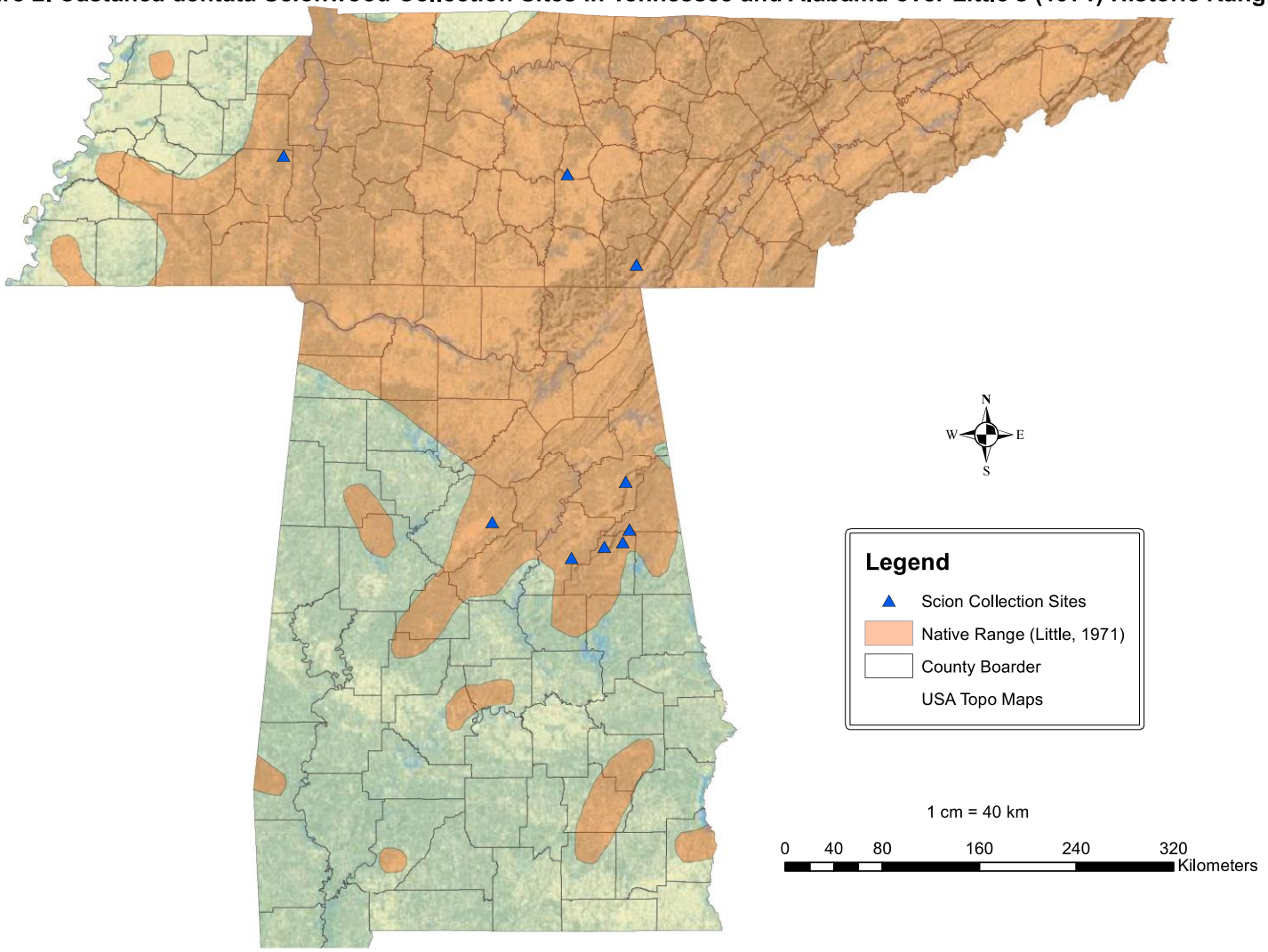
Tennessee/northern Alabama, and (4) western Tennessee/northern Mississippi. These sites were in Tennessee counties: Cannon, Hamilton, and Henderson, and Alabama counties: Calhoun, Clay, Cleburne, Jefferson, and Talladega (Figure 2). Tennessee collections included two (2) individuals from Signal Mountain, Hamilton County, TN in Prentice Cooper State Forest, 2 in Cannon County, TN on private land, and 6 in Henderson County, TN in Natchez Trace State Park. Alabama collections included 9 individuals from Talladega and Calhoun counties, all within Talladega National Forest and Cheaha State Park (surrounded by Talladega National Forest), 2 individuals in Cleburne County on private land, 1 individual in Clay County on private land, and 11 individuals in Jefferson County on a protected land trust, Ruffner Mountain Nature Preserve.

Table 1. Scion Collection Data

Tree Code/ID	State	County	Date	Site	Estimated Scions	Coordinates N	Coordinates W
ALJEFF81	AL	Talladega	12/12/17	Taladega NF	20	33.33595	-86.11632
ALJEFF82	AL	Talladega	12/12/17	Adams Gap	10	33.40292	-85.87519
ALJEFF83	AL	Talladega	12/12/17	Adams Gap	17	33.40313	-85.87519
ALJEFF84	AL	Talladega	12/12/17	Adams Gap	13	33.40272	-85.87561
ALCLEB06	AL	Cleburne	12/13/17	Frames Property	6	33.50892	-85.68871
ALCLEB06	AL	Cleburne	12/13/17	Frames Property	25	33.50951	-85.68905
ALCLAY01	AL	Clay	12/13/17	Sonny Clarke Property	25	33.43043	-85.7359
ALCALH22*	AL	Calhoun	12/13/17	Choccolocco Mtn	5	33.80471	-85.71583
ALCALH01*	AL	Calhoun	12/13/17	Choccolocco Mtn	4	33.80454	-85.71525
ALCALH02*	AL	Calhoun	12/13/17	Choccolocco Mtn	3	33.80454	-85.71525
ALCALH28*	AL	Calhoun	12/13/17	Choccolocco Mtn	25	33.80457	-85.71489
ALCALH27*	AL	Calhoun	12/13/17	Choccolocco Mtn	11	33.80457	-85.71521
ALJEFF76	AL	Jefferson	2/2/18	Ruffner Mtn	10	33.55648	-86.70345
ALJEFF74	AL	Jefferson	2/2/18	Ruffner Mtn	5	33.55593	-86.70357
ALJEFF79*	AL	Jefferson	2/2/18	Ruffner Mtn	5	33.55572	-86.70426
ALJEFF72	AL	Jefferson	2/2/18	Ruffner Mtn	10	33.55571	-86.70429
ALJEFF80*	AL	Jefferson	2/2/18	Ruffner Mtn	10	33.55565	-86.70423
ALJEFF78	AL	Jefferson	2/2/18	Ruffner Mtn	5	33.55543	-86.70387
ALJEFF70*	AL	Jefferson	2/2/18	Ruffner Mtn	5	33.55675	-86.70491
ALJEFF24*	AL	Jefferson	2/2/18	Ruffner Mtn	10	33.55811	-86.70392
ALJEFF21	AL	Jefferson	2/2/18	Ruffner Mtn	5	33.56953	-86.695
ALJEFF14*	AL	Jefferson	2/2/18	Ruffner Mtn	10	33.56963	-86.69503
ALJEFF25	AL	Jefferson	2/2/18	Ruffner Mtn	5	33.56971	-86.69593
TNCAN01*	TN	Cannon	12/18/17	Todd Property	20	35.67376	-86.14741
TNCAN02*	TN	Cannon	12/18/17	Todd Property	15	35.67378	-86.14742
TNHAM02*	TN	Hamilton	1/3/18	Signal Mtn	5	35.12777	-85.636228
TNHAM01	TN	Hamilton	12/21/17	Signal Mtn	7	35.1629	-85.37338
TNHEN01*	TN	Henderson	12/28/17	Natchez Trace SP	20	35.76633	-88.27876
TNHEN02*	TN	Henderson	12/28/17	Natchez Trace SP	12	35.76613	-88.27858
TNHEN03*	TN	Henderson	12/28/17	Natchez Trace SP	10	35.7829	-88.2503
TNHEN04*	TN	Henderson	12/28/17	Natchez Trace SP	10	35.78222	-88.2519
TNHEN05*	TN	Henderson	12/28/17	Natchez Trace SP	15	35.78388	-88.25566
TNHEN06*	TN	Henderson	12/28/17	Natchez Trace SP	12	35.78371	-88.25568

*Represents new germplasm collection: 19 of 33 (57.5%)

Figure 2. Castanea dentata Scionwood Collection Sites in Tennessee and Alabama over Little's (1971) Historic Range



Each site was to be visited twice: once in the fall of 2017 to confirm location and species, and again in winter to collect scionwood. However, due to poor logistical planning, only the Cannon County, TN site was visited in fall of 2017. This issue was mitigated by using winter identification characters and detailed GPS data and records generated by researchers, land stewards, and volunteers to confirm species of the remaining sites.

Despite the high genetic diversity of the region (Huang, 1994; Li & Dane, 2013), all of the counties represented in this study are under-sampled areas not well represented in TACF breeding program (Figure 1), where less than five trees have been utilized. No American chestnuts in Henderson County, TN have been incorporated into the TACF breeding program. Although collections have been made from the adjacent county to the north, Carroll County, diversity of this region is high, thus collections made in Henderson County may represent a novel source of genetic diversity for the program.

The other counties visited in this study have had collection accounted for in the TACF breeding program (Figure 1). I relied on records from land stewards and TACF members familiar with the site to determine if a tree had previously been collected in some way (i.e., pollen, seed, or scionwood). 19 of the 33 (~58%) American chestnuts collected in this study represent newly collected individuals. The remaining 14 have been collected prior, or I could not confirm whether they had been conserved previously (Table 1). As this study was to collect new and under-sampled sources of American chestnut, scionwood collected from previously sampled trees is justified given the high diversity of the region to increase the propagation of these diverse populations. For

example, previous research by Li and Dane (2013) had sampled chestnut at Ruffner Mountain Nature Preserve (Jefferson County, AL) and subsequently scionwood was collected from some individuals also collected in this study. However, as the Ruffner Mountain population contains rare alleles and haplotypes (Li & Dane, 2013), previously collected individuals were not excluded, and given the limited graft success reported by David Morris (Alabama Chapter, TACF; Personal Communication, 2018) collecting more scion for grafting was desired.

4.2. Rootstocks, Nursery Conditions, and Grafting

To date, 120 American (*C. dentata*) rootstocks, from Sara Fitzsimons, and 100 pure Chinese (*C. mollissima*), from Greg Miller of Route 9 Cooperative are ready for grafting as soon as they show signs of active growth (Craddock & Bassi, 1993). The remaining rootstocks, from James McKenna, are to be delivered in the coming weeks.

Originally, some 30-40 *C. mollissima* rootstocks grown on site at the Fortwood Street Greenhouse were to be used. However, due to sustained cold temperatures during the winter of 2017-2018 (two weeks of overnight lows ≤-9.4°C) the majority of the *C. mollissima* rootstocks sustained extensive freeze damage of the roots beginning at the root collar. Over-wintering potted nursery stock can be problematic given the limited amount of soil in each pot, which cannot retain heat sufficiently in extended cold temperatures (Greg Miller, Personal Communication, 2018). Although the nursery was winterized (plants were bunched together and covered with pine straw), it was not enough to insulate seedlings from the particularly cold winter. The freeze killed or

damaged *C. mollissima* rootstocks were not used and 100 replacements rootstocks were sourced from Route 9 Cooperative.

The total number of rootstocks are 366 (Table 2) and due to the limited and uneven number of rootstocks per type, some scion-rootstock combinations will be as few as one (Table 3). Because these scion-rootstock combinations are limited to one (BC3F2) and four (*C. dentata*) replications, a sufficient inference on graft compatibility cannot be made. Instead, this study was designed to increase the probability of graft success by using a variety of rootstocks to account for possible graft incompatibility as well as pathogen pressure by chestnut blight and PRR.

In addition to freeze damaged/killed rootstocks, the Fortwood Street Greenhouse was inoperable for a three-month period due to a disabled heater which resulted in frozen pipes. These mechanical issues caused a delay in greenhouse operations. Having been unable to introduce dormant rootstocks into a warmed greenhouse in order to accelerate budburst, grafting could not begin as originally scheduled. Thus, the results of graft success are not included in this paper. When the rootstocks have completed winter dormancy, grafting will begin following the scion-rootstock combinations shown in Table 3, and depending on scion diameter, the whip-and-tongue or bark flap graft techniques will be used.

Table 2. Rootstock Species/Hybrids

Species/Hybrid	**Quantity**
Castanea dentata	120
Castanea mollissima	130
F1 *C. dentata* X *C. mollissima*	78
BC3F2 *C. dentata* X *C. mollissima*	38
TOTAL	366

Table 3.5 Scion-Rootstock Combinations

Tree Code/Name	Scions Collected	*Castanea dentata*	*Castanea mollissima*	F1 *C. dentata* X *C. mollissima*	BC3F2 *C. dentata* X *C. mollissima*
ALJEFF81	20	4	4	3	2
ALJEFF82	10	4	4	3	2
ALJEFF83	17	4	4	3	2
ALJEFF84	13	4	4	3	2
ALCLEB06	6	4	4	3	2
ALCLEB06	25	4	4	3	1
ALCLAY01	25	4	4	3	1
ALCALH22	5	4	4	3	1
ALCALH01	4	4	4	3	1
ALCALH02	3	4	4	3	1
ALCALH28	25	4	4	3	1
ALCALH27	11	4	4	3	1
ALJEFF76	10	4	4	2	1
ALJEFF74	5	4	4	2	1
ALJEFF79	5	4	4	2	1
ALJEFF72	10	4	4	2	1
ALJEFF80	10	4	4	2	1
ALJEFF78	5	4	4	2	1
ALJEFF70	5	4	4	2	1
ALJEFF24	10	4	4	2	1
ALJEFF21	5	4	4	2	1
ALJEFF14	10	3	4	2	1
ALJEFF25	5	3	4	2	1
TNCAN01	20	3	4	2	1
TNCAN02	15	3	4	2	1
TNHAM02	5	3	4	2	1
TNHAM01	7	3	4	2	1
TNHEN01	20	3	4	2	1
TNHEN02	12	3	4	2	1
TNHEN03	10	3	4	2	1
TNHEN04	10	3	4	2	1
TNHEN05	15	3	3	2	1
TNHEN06	12	3	3	2	1
Total Rootstocks:		120	130	78	38

As each scion contains multiple buds and only one bud is needed for grafting, it will be possible to graft more trees than indicated by the number of scions.

5. Discussion

5.1. Overview

The American chestnut, *Castanea dentata*, which once held a distinct presence in the eastern hardwood forest of North America has been dramatically reduced in both form and abundance due to the exotic invasive pathogens *Cryphonectria parasitica* and *Phytophthora cinnamomi* (Anagnostakis, 2001). Chestnut blight, caused by *C. parasitica*, spread rapidly though the entire range of the American chestnut, Maine to Alabama, causing cultural and economic impacts in many communities which relied heavily upon it. The demise of *C. dentata* was so concerning that it spurred political action resulting in the Plant Quarantine Act of 1912, aimed at preventing such a disaster from happening again (Waterworth & White, 1982)

The American Chestnut, having no resistance to these pathogens is now relegated to the understory, primarily persisting as a small tree in a repeating cycle of growth, infection by blight, and die-back (Paillet, 2002). Nevertheless, efforts led by TACF and other research organizations are making progress towards restoring this tree to its former place in eastern North American forests. TACF's backcross breeding program, designed to introgress disease resistance from *C. mollissima* while maintaining American form and genetic diversity, is coming to a head in the BC3F3 progeny (Diskin et al., 2005; Hebard, 2005). Pending selections made by chestnut breeders, this generation of chestnut hybrids offer a promising vision for lofty goals set by the organization. However, this program could be advanced by incorporating vegetative propagation methods to expand and accelerate breeding efforts.

This study has demonstrated how graft propagation may allow the inclusion of individuals previously excluded from the breeding program. As most of the individuals collected in this study were confined to the understory, it is unlikely that they will receive the necessary sunlight to reach sexual maturity (Paillet, 2002). Relying solely on *in situ* conservation of these shade-dominated individuals is not an adequate approach for broadening the genetic base of the TACF breeding program. Additionally, as pathogen pressure increases (particularly by PRR), the loss of these individuals and their alleles are likely if *ex situ* methods are not emphasized. While each individual will be grafted and maintained here at the Fortwood Street greenhouse, scions from each individual were sent to Jim McKenna (Purdue University, Indiana) to be grafted and grown north of the latitude suitable for *Phytophthora cinnamomi* (JH Craddock, Personal Communication, 2018). Maintaining germplasm north of PRR will increase survival and promote longer-term conservation. Having conserved these 33 American chestnut trees through grafting, successful grafts can be grown in conditions that allow flower production. Pollen produced from these container-grown grafted plants can be used in by TACF plant breeders.

As the refugium location during the last glacial event, these ancient southern populations of American chestnut have shown higher genetic diversity and more frequent occurrence of rare alleles (Davis, 1983; Huang, 1994; Kubisiak & Roberds, 2006; Li & Dane, 2013; Perkins, 2016; Shaw et al., 2012). Successful grafts of these 19 (of 33) newly sourced individuals from highly diverse populations may offer novel genetic diversity to the restoration of the American chestnut. The introduction of potentially new, regionally sourced alleles will reinforce the TACF breeding program

and further protect it against interbreeding, while also increasing the effective population size for reforestation efforts (Hebard, 2005).

5.2. Critique of Methods and Limitations

5.2.1. Locating American Chestnuts

This study relied on known locations of naturally occurring American chestnut in the southeastern U.S. They were primarily sourced (10 of 12) through communication with TACF members, while only two were identified by an external source (SERNEC herbarium database). Though these methods proved fruitful, future collections may be expanded to include other data (i.e., GIS modeling) to locate new American chestnut individuals. Additionally, because each location was not visited during the growing season identification was limited to winter characters and proper voucher specimens were not obtained. The consequence of this may be in the accidental collection other species mistaken as *C. dentata* during winter identification. Although this is unlikely for collection sites in Tennessee, areas in Alabama where both *C.* dentata and *C. pumila* occur, such as Ruffner Mountain, Choccoloco Mountain, and Adams' Gap, some individuals collected may be hybrids of the two species. Any collection errors will be mitigated by observing the phenotype of each grafted plant and nontarget species will be removed.

5.2.2. Scionwood Storage

As grafting has not taken place, it remains to be seen whether the scion storage methods could be improved. However, the main obstacle to scion viability is desiccation and at last check, the scions appear to be in good condition. Properly sealed and double-bagged scions, stored at 0^{o} – 1^{o}C should remain dormant, and I do not expect any significant loss of the material collected.

5.2.3. Greenhouse Issues

Another limitation to this study included the loss of functionality of the Fortwood Street greenhouse. This malfunction caused a delay in this study, pushing back the estimated grafting timeline until repairs were made. Although, it may have been warm enough in the greenhouse to reduce dormancy of the rootstocks, burst pipes negated the ability to water. Actively growing plants could not have been watered regularly, thus risking their survival for grafting. It was determined that rootstocks should remain in the nursery, where they would continue dormancy until the greenhouse was operating. However, other actions such as locating another greenhouse or water source could have been taken to prompt active growth and begin grafting sooner. This delay prevents the reporting of graft success in this paper.

5.3. Future Direction

5.3.1. Expanding Collections

This study could be expanded in the future to include predictive GIS modeling to source new American chestnut individuals. While this study was able to collect from trees not utilized in TACF breeding program, most were already known to researchers. In order to better conserve genetic diversity, conservation efforts should be expanded to include new American chestnut individuals. GIS could be used to build prediction models that would allow targeted collection into areas not previously sampled.

5.3.2. Accelerating Flower Induction

Using the methods outlined by Baier et al. (2016), successfully grafted plants could be exposed to a high light environment in order to accelerate flower production. As these grafted plants were collected from mature trees (though not sexually mature), rather than starting from seed as in the Baier et al. (2016) study, flower induction may be accelerated even further. Additional lighting equipment installed in the Fortwood Street greenhouse would offer ideal conditions for studying the effects of increased light and photoperiod on flower production. Pollen produced, as well as female flowers, would also allow for the conservation of cytoplasm of the grafted plants. Additionally, earlier flower production would allow chestnut breeders access to pollen sooner, which may offer more time to conduct costly pollination of other chestnut in the breeding program. Further, growing sexually mature grafted plants in the greenhouse and/or nursery would minimize the logistics required to collect pollen from the field.

5.3.3. Germplasm Conservation Orchards

Increasing the number American chestnut individuals conserved through grafting would allow the concentration of diversity into a germplasm conservation orchard (GCO). Successful grafts from this study, as well as those collected by other means and future collections, could be planted in orchards managed by TACF. Concentrating diversity in a GCO would complement *in situ* conservation and, pending approval by regulators, for the use of OxO transgenic pollination.

6. References

3BUR Proposal for Integrated Research. 2016. TACF Science Oversight Committee. Webpage. https://www.acf.org/our-work/. Accessed 12 March 2018.

Alexander S, Alexander M, Houck J, Craddock JH. 2003. Geographic information systems (GIS) mapping of *Castanea dentata* genetic resources in Tennessee. Southeastern Biology 50(2): 111

Anagnostakis, SL. 1977. Vegetative incompatibility in *Endothia parasitica*. Experimental Mycology 1: 306–316

Anagnostakis, SL. 1987. Chestnut blight: the classical problem of an introduced pathogen. Mycologia, 79(1): 23-37.

Anagnostakis, SL., 2001. The effect of multiple importations of pests and pathogens on a native tree. Biological Invasions 3: 245–254.

Anderson PJ. 1914. The morphology and life history of the chestnut blight fungus. Bulletin 7, Pennsylvania Chestnut Tree Blight Commission, Harrisburg, Pennsylvania. 3-44

Ashe, WW. 1911. Chestnut in Tennessee. Tennessee Geological Survey Bulletin 10-B, Nashville, TN: Tennessee Division of Geology. 35 p.

Baier, K, Maynard C, and Powell W. 2012. Chestnuts and Light. Journal of the American Chestnut Foundation. 3(26): 8-10

Burnham, CR. 1981. Blight-resistant American chestnut: there's hope. Plant Disease, 65, 459-460.

Burnham, CR. 1988. The restoration of the American chestnut. American Scientist.

76(5):478-87.

Burnham, CR, Rutter PA, and French DW. 1986. Breeding blight-resistant chestnuts. Plant Breeding Reviews 4: 347–397.

Chmielewski FM and Rotzer T. 2002. Annual and spatial variability of the beginning of the growing season in Europe in relation to air temperature changes. Climate Research, 19: 257–264.

Craddock, JH and Bassi G. 1993. Nursery application of whip and tongue grafting on chestnut. p. 195-198. *In:* Proceedings of the International Congress on Chestnut. Spoleto, Italy. 20-23 October 1993. Comunita Montana Monti Martani e Serano, Spoleto. 687p.

Craddock, JH and Bassi, G. 1999. Effect of clonally propagated interspecific hybrid chestnut rootstocks on short-term graft compatibility with four cultivars of Italian "Marrone." Acta horticulturae 494 (7): 207

Crandall, BS and Gravatt GF. 1967. The distribution of *Phytophthora cinnamomi*. Ceiba 13(2): 57-78.

Crandall, BS, Gravatt GF, and Ryan MM. (1945) Root diseases of *Castanea* species and some coniferous and broadleaf nursery stocks, caused by *Phytophthora cinnamomi*. Phytopathology. 35:162-180.

Critchfield, WB. 1984. Impact of Pleistocene on the genetic structure of North American conifers. In R. M. Lanner [ed.], Proceedings of the Eighth North American Forest Biology Workshop, 70–118. Utah State University, Logan, UT.

Davis, MB. 1983. Quaternary history of deciduous forests of eastern North American and Europe. Annals of the Missouri Botanical Garden 70: 550–563.

Day PR, Dodds JA, Elliston JE, Jaynes RA and Anagnostakis SL. 1977. Double-stranded RNA in *Endothia parasitica*. Phytopathology 67: 1393–1396

Delcourt, H.R. 1979. Late Quaternary vegetation history of the eastern highland rim and adjacent Cumberland Plateau of Tennessee. Ecol. Monogr. 49: 255-280.

Delcourt, PA, Delcourt HR, Brister RC. and Lackey L. E. 1980. Quaternary vegetation history of the Mississippi Embayment. Quaternary Research 13: 111-132.

Diller, JD, and Clapper RB. 1965. A progress report on attempts to bring back the chestnut tree in the Eastern United States, 1954-1964. Journal of Forestry, 63(3), 186-188.

Diskin, M, Steiner KC, and Hebard FV. 2006. Recovery of American chestnut characteristics following hybridization and backcross breeding to restore blight-ravaged *Castanea dentata*. Forest Ecology and Management 223 (1): 439-447. doi:10.1016/j.foreco.2005.12.022.

Fei, S, Schibig J, and Vance M. 2007. Spatial habitat modeling of American chestnut at Mammoth Cave National Park. Forest Ecology and Management 252 (1): 201-207. doi:10.1016/j.foreco.2007.06.036

Gailing, O. and Nelson, CD. 2017. Genetic variation patterns of American chestnut populations at EST-SSRs. Botany, 95(8): 799-807.

Garner, RJ. 1947. The Grafter's Handbook. London: Faber & Faber.

Gravatt, GF. 1949. Chestnut blight in Asia and North America. Unasylva 3: 3-7

Gravatt, GF and Marshall RP. 1926. Chestnut blight in the southern Appalachians. U.S. Dept. Agric. Circular 370. *In* Anagnostakis, SL. 1987. Chestnut blight: the classical problem of an introduced pathogen. Mycologia, 79(1): 23-37.

Graves, AH. 1926. The cause of the persistent development of basal shoots from blighted chestnut trees. Phytopathology 16: 615-621

Grente J and Sauret S. 1969. L'hypovirulence exclusive, phenomene original en pathologie vegetale. Comptes Rendus Hebdomadaire des Seances de l'Academie d'Agriculture de France, S´erie D 286: 2347–2350 *In* Anagnostakis, SL., 2001. The effect of multiple importations of pests and pathogens on a native tree. Biological Invasions 3: 245–254.

Grente J and Berthelay-Sauret S (1978) Biological control of chestnut blight in France. In: MacDonald WL, Cech FC, Luchock J and Smith C (eds) Proceedings of the American Chestnut Symposium, pp 30–34. West Virginia University, Morgantown, West Virginia

Hebard, FV. 2005. Meadowview Notes 2004-2005. Journal of American Chestnut Foundation 19(2): 16-29.

Huang, H, Norton JD, Boyhan GE, Abrahams BR. 1994. Graft compatibility among chestnut (*Castanea*) species. Journal of American Society of Horticulture Science 119(6):1127–1132.

Huang, H, Dane F, and Kubisiak TL. 1998. Allozyme and RAPD analysis of the genetic diversity and geographic variation in wild populations of the American chestnut (Fagaceae). Am. I. Bat. 85: 10 13- 102

Jaynes, RA. 1979. Chestnuts, p. 124. In: R.A. Jaynes (ed.). Nut trees in North America. Northern Nut Growers Assn.

Jeffers, SN, James FB, and Sisco PH. 2008. Screening for resistance to *Phytophthora cinnamomi* in hybrid seedlings of American chestnut. Clemson University,

Chestnut Return, The American Chestnut Foundation: 1-23.

Keys, RN. 1978. Prospects for vegetative propagation of the genus *Castanea*. In The American Chestnut Symposium (W.L. MacDonald, F.C. Cech, J. Luchok, and C. Smith, eds): West Virginia University, pp 10-16.

Kubisiak, T, and Roberds JH. 2006. Genetic structure of American Chestnut populations based on neutral DNA markers. *In* Restoration of American Chestnut to forested lands. National Park Service, Washington, D.C. pp. 109-122.

Li, X. and Dane, F., 2013. Comparative chloroplast and nuclear DNA analysis of *Castanea* species in the southern region of the USA. Tree genetics & genomes, 9(1): 107-116.

Maurel M, Robin C, Capdevielle X, Loustau D, and Desperez-Loustau ML. 2001. Effects of variable root damage caused by *Phytophthora cinnamomi* on water relations of chestnut saplings. Annals of Forest Science 58: 639–651.

McIlwrick, K, Wetzel S, Beardmore T, and Forbes K. 2000. Ex situ conservation of American chestnut (*Castanea dentata* [Marsh.] Borkh.) and butternut (*Juglans cinerea* L.), a review. The Forestry Chronicle, 76(5): 765-774.

McKay, JW and Jaynes RA. 1969. Chestnuts, p. 281–285. In: R.A. Jaynes (eds). Handbook of North American nut trees. Northern Nut Growers Assn., Knoxville, Tenn.

Merkel, HW. 1905. A deadly fungus on the chestnut. New York Zool. Soc. Tenth Ann. Rep 97-103.

Nixon KC. 1997. Fagaceae Dumortier, Beech Family. in *Flora of North America North*

of Mexico (ed. Flora of North America Editorial Committee), pp. 436-437. Oxford University Press, New York, New York.

Paillet, FL. 2002. Chestnut: history and ecology of a transformed species. Journal of Biogeography 29: 1517–1530

Palmer, JD, Jansen RK, Michaels HJ, Chase MW, and Manbart JR. 1988. Chloroplast DNA variation and plant phylogeny. Ann. Miss. Bot. Gar. 75: 1 180- 1206

Roane, MK, Griffin GJ, and Elkins JR. 1986. Chestnut blight, other *Endothia* diseases, and the genus *Endothia*, St. Paul, MN.

Robinson, A. 2016. Measuring *Phytophthora* resistance phenotypes in segregating testcross families of hybrid American chestnut trees. Honors Theses. https://scholar.utc.edu/honors-theses/57

Rutter PA, Miller G, Payne JA. 1991. Chestnuts (*Castanea*). Genetic resources of temperate fruit and nut crops. Acta Horticult 90:761–788

Santamour, FS, Jr., McArdle AJ, and Jaynes RA. 1986. Cambial isoperoxidase patterns in *Castanea*. J. Environ. Hort. 4:14–16.

Sanz-Pérez, V, Castro-Díez P, and Valladares F. 2009. Differential and interactive effects of temperature and photoperiod on budburst and carbon reserves in two co-occurring Mediterranean oaks. Plant Biology 11(2): 142-151. doi:10.1111/j.1438-8677.2008.00119.x.

Shaw, J, Craddock JH, and Binkley M. 2012. Phylogeny and Phylogeography of North American *Castanea* Mill. (Fagaceae) Using cpDNA Suggests Gene Sharing in the Southern Appalachians (*Castanea* Mill., Fagaceae). Castanea 77(2):186-211. https://doi.org/10.2179/11-033

Soltis, DE, Morris AB, McLachlan JS, Manos PS, and Soltis PS. 2006. Comparative phylogeography of unglaciated eastern North America. Molecular ecology 15(14):4261-4293.

Steiner, KC, Westbrook JW, Hebard FV, Georgi LL, Powell WA, and Fitzsimmons SF. 2017. Rescue of American chestnut with extraspecific genes following its destruction by a naturalized pathogen. New Forests, 48(2): 317-336.

Weber, GC and MacDaniels LH. 1969. Propagation, p. 29. In: R.A. Jaynes (eds). Handbook of North American nut trees. Northern Nut Growers Assn., Knoxville, Tenn.

Wang, BSP, Charest, PJ, and Downie, B. 1993. Ex situ storage of seeds, pollen and in vitro cultures of perennial woody plant species. FAO Forestry Paper, No. 113: 83.

Wang, GG, Bauerle WL, and Mudder BT. 2006. Effects of light acclimation on the photosynthesis, growth, and biomass allocation in American chestnut (*Castanea dentata*) seedlings. Forest Ecology and Management 226:173–180

Waterworth HE and White GA (1982) Plant introductions and quarantine: the need for both. Plant Disease 66: 87–90

Wright, JW. 1976. Introduction to forest genetics. Academic Press. New York. 463p.

Yoshida, K, Schuenemann VJ, Cano LM, Pais M, Mishra B, Sharma R, Lanz C, Martin FN, Kamoun S, Krause J, and Thines M. 2013. The rise and fall of the *Phytophthora infestans* lineage that triggered the Irish potato famine. Elife, 2.

Zhang, X, Nuss DL. 2016. Super mycovirus donors of chestnut blight fungus Proceedings of the National Academy of Sciences 113 (8) 2062-

2067; DOI:10.1073/pnas.152221911