

Synthesis of American chestnut (*Castanea dentata*) biological, ecological, and genetic attributes with application to forest restoration

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Introduction

American chestnut (*Castanea dentata* (Marsh.) Borkh. once occurred over much of the eastern deciduous forests of North America (Russell, 1987), with a natural range exceeding 800,000 km² (Braun, 1950) (Figure 1). *Castanea dentata* was a dominant tree species throughout much of its range, comprising between 25-50% of the canopy (Braun, 1950; Foster et al., 2002; Russell, 1987; Stephenson, 1986). Particularly in the Appalachian region, *C. dentata* filled an important ecological niche (Ellison et al., 2005; Youngs, 2000). The wood of *C. dentata* has a straight grain, is strong and easy to saw or split, lacks the radial end grain found on many hardwoods and is extremely resistant to decay (Youngs, 2000). Historically, *C. dentata* wood served many specialty use purposes including telephone poles, posts, and railroad ties, as well as construction lumber, siding, and roofing (Smith, 2000; Youngs, 2000). Due to the high tannin content, both the wood and bark were used to produce tannin for leather production. The nuts, which are edible raw or roasted, were collected throughout the fall to provide a dietary supplement and were also used as a commodity for sale or trade on U.S. streets (Steer, 1948; Youngs, 2000).

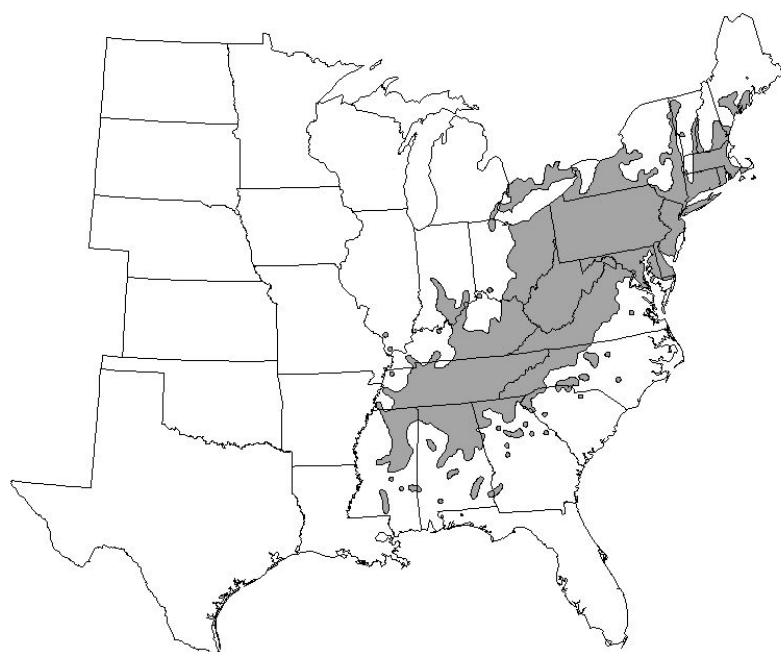


Figure 1: Original natural range of *Castanea dentata* in eastern North America, as adapted from Little (1977).

500 0 500 1000 1500 2000 Kilometers

Chestnut blight disease, caused by *Cryphonectria parasitica* (Murr.) Barr (= *Endothia parasitica* (Murr) Anderson and Anderson) (Anagnostakis, 1987), rapidly annihilated *C. dentata* throughout its range (Roane et al., 1986). The introduced pathogen is thought to have been

imported on *Castanea* spp. seedlings from Asia and was first discovered in 1904 on infected chestnut trees at the Bronx Zoological Park in New York City (Anderson and Rankin, 1914; Murrill, 1906; Roane et al., 1986). By 1950, the disease had spread throughout the range of *C. dentata*, and by 1960 had killed an estimated 4 billion trees; essentially extirpating the species from the canopy (Anagnostakis, 1987; Hepting, 1974; McCormick and Platt, 1980). Since the discovery of chestnut blight, many groups have worked to develop blight-resistant *C. dentata* through diverse strategies including biocontrol of the fungus, breeding and selection of large surviving *C. dentata* trees, inter-species backcross breeding with resistant Asian chestnut species, and genetic modification. Continuing and recent progress in these areas suggest a large-scale re-introduction program is imminent (Diskin et al., 2006; Jacobs, 2007).

Because *C. dentata* disappeared decades before the development of modern principles of forest ecology (Paillet, 2002), our knowledge of basic biological and ecological characteristics of the species is rudimentary (Jacobs, 2007; Paillet, 2002). Much of our understanding regarding establishment and growth of *C. dentata* originates from historical observations or growth of stump sprouts (Paillet, 1982; 1984; 2002). With the successful advancement of *C. dentata* breeding programs leading to the verge of reintroduction, there has been increased prioritization for research examining *C. dentata* establishment and growth in planted and natural forests (Jacobs, 2007). This progress, combined with continued advances in genetic technologies for production of blight-resistant *C. dentata* trees for reintroduction, implicates the need for an updated critical synthesis to aid in further developing protocols for disease resistance breeding and subsequent germplasm deployment. Thus, the purpose of this technical review is to synthesize the current state of knowledge regarding 1) *C. dentata* biology and natural history 2) the development of blight-resistant *C. dentata* trees and 3) the ecology of *C. dentata* pertinent to pending restoration programs. These knowledge areas as well as understanding of their considerable overlap will contribute to the formulation of a viable restoration plan for the ecologically and socially important *C. dentata* (Figure 2).

Part 1: Biology and Natural History

Taxonomy

Castanea dentata belongs to the Beech family, Fagaceae, and the chestnut genus, *Castanea* (Mill.). Three subgenera have been identified: 1) *Castanea* contains *C. dentata*'s closest relatives including the European chestnut (*C. sativa* Mill.), Chinese chestnut (*C. mollissima* Blume), and Japanese chestnut (*C. crenata* Siebold & Zucc.) (Lang et al., 2006); 2) *Balanocastanon* contains two varieties of *C. pumila* (L. Mill.): the Ozark chinkapin (var. *ozarkensis* (Ashe) Tucker) and chinkapin (var. *pumila*) both native to the eastern U.S.; 3) *Hypocastanon* contains only a single species (*C. henryii*) of Asian origin. Within the subgenus, *Castanea*, *C. dentata* is morphologically distinguished from European and Asian species by its larger and more widely spaced saw-teeth on the edges of its leaves (i.e., *dentata*). The two species within *Balanocastanon*, called the chinkapins, (*C. pumila* (L.) Mill.) grow as spreading shrubs or small trees and vary in habitat, range, and susceptibility to chestnut blight.

Castanea dentata is able to outcross with some other *Castanea* species (Jaynes, 1974). Interspecies crosses can be made between all species within both the *Castanea* and *Balanocastanon* subgenera (Jaynes, 1964). Crosses between members from different subgenera are also possible but with lesser success rates. In all interspecies crosses, at least partial incompatibilities (i.e., reduced seed set compared to within species crosses) have been observed between various pairs of trees (Jaynes, 1964). This indicates wide variability within species for factors controlling sexual compatibility. *Castanea dentata* appears most compatible with *C. sativa* with some levels of partial incompatibility observed with *C. mollissima* and *C. crenata*. *Castanea dentata* is sexually compatible with its allopatric chinkapin congener *C. pumila* var. *pumila*, with too little

information on the other chinkapin species to draw conclusions (Jaynes, 1964). In addition, *C. dentata* appears compatible with *C. henryii*.

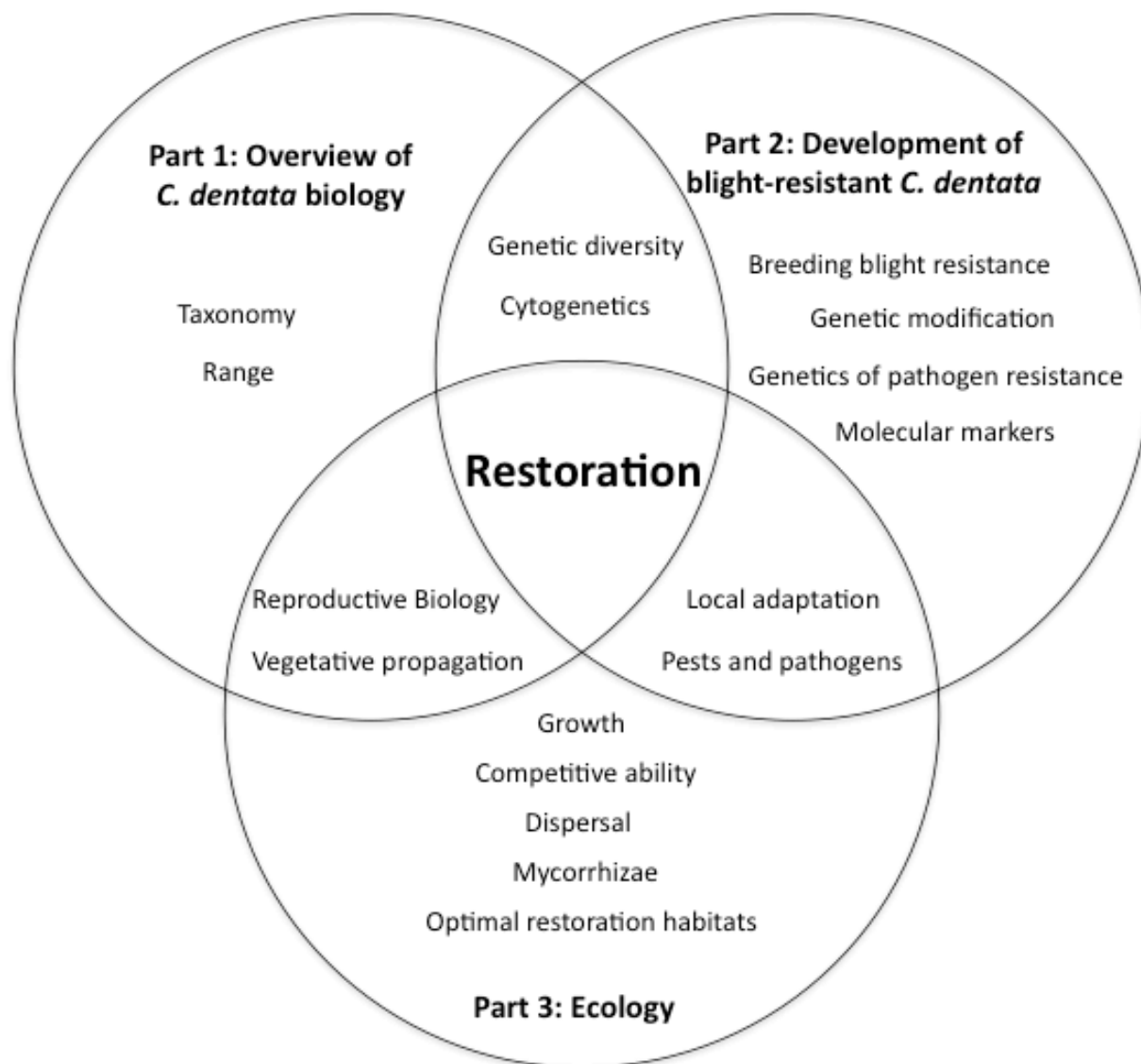


Figure 2: The three parts of this paper correspond to three overlapping spheres of knowledge that will influence the potential success of *C. dentata* restoration.

Historical Range

The pre-blight distribution of *C. dentata* in North America ranged from Mississippi north to Maine, west through Ohio and Tennessee, and north into Ontario (Little, 1977; Russell, 1987) (Figure 1). The species frequently dominated upland habitats composed of non-calcareous, acidic to moderately acidic (pH 4-6), and moist but well-drained sandy soils in mixed forests (i.e., submesic or subxeric sites) (Abrams and Ruffner, 1995; Burke, 2011; Russell, 1987; Stephenson et al., 1991). *Castanea dentata* has also been documented as a lesser component of many forest types, varying in soil characteristics and landscape position (Abrams and Ruffner, 1995; Fei et al., 2007; Whitney and DeCant, 2003). However, the range was notably truncated in areas of high pH, limestone-derived soils (Russell, 1987); additionally, frost sensitivity (Parker et al., 1993) may have limited its proliferation at higher latitudes in some northern forests

(Russell, 1987). Susceptibility to frost may have also restricted its spread in ravines or valleys over portions of its range, though its prominence in riparian zones of pre-blight stands in southern Appalachia has been reported (Vandermast and Van Lear, 2002).

Evidence suggests the dynamic nature of the pre-blight range of *C. dentata* during post-glacial expansion. The range expansion of *C. dentata* during the Holocene from glacial refugia was the most recent of wind-pollinated trees (Paillet, 1982; 2002; Russell, 1987). An outbreak of the introduced soil borne oomycete pathogen, *Phytophthora cinnamomi* Rands, during approximately 1825-1875 may have been responsible for permanently retracting the southern portion of the range of *C. dentata*, which once extended as far south as Florida (Anagnostakis, 2001; Crandall et al., 1945). In the late 1800's most *C. dentata* in the Piedmont region of North Carolina had disappeared, while its natural range was still expanding before the introduction of the blight in other areas (Russell, 1987). For example, *C. dentata* was still spreading northwestward into Michigan at the time of blight introduction (Brewer, 1995).

Castanea dentata is still a common component of eastern North American forests, but nearly all individuals are sprouts that originated from blight-killed trees (Paillet, 2002; Russell, 1987; Stephenson et al., 1991). Cycles of sprouting, infection, dieback, and re-infection may persist for decades (Paillet, 1984), yet sprouts rarely exceed small tree size or grow to reproductive maturity (Paillet, 2002). The species is now classified as endangered in its native range in Canada, as well as in the U.S. States of Kentucky and Michigan; it is listed as being of special concern in Tennessee and Maine.

Reproductive Biology

Castanea dentata is a monoecious, self-incompatible species (Clapper, 1954a; Russell, 1987) that generally flowers from June to July (Horton, 2010; Paillet, 2002). Trees have been reported to begin flowering after only 8-10 years (Zon, 1904), though plantation-grown stock can begin flowering much earlier. The male and female inflorescences differ, with males being unisexual and proximally located on the shoot and the females being bisexual (i.e., pistillate proximal, staminate distal) and distally located on the shoot (Jaynes, 1974). Although the male inflorescence has characteristics of an insect pollinated form, most evidence (Clapper, 1954a) supports that wind-pollination is the primary mechanism (Jaynes, 1974). Flowering after leaf out reduces the dissemination distance of chestnut pollen compared to other spring-flowering, wind-pollinated species (Paillet, 2002). In addition pollen release typically occurs in two phases, effectively extending pollination time, with the unisexual inflorescences releasing pollen somewhat before female receptivity and the bisexual inflorescences somewhat after receptivity (Clapper, 1954a). Self-incompatibility and short distance of pollen dissemination requires that trees be within about 100 m of each other for successful pollination (Paillet, 2002). Fertilization produces one to three large nuts encapsulated in a spiny bur (i.e., involucre).

Nuts of *C. dentata* possess several unique characteristics. The nuts themselves have thin shells. Although formidable, the burr only protects the seeds until they are ripe and then opens widely, making the nuts readily available (Steele et al., 2005). While acorns, hickories, and walnuts all contain a higher percentage of lipids, *C. dentata* nuts have a higher percentage of carbohydrates and much lower levels of tannins (Steele et al., 2005). Higher carbohydrates combined with lower tannin likely made *C. dentata* nuts sweeter and more palatable than acorns as well as a better protein source (Steele et al., 2005). Diamond et al. (2000) present estimates of average *C. dentata* nut production rates at 28 kg m⁻² tree basal area. Data indicate that nut production was much more consistent from year-to-year in *C. dentata* than in many oak (*Quercus* L.) species (Dalglish and Swihart, *in press*). Such consistent seed crops are likely a result of summer flowering because flowers are not susceptible to late-spring frosts (Horton, 2010). Regular nut production, lack of defenses against consumption, and young age to nut production all indicate that *C. dentata* was a key resource for wildlife.

Heavy seed consumption by wildlife, insects, and livestock likely limited seedling establishment (Dalglish et al., in review; Steele et al., 2005). Thus, sexual reproduction may have contributed only nominally to historical regeneration of *C. dentata*, suggesting that regeneration success may have largely been dependent upon its capacity to sprout vigorously from the root collar following disturbance (Paillet, 2002; Russell, 1987). Sprouts have been reported to reach 2-3 m height in the first year and trees aged > 100 years still commonly retain sprouting ability (Russell, 1987). Thus, even when reproduction by seed is limited or absent, *C. dentata* can maintain itself in a stand and even increase in volume and density through sprouting (Paillet, 2002). Historically, foresters noted the rarity of *C. dentata* reproduction by seed and specifically designed silvicultural operations to promote *C. dentata* regeneration by sprouting (see citations in Paillet, 2002).

Vegetative Propagation

Castanea dentata is difficult to vegetatively propagate, with only limited success achieved using various techniques (Cummins, 1970; Elkins et al., 1980; Keys, 1978) including softwood and hardwood rooted cuttings, ground- and air-layering, grafted scions on seedling or sapling rootstocks, rooted micropropagules (i.e., microcuttings), (e.g., Keys and Cech, 1982; Serres et al., 1990; Xing et al., 1997) and germinated somatic embryos (e.g., Merkle et al., 1991; Xing et al., 1999). Problems with rooting cuttings (both macro- and micro-propagation) can be circumvented by using juvenile source plants instead of more mature plants, or with stump sprouts instead of shoots from the higher parts of the tree (Sanchez and Viéitez, 1991). Serially grafting onto juvenile rootstocks as a means to rejuvenate mature *Castanea* genotypes has shown limited and only short-term (i.e., transient) positive effects (Giovannelli and Giannini, 2000). Stooling seedling stock plants (i.e., macrottage) (Solignat, 1964) and inarching (Jaynes, 1961) are two other rooting techniques that have been used in various situations. Splice grafting seems to work best compared to whip, cleft and side grafts (Nienstadt and Graves, 1955) but in all methods matching sizes of rootstocks and scions is important as well as using closely related rootstocks and scions. Using juvenile rootstocks for mature scions in which the rootstocks are progeny of the ramet being propagated is recommended (McKay and Jaynes, 1969), although successful grafts can be made using scion and rootstocks of unrelated genotypes and even different species (Clapper, 1954a). More recently, bark grafting for propagating *C. dentata* scions onto juvenile *C. dentata* rootstocks has achieved up to 10% and 50% success rates for mature and juvenile scions, respectively (Elkins et al., 1992). Current state-of-the-art methods including micropropagation and somatic organogenesis and somatic embryogenesis are summarized by Viéitez and Merkle (2004) and Maynard et al. (2008); these methods offer increasing potential for large scale propagation of *C. dentata*, especially if starting with juvenile explants.

Genetic Diversity and Population Structure

Studies have estimated genetic diversity in *C. dentata* and *C. mollissima* (the primary species used in backcross breeding efforts) using protein (isozymes) and non-coding (i.e., neutral) DNA markers (Dane et al., 1999; Huang et al., 1994; Huang et al., 1998; Lang and Huang, 1999; Tanaka et al., 2005; Villani et al., 1991). Isozyme studies show *C. dentata* to contain low to moderate levels of genetic diversity relative to other species with large geographic ranges and similar life history traits (Dane et al., 2003). In most direct comparisons with other *Castanea* species *C. dentata* exhibits the least diversity (0.151-0.183, range mean expected heterozygosity over loci) and *C. mollissima* the most (0.305-0.311). It remains unclear whether the low genetic diversity (as measured by expected heterozygosity) predisposed *C. dentata* to rapid population decline in response to the blight epidemic or whether it is a consequence of blight-induced population decline (Dane et al., 2003). An apparent consequence of the blight and *C. dentata*'s resiliency through stem-collar sprouting is the moderately high level of observed heterozygosity relative to what might be expected for such decimated populations. The most persistent genotypes tend to be more heterozygous than average although seedling reproduction is rare due to the blight

and other competing environmental factors (Stilwell et al., 2003). Another possible source of persistence is somatic mutation towards blight resistance, because the source of the sprouts is comprised of only a few cells (Anagnostakis and Hillman, 1992).

Most of the genetic variation observed in *C. dentata* resides within populations (>~90% for isozymes; >~95% for DNA markers), with evidence of clinal trends in overall allele diversity and allele frequencies for some loci (Huang et al., 1994; Kubisiak and Roberds, 2006). More isozyme diversity is apparent in the southern parts of the *C. dentata* and *C. mollissima* ranges, with amounts gradually declining to the north. Two exceptions to this general pattern include less diversity found in some lower and intermediate latitude populations of *C. dentata* (Huang et al., 1998) and higher diversity found in *C. mollissima* populations in the Changjian River region, Shennongjia district (Huang et al., 1994; Lang and Huang, 1999). Two apparent clinal trends in allele frequencies have been noted in *C. dentata*, suggesting the possibility of two glacial refugia (Huang et al., 1998)—one south of the Appalachian spine (towards the Gulf of Mexico) and one to the east of the southern part of the Appalachians (towards the Atlantic Ocean). Additional neutral DNA markers and population sampling strongly support the southwest to northeast clinal trend in decreasing genetic diversity with no indication of regional boundaries (Kubisiak and Roberds, 2006). This study also found low but positive correlations between genetic and geographic distances, suggesting that *C. dentata* was a single metapopulation established by high gene flow and genetic drift and is apparently maintained by persistence of a large sample of pre-blight genotypes.

Local Adaptation

Although gene diversity studies have measured genetic variation in neutral allele frequencies over large areas of the *C. dentata* native range and found little genetic structure (Huang et al., 1998; Kubisiak and Roberds, 2006), essentially no information is available on geographic variation or genetic structure within adaptive traits such as bud flushing date, cold tolerance, or growth rate (Steiner, 2006). General patterns from species that share many life history and geographic range characteristics with *C. dentata*, can be tentatively applied to *C. dentata*. For example, trees from colder climates tend to flush leaves later, be more cold tolerant, and produce less stem wood per growing season than trees from warmer climates. The strength of the relationships varies from strong to moderate to weak, respectively, for these three traits. For bud flush, clinal gradients were detectable down to 100-300 km and pollen shed responded similarly (Steiner, 2006). Pollen shed for *Castanea* spp. at Glenn Dale, Maryland, (N38°59' W76°49') was strongly affected by spring and early summer temperatures, with warmer temperatures generally causing earlier flowering (Clapper, 1954b). However, nut drop date was not affected by spring or summer temperatures, but was a fixed feature of genotype. From a genetic standpoint, earlier flushing was dominant to later flushing and length of nut maturation period was additive, with progeny values being intermediate to parents flowering (Clapper, 1954b). Accordingly, one would expect the early flushing genotypes observed at Glenn Dale to be from colder climates, but sample size was not sufficient to test this hypothesis.

Although examples of short-range (<100 km) adaptation are rare, the case of cold tolerance and pitch pine (*Pinus rigida* Mill.) illustrates a precaution against broad generalizations. In this case, *P. rigida* families from colder and warmer locales within 8 km of each other were shown to have significantly different cold tolerance levels (Steiner and Berrang, 1990). Substantial within population variation in growth rate exists, creating the observed weaker trends in which trees from warmer climates grow faster in common gardens than those from colder climates. Similar patterns have been shown for wide ranging pine species, such as loblolly pine (*Pinus taeda* L.) and longleaf pine (*P. palustris* Mill.) in the southern United States (Schmidting, 1994; Schmidting and Sluder, 1995): warmer climate-adapted sources suffer increased mortality and

slower growth in colder common garden experiments. Understanding the limits of seed source movements relative to climate is critically important for restoring *C. dentata*. Lacking empirically-derived genetic information, the logical “rule of thumb” is that adaptation is driven primarily by average minimum winter temperatures as is implemented in the USDA Cold Hardiness Zones and for forest trees such as the southern pines (Schmidting, 2001).

Cytogenetics

Castanea spp are diploid with haploid (n) and monoploid (x) numbers of 12 chromosomes ($2n=2x=24$) (Jaynes, 1962). Estimates of the genome size of *C. sativa* include 0.98 pg (943 Mbp) (Barow and Meister, 2003) and 0.81 pg (774 Mbp) (Kremer et al., 2010) per 1C or haploid content, making the average chromosome length around 70 Mbp (or about one-half the size of the *Arabidopsis* genome). Genome size estimates for *C. dentata* and *C. mollissima* (Kremer et al., 2010) are closer to the lower figure for *C. sativa* and a whole genome sequencing project for *C. mollissima* is underway (J. Carlson, personal communication). Standard root tip (mitotic) cytology has been practiced for some time (e.g., Jaynes, 1962; McKay, 1942), but only recently have techniques, including fluorescent in situ hybridization (FISH), improved to the point of developing chromosome-specific karyotypes. Chromosomal locations of the ribosomal DNA loci (18S-26S and 5S) have been established as well as the presence of the *Arabidopsis* telomere repeat sequences (Islam-Faridi et al., 2009). Anthers and their microspore mother cells are extremely small (pollen grain diameter ~ 14 μ m) making meiotic stage cytology difficult (Dermen and Diller, 1962; Jaynes, 1962), although recent progress has been made with *C. mollissima* \times *C. dentata* hybrids (Islam-Faridi, unpublished data). Evidence for translocations and inversions with respect to these two species were suspected based on genetic linkage map data (Kubisiak et al., 1997; Sisco et al., 2005) and supported by species crossability studies (Jaynes, 1962) and recent cytogenetics (Islam-Faridi et al., 2008). Further resolution is needed to determine the effect of these rearrangements on the ability of interspecies backcross breeding programs to introgress *C. mollissima* resistance genes into *C. dentata* (Ellingboe, 1994). Some isozyme loci are present in *C. mollissima* and absent in *C. dentata* and vice versa (Dane et al., 2003), suggesting that post-divergence deletions and insertions will provide additional genetic variation within interspecies backcross breeding populations (described below).

Part 2: Development of Blight Resistance

Chestnut Blight Disease

Cryphonectria parasitica, a filamentous ascomycete fungus, is a necrotrophic pathogen that incites the disease, chestnut blight (reviewed by Anagnostakis, 1987). The pathogen infects primarily through wounds on stems. Once established as germinating conidia or ascospores (or mycelial plugs in artificial inoculation), the fungus grows rapidly through the bark and colonizes the cambial zone (Beattie and Diller, 1954). Resistant reactions slow this growth, maintaining the fungus in a superficial canker (Griffin et al., 1983). Susceptible reactions continue development unimpeded, encircling the stem and causing vascular dysfunction, resulting in death of distal tissues and stem dieback.

Resistance reactions are thought to be primarily chemical, where mycelial fans are unable to develop and grow rapidly in resistant trees (Griffin et al., 1983). More susceptible reactions allow the fungus to develop to the reproductive stage in which two types of spores can be formed to cause additional infections and expand the epidemic (Beattie and Diller, 1954). Conidia are single-celled spores, produced asexually and thus carry the same haploid genotype as the parental culture (thallus). They are formed within pycnidia from which they are extruded in a gummy paste (cirrhous) and are efficiently transported through water or animal (insects, mites, birds, mammals) movement (Anagnostakis, 1987). Conidia can serve as vegetative propagules when infecting wounds on chestnut stems or serve as donor gametes (spermatia) when mating with fruiting bodies (protoperithecia) of sexually compatible genotypes.

Fertilization results in the formation of dikaryotic ($n + n$) ascus initials within the perithecium where diploidization ($2n$) occurs followed by meiosis and one mitoses leading to eight (two per meiotic product) ascospores (n) per ascus (Marra and Milgroom, 2001). The ascospores subsequently undergo another mitosis, becoming two celled. Ascus contents are forcibly ejected into the air and wind-disseminated to fresh wounds where they may cause new infections. Conidia can form within one month of infection and ascospores by four months, resulting in rapid spread of the disease. Because it forms a perennial canker, the fungus has ample opportunity to sporulate whenever temperatures are above freezing. Conidia can persist at least one year in soil and the net result is persistence over winter and other harsh environments.

The mating system of *C. parasitica* is bipolar (one locus, two alleles (MAT-1, MAT-2)) self-incompatible (i.e., heterothallic) (Marra and Milgroom, 2001). However, the genetic basis of self-incompatibility is not entirely clear and exceptions occur and have been observed in both the laboratory and field (Marra et al., 2004). In these cases of mixed mating both self- and cross-fertilization occur, providing *C. parasitica* with additional opportunity for successful reproduction and continued disease development. Vegetative (or heterokaryon) incompatibility is commonly observed between *C. parasitica* cultures (Cortesi and Milgroom, 1998). The genetic basis for this is fairly well understood with several vegetative incompatibility (vic) genes identified and mapped (Anagnostakis, 1982; Cortesi and Milgroom, 1998; Kubisiak and Milgroom, 2006). *Cryphonectria parasitica* genotypes are essentially vegetatively incompatible (anastomosis prevented) when any one of the vegetative incompatibility (vic) genes does not match, although exceptions occur such as epistasis (Huber, 1996). Such incompatibility effectively limits hyphal anastomoses and the potential for cytoplasmic transfer including the transmission of virulence-attenuating (hypovirulent) mycoviruses (discussed below).

Approaches to Control Chestnut Blight

Over the decades, scientists have pursued three genetic approaches to control blight disease: i) biological control, inoculation of *C. dentata* with hypovirulent strains of the blight fungus; ii) breeding *C. dentata* using both intra- and inter-species methods; and iii) genetic modification of *C. dentata* using genes having resistance-like properties. Each of these approaches will be reviewed below. Some have argued that successful restoration of *C. dentata* will require a combination of approaches combined with sound silvicultural practice. For example, Griffin (2000) promotes combining appropriate site selection and optimal silviculture to minimize stress on *C. dentata* with trees selected through natural variants, breeding or engineering for partial blight resistance along with hypovirulence treatments, or restricting planting to areas harboring less virulent strains of *C. parasitica*. Only time will tell how these different approaches will individually and collectively contribute to establishing and increasing *C. dentata* populations capable of surviving and sexually reproducing in contemporary eastern forests.

Biological Control with Hypovirulence

Hypovirulence is the reduction in (attenuation of) virulence of the blight fungus caused by a mycovirus in the family Hypoviridae (Milgroom and Cortesi, 2004). *Cryphonectria parasitica* strains that are infected with these hypoviruses will create superficial or 'healing' cankers that are not lethal for the tree (Griffin, 2000; Milgroom and Cortesi, 2004). In many areas of Europe, hypovirulence has effectively controlled blight spread (Griffin, 2000; Milgroom and Cortesi, 2004). Hypoviruses in Europe have dispersed naturally and through management that directly inoculates cankers, though it remains unclear whether human-aided deployment has significantly increased the dispersion of hypoviruses (Milgroom and Cortesi, 2004). In different areas of Europe, *C. sativa* is managed either for coppice forests for timber or nut orchards, or some combination of both (Milgroom and Cortesi, 2004). The incidence of blight infection, hypovirulence, and tree mortality all vary with management, environmental conditions and the age of the trees within a stand or orchard (Milgroom and Cortesi, 2004).

The discovery of hypoviruses affecting blight cankers in *C. dentata* populations outside the native range in Michigan fueled hopes for using hypovirulence to control blight throughout North America (MacDonald and Double, 2006). Between the 1970s and 1990s, several attempts were made to use hypoviruses for biocontrol, with limited success in plantation settings in Connecticut and Virginia (Griffin, 2000) and in a natural stand in Wisconsin (Milgroom and Cortesi, 2004). Michigan remains the only success story for hypovirulence in North America: in some Michigan populations trees grow large with only few healing cankers and reproduce via seed (Milgroom and Cortesi, 2004). However, while blight control at the individual canker level with hypoviruses is often highly successful, in most North American *C. dentata* stands where biocontrol has been tried, viruses fail to spread among trees and sometimes even among cankers within a tree, severely limiting the use of mycoviruses as biocontrol agents (Griffin, 2000; MacDonald and Double, 2006; Milgroom and Cortesi, 2004). While vegetative incompatibility of the fungus is often cited as the mechanism preventing hypovirus spread, many questions remain concerning the environmental and biological conditions necessary to promote the establishment and spread of hypoviruses (Milgroom and Cortesi, 2004).

Breeding for Blight Resistance

Chestnut breeding in the eastern U.S. began as early as 1894 with work at Beltsville, Maryland (van Fleet, 1914). The USDA breeding program began under van Fleet in 1909 in direct response to the chestnut blight epidemic, with an important experimental test site at Glenn Dale, Maryland, added in 1911. The primary goal of the USDA breeding program was producing blight resistant forest trees for timber, tannins, and wildlife as well as orchard trees for nuts (Clapper, 1954a). Van Fleet first observed blight in his material in 1907, causing him to terminate work on *C. dentata* (= *C. americana*) and concentrate on Asian chestnuts and chinkapins. By 1925, the USDA program was being led by G.F. Gravatt and R.B. Clapper, when Clapper's first *C. dentata* × *C. mollissima* hybrid crosses were made utilizing materials collected in Asia by R.K. Beattie (Beattie and Diller, 1954; Diller and Clapper, 1965). Clapper led the program through 1949 when F.H. Berry and J.D. Diller assumed responsibility. In 1960 the USDA program was discontinued; some materials were transferred to the Connecticut breeding program (Berry, 1978).

Chestnut breeding work at the Connecticut Agricultural Experiment Station (CAES) began in 1930 with A.H. Graves working at the Brooklyn Botanical Garden and conducting field tests near Hamden, Connecticut, through 1962. Following Graves in 1962, R.A. Jaynes led the CAES breeding program until 1983 when S.L. Anagnostakis assumed responsibility through to the present (<http://www.ct.gov/caes/cwp/view.asp?a=2815&q=376752>). Work at CAES was highly collaborative with the USDA program, using similar strategies of species hybridization and resistance testing in anticipation of finding and cloning the ideal combination of resistance from Asian chestnut species and fast growth and forest tree form from *C. dentata*. One extensive forest test planting of CAES hybrid material was made between 1969 and 1975 at the Lesesne State Forest in Virginia (Jaynes and Dierauf, 1982). Most trees planted were open-pollinated seeds/seedlings of selected first- and second-generation hybrid parents (with resistance sources from *C. mollissima* and *C. crenata*), thus comprising third and fourth generation trees where selection for blight resistance had been practiced. By 1980, eleven of the nearly 12,000 planted trees were selected and propagated into two orchards in Connecticut and Virginia. However, Jaynes and Dierauf (1982) concluded that adequate field resistance was not obtainable among trees that are predominantly (presumably >50%) *C. dentata*. Later, Anagnostakis (2001) found this strategy limiting in terms of producing timber quality forest trees and is now actively backcrossing both *C. mollissima* and *C. crenata* sources of resistance to *C. dentata* as originally outlined by Burnham et al. (1986), discussed below.

In the early 1980s, a backcross breeding program was proposed to introgress blight resistance genes from Asian chestnuts into *C. dentata* (Burnham, 1981; Burnham et al., 1986). The specific steps include making three backcross generations with selection for resistance at each generation to ensure retention of Asian resistance genes, intercrossing the selected BC₃ trees to produce BC₃F₂ populations fully segregating (i.e., all of homozygote and heterozygote classes) for resistance, selecting in the BC₃F₂ populations for high resistance (i.e., tree being homozygous for the Asian alleles at all resistance genes), and establishing the selections in seed orchards to produce planting stock for forest planting. In this selection program, two types of orchards are maintained, isolated from each other—Type A and Type B. In the Type A orchard only backcross progeny are grown, exposed to blight, susceptible trees removed, resistant (i.e., moderately resistant due to heterozygous state of resistance genes) trees used for control-crossing to *C. dentata* to form next generation backcross or open-pollination among selected backcrosses to produce segregating F₂ population. Seeds from the open-pollination in the Type A orchard are planted in the Type B orchard and again exposed to blight, susceptible and intermediate resistant trees are removed, highly resistant trees are allowed to open-pollinate each other to produce highly resistant backcross bred *C. dentata* nuts for planting in the forest.

The American Chestnut Foundation (TACF) was founded to breed *C. dentata* capable of surviving and reproducing in the forest using the backcross breeding method proposed by Burnham (Burnham et al., 1986; Ellingboe, 1994). Several lines from both the USDA and the CAES breeding programs served to jump-start TACF's breeding program. Prior to closing the USDA program, Clapper and Diller established two wide-ranging series of test plots (1936-1939 and 1947-1955) of *C. mollissima* and various first- and second-generation hybrids, including material from the CAES program (Berry, 1980; Diller and Clapper, 1969; Diller et al., 1964). A few of the individual backcross chestnut trees survived and grew well in the test plot in southern Illinois (Crab Orchard Wildlife Refuge) with the best tree being cloned by grafting and eventually named 'Clapper' (Clapper, 1963; Little and Diller, 1964) (ancestry *C. mollissima* seedling M16 selected at Glenn Dale, Maryland, from PI 34517, Tianjin, China and *C. dentata* FP.555 used as grandparent and parent). Additional important named *C. mollissima* selections included 'Crane', 'Kuling', 'Meiling', 'Nanking', and 'Orrin' (Berry, 1978). Similar to the USDA program's 'Clapper' tree, CAES produced and identified a highly desirable BC₁ named 'Graves' (ancestry *C. mollissima* seedling 'Mahogany' selected by A. H. Graves at Hamden, Connecticut, from PI 70315, northeastern China, *C. dentata* FP.551, pollen received from Bell, Maryland, and a *C. dentata* tree from Clinton Corners, New York, used as grandparent and parent, respectively). A. H. Graves made the M16 x FP.551 F₁ cross and H. Neinstaedt selected the F₁ parent tree at Hamden and made the backcross (see Burnham et al., 1986, for a detailed summary of all crosses made in both CAES and USDA programs and their performances in various tests). Hebard (1994; 2006) describe the maturation of the TACF breeding program (also see www.acf.org/r_r.php), including breeding, planting, growing, and inoculating techniques.

There are many important features of the backcross breeding program associated with genetics, plant breeding, and restoration (Burnham et al., 1986). For example, it is important to use many unrelated *C. dentata* trees at each generation to properly sample the native species alleles. Parent trees should originate within the region where the progeny trees will be planted to promote local adaptation. Recent evidence for uncertainty regarding cold tolerance of hybrid-backcross chestnut used in breeding programs for reintroduction in the northeastern U.S. (Gurney et al., 2011) emphasizes the importance of adaptation for successful reintroduction. In addition, sources of resistance should include parent trees of both *C. mollissima* and *C. crenata*, because it is likely that trees within and among species will carry different resistance genes. These features are especially important when breeding many locally adapted populations to reintroduce and restore a wide-ranging species (Worthen et al., 2010). To achieve these goals, multiple *C. mollissima* genotypes are being used as resistance sources, with several being advanced to the BC₂ and BC₃ stages. The basic plan of using 20 sets (i.e., recurrent lines or lines) of four unrelated

C. dentata trees as parents (producing the F₁, B₁, B₂, and B₃ generations) with each *C. mollissima* resistance source has proven to have substantial practical limitations, because the *C. dentata* individual serving as a parent typically dies before enough flowers and progeny can be produced. Thus, most of the lines contain more than four *C. dentata* parents, providing the potential for additional genetic diversity among the selections.

Leffel (2004b) provided a thorough discussion of additional breeding techniques and methods to produce blight-resistant *C. dentata*. Some can be considered modifications of the basic backcross breeding plan, while others appear novel to *C. dentata*. The modifications are aimed at making the backcross breeding more efficient by utilizing naturally selected BC₂F₂ trees to make the BC₃ generation and/or using cytoplasmic male sterility (CMS) to produce the backcross generations. The former modification allows for much smaller backcross families, as all BC₃ trees should be equally resistant being heterozygous for most if not all resistance genes. Allowing these BC₃ trees to intercross provides a BC₃F₂ generation that can be planted in seed orchards at close enough spacing to allow natural blight infections to cull the less than fully resistant trees. The selected trees should then be mostly homozygous for resistance, providing resistant planting stock for forest planting. Leffel (2004a) provides information suggesting that male sterility is controlled by a cytoplasmic and a nuclear factor and that *C. mollissima* × *C. dentata* F₁ trees are male fertile while the reciprocal crosses are male sterile. If this proves correct, male sterile hybrids and backcrosses can be selected allowing surrounding *C. dentata* to open-pollinate to provide the next generation of BC seeds. Allowing natural selection for blight resistance further reduces the workload. A third modification specifically recommends using *C. mollissima* and *C. crenata* as sources of resistance and crossing to avoid male sterility. But instead of backcrossing, the program proceeds as follows: 1) F₁ selected for blight resistance and open-pollination to produce F₂, 2) select for blight resistance in F₂ and allow open-pollination to produce F₃, and 3) plant F₃ in seed orchard at close enough spacing to allow for natural selection for most resistant genotypes. These trees should breed fairly true for resistance and can be used to produce planting stock for forest planting.

Many workers have noted low levels of blight resistance at very low frequencies in naturally-occurring populations of *C. dentata*, suggesting that if this phenotype is genetically based then it should be possible to use within species recurrent selection and breeding to produce populations with resistance levels adequate for forest planting (Griffin et al., 1983). The American Chestnut Cooperators Foundation (ACCF) is actively pursuing an intra-species recurrent selection and breeding program starting with a sizeable base of large surviving American (LSA) chestnut trees (<http://ipm.ppws.vt.edu/griffin/accf.html>). Breeding programs in Tennessee (Thor, 1978), West Virginia (Given and Haynes, 1978), and Virginia (Griffin, 2000) are committed to identifying large surviving *C. dentata* trees, screening their progeny (open- and control-pollinated) for resistance, selecting and grafting the most resistant progeny for producing improved seed orchards and breeding parents for another cycle of screening and selection. An attempt at mutation breeding was carried out by various individuals and organizations starting in 1956 and running into the 1970s (Burnworth, 2002; Dietz, 1978). Native *C. dentata* seeds were irradiated with gamma radiation (3000 rads) and then planted to evaluate their phenotypes. Seeds were collected from selected first mutant generation (M₁) trees and planted to produce a M₂ generation. Some of these on Sugarloaf Mountain near Dickerson, Maryland, have shown potential for blight-resistance (D.W. Fulbright and W.L. MacDonald in Burnworth, 2002). A breeding program to continue working with these trees was initiated in 2002 by the American Chestnut Research Foundation sponsored by Stronghold, Inc. (Burnworth, 2002).

Genetic Modification

It has been argued that the first application of genetically modified organisms (GMO) in forest trees will be for restoration of species decimated by invasive pathogens or pests (Adams et al.,

2002; Merkle et al., 2007). *Castanea dentata* certainly falls into this category and much progress has been made in developing the prerequisite technologies for genetic modification (GM). *In vitro* propagation in *Castanea* spp. was studied over decades in Spain with results summarized by Viéitez and Merkle (2004) and Maynard et al. (2008). Key achievements include derivation of somatic embryogenic cultures from seedling leaf explants (Corredoira et al., 2003) and stable gene transformation using *Agrobacterium* co-cultivation with leaf-derived embryogenic cultures and eventual plantlet formation (Corredoira et al., 2004). Work in *C. dentata* has progressed through similar stages under long-running programs at the University of Georgia (UGA) and the State University of New York, College of Environmental Science and Forestry (SUNY-ESF). In the UGA program, somatic embryogenic cultures were matured into cotyledon-stage embryos (Merkle et al., 1991) and stably transformed embryogenic cultures were obtained using biolistics (Carraway et al., 1994). This was followed by plantlet formation *in vitro* (Carraway and Merkle, 1997), but survival of trees through acclimation and transfer to greenhouse was not achieved for several more years (Robichaud et al., 2004). Later, application of suspension culture and other cultural changes resulted in 100-fold improvement in efficiency of plantlet formation (Andrade and Merkle, 2005). Use of antibiotic selection in suspension cultures following co-cultivation of embryogenic cultures with *Agrobacterium* led to production of transgenic *C. dentata* plants that grew to the male flowering stage (Andrade et al., 2009). In the SUNY-ESF program, plantlets derived from somatic embryogenic cultures were successfully produced and transferred to the nursery (Xing et al., 1999) and stably transformed cultures and plantlets were produced using *Agrobacterium*-mediated transformation of an antifungal gene (Polin et al., 2006; Rothrock et al., 2007).

Obtaining blight resistant *C. dentata* plants through GM, followed by crossing those plants to a wide array of *C. dentata* trees to produce a blight-resistant, genetically-variable population for reforestation is the goal of the program at SUNY-ESF (W.A. Powell, personal communication). Substantial progress has been made in designing and selecting small proteins, with antimicrobial activity against *C. parasitica* and other necrotrophic pathogens, while showing little or no toxicity to *Castanea*, *Malus*, or *Salix* pollen (Powell et al., 1995; Powell et al., 2000; Powell and Maynard, 1997; Powell et al., 2006). This suggests a potential path forward in engineering pathogen resistance for plants as demonstrated in transgenic poplar with enhanced resistance to the necrotrophic pathogen *Septoria musiva* (Liang et al., 2002). Another promising lead for chestnut blight resistance is the oxalate oxidase gene (*OxO*) (Polin et al., 2006; Welch et al., 2007). Oxalate production has been shown to be a significant *virulence* factor in the blight fungus, *C. parasitica* (Chen et al., 2010; Havir and Anagnostakis, 1986). The *OxO* gene, when transformed into poplar, provides increased tissue tolerance to oxalate and enhanced resistance to *S. musiva* (Liang et al., 2001). Co-transformation of two or three genes is a strategy that may prove useful where post-transformation breeding is required. In *C. dentata* this is routinely accomplished with three genes-- a visual selectable marker (such *GFP*), antibiotic resistance (such as *nptII*) for selection in culture, and the candidate resistance gene (Newhouse et al., 2010; W. A. Powell, personal communication). Because the marker and selection genes are not linked to the resistance gene, they can be removed from the segregating breeding population while progeny inheriting only the candidate resistance genes are maintained. Although potentially useful, co-transformation has limitations such as high variation in gene expression and gene silencing (see Halpin et al., 2001). One way around these limitations is the co-expression of multiple genes in a single open reading frame (i.e., Liang et al., 2005).

Another important consideration for GM trees is the source and tissue specificity of resistance genes and their promoters and regulators. Researchers are identifying and isolating candidate resistance genes from the relatively resistant *C. mollissima* (Forest Health Initiative, FHI, unpublished data) and efforts to clone promoters from *C. dentata* have been successful (Connors et al., 2002). Within the FHI, candidate genes are identified by their presence in genomic regions identified as QTLs for resistance, their up-regulation in inoculated vs. non-inoculated stems in

C. mollissima, and their presence or absence in suppressive subtraction hybridization (SSH) libraries (Baier and Powell, personal communication) and transcriptomic screens (Barakat et al., 2009). One such gene of interest is a laccase gene that is highly expressed in *C. mollissima* stem tissues and very lowly expressed in *C. dentata*. It also appears that this gene maps to a blight resistance QTL and as such is considered a candidate resistance gene. Utilizing genes from a closely related species in GM, so called intragenics (including cisgenics), has similarities to interspecies backcross breeding (Schouten and Jacobsen, 2008) and may offer new opportunities for restoring species on the verge of extirpation. An example is *Tsuga canadensis*, where the exotic hemlock woolly adelgid (*Adelges tsugae*) is decimating the species and no crossable species with resistance exists. However, non-crossable congenic species with co-evolved resistance do exist, e.g., *T. chinensis* (see Montgomery et al., 2009) and offer hope for a form of intragenic technology to intervene on behalf of *T. canadensis*. A recent intragenesis example in poplar, the model forest tree for genetic transformation, utilized genomic copies (i.e., cisgenes) of five protein-encoding genes (involved in gibberellin metabolism or signaling) to demonstrate increased genetic variation in growth and wood anatomy traits, including variants that showed either faster growth with no change in wood fiber quality or higher fiber quality with no change in growth rate (Han et al., 2010).

Molecular Marker Applications

Molecular markers have improved our understanding of *C. dentata* genetics by delineating patterns of genetic diversity and dissecting quantitative trait variation (e.g., Casasoli et al., 2006; Dane et al., 2003; Huang et al., 1998; Kubisiak et al., 1997; Kubisiak and Roberds, 2006; Pigliucci et al., 1990). In a similar manner, molecular markers revealed detailed information on the chestnut blight fungus focusing on genetic diversity and mating system mechanics (Marra and Milgroom, 1999; Marra and Milgroom, 2001; Milgroom et al., 1992a; Milgroom et al., 1992b). In the near future marker genotyping a mapping population (i.e., a single cross of 100 progeny) of the fungus scored for canker development in a sample of *C. mollissima* x *C. dentata* host trees may provide QTL for *virulence* (F. Hebard, personal communication, unpublished data). Early in the DNA marker era Bernatzky and Mulcahy (1992) and Ellingboe (1994) suggested using a large number of restriction fragment length polymorphisms (RFLP) markers to map resistance genes in *C. mollissima* and use the markers to facilitate their introgression into *C. dentata* through backcross breeding. Conceptually, this is an excellent idea that was proven in numerous systems (Collard and Mackill, 2008; Moose and Mumm, 2008); however, only a few RFLP markers were developed for *Castanea* spp. Random amplified polymorphic DNA (RAPD) markers proved more cost effective for producing larger numbers of markers and they, along with the few RFLPs and the previously developed isozyme markers, were used to map the *C. dentata* genome as well as identify quantitative trait loci (QTL) (Kubisiak et al., 1997). However, RAPD and the later developed amplified fragment length polymorphisms (AFLP) markers (Sisco et al., 2005), both being dominant and difficult to track among different families were not optimal or cost effective for operational use in large breeding programs (F. Hebard, personal communication).

The recent development of large sets of short sequence repeat (SSR or microsatellite) and single nucleotide polymorphism (SNP) markers (TL Kubisiak, CD Nelson, RR Sederoff *in prep*; also see www.fagaceae.org) are likely to provide the practical application envisioned early on by Bernatzky, Mulcahy, Ellingboe and others (e.g., Nance et al. 1992). These markers are codominant and much higher in sequence specificity (providing data on the same loci across families) effectively overcoming the two major problems encountered with RAPD and AFLP. However, cost effectiveness could still be an issue at least in the near-term. Fortunately newly funded research (i.e., FHI) is fully testing these markers in backcross breeding as well as in assisting with higher-density and higher-resolution mapping for candidate gene discovery. The candidate genes will be isolated from *C. mollissima* and used to transform *C. dentata* to directly test their effectiveness in providing blight resistance. The highly informative maps enable the

tracking of introgressed *C. mollissima* genes in early and later generation backcross families. In addition, these maps will provide estimates of the remaining *C. mollissima* genome at various backcross generations, facilitating the dual selection of resistance provided by *C. mollissima* genes and *C. dentata* silvical traits provided by the *C. dentata* genome. The use of markers in selection for recurrent (*C. dentata*) type (i.e., genomic regions) provides up to a 3X improvement in recovery of recurrent type (Tanksley and Rick 1980; Soller and Beckmann 1986).

Traditionally, many backcross programs used six backcross generations as a standard (Allard, 1960); however, with informative, well-spaced markers two generations provide similar results (e.g., Visscher et al., 1996). Given that TACF backcross program was planned for three backcross generations (Burnham et al., 1986), markers may reduce this to one allowing for additional resistance sources to be introgressed with a similar level of effort.

Other potentially useful applications for highly polymorphic DNA markers include fingerprinting, paternity analysis, and species classification. Fingerprinting has been used to identify mislabeled individuals in breeding populations and research crosses (Kubisiak, personal communication; Sisco et al., 2005) and to unravel clonal identities in germplasm banks (Coggeshall et al., 2009). A form of paternity analysis was used to identify trees with non-*C. dentata* cytoplasm in a large set of trees sampled from across the *C. dentata* range (Kubisiak and Roberds, 2006). This test relied on a single marker difference for the chloroplast genome, differentiating *C. dentata* from all other *Castanea* spp. Highly informative nuclear genome SSR marker sets can be developed for routine fingerprinting and paternity analysis (Jeanne Romero-Severson, personal communication). When fully developed, these tools will open new breeding opportunities such as pedigree-controlled breeding without control-pollination (El-Kassaby and Lindgren, 2007; El-Kassaby and Lstiburek, 2009; Lambeth et al., 2001) and efficient tracking of clonal lines in tissue culture and genetic modification programs. Species classification relies on a large number of markers distributed across the genome where data are collected on representative trees of each species and on samples of unclassified trees. Computer algorithms (Falush et al., 2003; Pritchard et al., 2000) are then used to classify the individuals based on their genetic marker composition. This has been successfully utilized in loblolly pine (*Pinus taeda* L.) and shortleaf pine (*P. echinata* Mill.) to determine past and current rates of natural hybridization and introgression (Stewart et al., 2010; Stewart et al., *in press*).

Genetics of Blight Resistance

The inheritance of chestnut blight resistance has been studied extensively, especially in interspecies first- (F_1) and second-generation (F_2 and BC_1) crosses (Clapper, 1952; Graves, 1942; Graves, 1950). Burnham et al. (1986) analyzed and summarized the existing knowledge, confirming that a two-gene pair model of resistance seemed reasonable as first suggested by (Clapper, 1952) with the resistant *C. mollissima* or *C. crenata* parents providing partially dominant alleles for resistance. Graves (1942) had actually proposed a one-gene model, quickly discounting it due to the intermediate nature of the resistance reaction. Stem canker data from controlled inoculation trials of several segregating (i.e., F_2) families (as outlined in Ellingboe, 1994) provided support for the two-gene model and indicated that the Asian parents appear homozygous for their resistance genes. In addition, the two Asian species may have different gene pairs (i.e., loci) for resistance suggesting that combining parents from each species in interspecies breeding may lead to enhanced resistance. This variation is more likely due to allelic differences (resistant vs. susceptible alleles) at the resistance gene loci. Thus, a few major gene loci for resistance may exist that differ among species and are completely lacking or defective in *C. dentata*. There may also be allelic variation at various loci for factors that further affect resistance expression. Although it appears that homozygosity for resistance alleles at two major gene loci is sufficient for survivability to the blight, additional genes will likely improve survivability and increase resistance diversity against a potentially changing (i.e., mutating) pathogen (Ellingboe, 1994).

The most definitive research on blight resistance genetics further supports a two or possibly three gene model as detected by QTL mapping in a *C. mollissima* × *C. dentata* F₂ cross (Kubisiak et al., 1997). The resistance genes from *C. mollissima* are partially dominant and obtaining individuals homozygous for two genes provided resistance reactions (i.e., stem canker area at about 8 weeks post-inoculation) on par with *C. mollissima*. The three-locus model accounted for about 70% of the genetic variation, further implicating a combination of major and minor genes as contributing to resistance. This F₂ cross included one *C. mollissima* grandparent (cv. ‘Mahogany’) and two related *C. dentata* parents; thus, they were investigating a rather narrow sample of the potential genetic variation in resistance, yet this was still instructive for revealing the genetic architecture of blight resistance (Hebard, 2006). New work funded by NSF (www.fagaceae.org) and a partnership developing and utilizing genetic transformation in forest health (www.foresthealthinitiative.org) are expanding genomic tools for more precisely and comprehensively mapping resistance genes (Kremer et al., 2010). New higher density maps using SSR and SNP markers developed from large-scale expressed gene sequencing (Barakat et al., 2009) has confirmed and slightly refined the genomic locations of the blight QTLs originating in cv. ‘Mahogany’ (Kubisiak et al., unpublished data). Collaboration with TACF’s breeding program will provide much larger population sizes in BC₃ and BC₃F₂ generations as well as including additional sources of resistance. These features combined with the higher-density genetic maps will allow increased precision in locating blight resistance loci, greater sensitivity in finding smaller effect loci, and the possibility of determining whether different *C. mollissima* trees contribute different resistance loci.

Part 3: Ecology and Restoration

Environmental Controls on Growth

Current knowledge implicates *C. dentata* as an intermediate shade tolerant to shade tolerant species (Joesting et al., 2007; Joesting et al., 2009; McCament and McCarthy, 2005; Wang et al., 2006). Shading produces either a neutral (Rhoades et al., 2009; Wang et al., 2006) or positive (Anagnostakis, 2007; McCament and McCarthy, 2005) effect on germination and/or survival of young *C. dentata*. Once established, seedlings and saplings may persist for years under low light conditions beneath a forest canopy (McEwan et al., 2006; Paillet and Rutter, 1989), exhibiting plasticity by increasing leaf mass per area with greater light availability (Joesting et al., 2009; King, 2003; Wang et al., 2006). *Castanea dentata* seedlings, saplings, and mature trees in a forest in southwestern Wisconsin exhibited light compensation points, quantum efficiency, leaf mass per area, and percent nitrogen content similar to those of shade tolerant species (Joesting et al., 2009). Interestingly, however, understory trees measured in this same study had high maximum rates of photosynthesis, similar to that of fast growing, shade intolerant species such as yellow-poplar (*Liriodendron tulipifera* L.) and eastern cottonwood (*Populus deltoides* Bartram ex Marsh.) (Joesting et al., 2009). Nevertheless, *C. dentata* exhibits greater growth and photosynthesis with increasing light availability (Joesting et al., 2007; McCament and McCarthy, 2005; Wang et al., 2006) and growth rates of *C. dentata* under high light availability may exceed or equal that of other species exhibiting strong positive responses to light (Boring et al., 1981; Griffin, 1989; King, 2003; Latham, 1992). These ecological attributes distinguish American chestnut from oaks and other co-occurring species (Paillet, 2002).

Increasing light availability was shown to have a greater influence on *C. dentata* growth than soil parameters (McCament and McCarthy, 2005) or site type (i.e., xeric vs. mesic; Rhoades et al., 2009). This combined evidence reflects the capacity of *C. dentata* to survive for prolonged periods as stump sprouts or advance regeneration under suppressed conditions, while maintaining the ability to rapidly respond to release following disturbance. *Castanea dentata* sprout growth may exceed that of any other hardwood species following clearcutting (Mattoon, 1909; Smith, 1977) with radial growth rates approaching 5 mm year⁻¹ in plantation or natural stand settings, with maximum values of 10-12 mm year⁻¹ (Jacobs and Severeid, 2004; McEwan et

al., 2006; Paillet and Rutter, 1989; Zeigler, 1920). Productivity of mature *C. dentata* trees in Connecticut was measured to be at least 25% greater than that of oak species (Frothingham, 1912). A productivity of 2.9 m ha⁻¹ year⁻¹ was reported for *C. dentata* stands on 60-year rotations in the Blue Ridge Mountains (Buttrick et al., 1925).

The former dominance of *C. dentata* in upland habitats suggests greater drought tolerance compared to co-occurring species (Jacobs, 2007). For example, *C. dentata* exhibited higher instantaneous water use efficiency relative to several species of upland oaks (*Quercus* spp.) and dry site red maples (*Acer rubrum* L.) subjected to drought under controlled conditions (Bauerle et al., 2006). Additionally, sprouts of *C. dentata* had higher leaf water potential than several species of upland oaks during an early summer drought in Pennsylvania (Abrams et al., 1990). The oaks in this study were newly planted, however, whereas the *C. dentata* were of sprout origin, suggesting potential bias associated with root system age. *Castanea dentata* resists high pH soils (Russell, 1987), and growth may be negatively correlated with pH (Tindall et al., 2004). Specific responses to varying nutrient availability are less well documented, although *C. dentata* has been shown to increase leaf, shoot, and root biomass with increasing availability of specific nutrients including nitrogen, potassium, and magnesium (Latham, 1992; McCament and McCarthy, 2005; Rieske et al., 2003).

Mycorrhizae

Similar to other members of Fagaceae, *C. dentata* forms associations with ectomycorrhizal fungi of both Ascomycota and Basidiomycota (Bauman, 2010; Dulmer, 2006; Palmer et al., 2008). *Castanea* spp, including *C. dentata*, have been noted to form associations with arbuscular mycorrhizae as well, though the ecological role of arbuscular vs. ectomycorrhizal associations in *C. dentata* is not well understood (Dulmer, 2006; Molina et al., 1992). Colonization by ectomycorrhizae has been shown to increase seedling survival and total seedling biomass in plantings on mine reclamation sites, as long as over story competition for light is not too high (Bauman, 2010). Management actions, such as plowing or disking, increase percent colonization of ectomycorrhizae on planted *C. dentata* seedlings (Bauman, 2010). In addition, Bauman (2010) found that inoculating seedlings in the nursery increases seedling survival after outplanting, even though the initial ectomycorrhizae species are replaced with field-available fungal species after the first year.

Competitive Ability

Castanea dentata historically grew with many forest tree species due to its occurrence in a wide variety of mixed forest types. Under the submesic or subxeric sites on which *C. dentata* dominated, it was primarily associated with upland oaks (*Quercus* spp.), maples (*Acer* spp.), hickories (*Carya* spp.), and other mixed hardwoods depending on region (McEwan et al., 2006; Russell, 1987). Historical and more recent observations have reported on the rapid early growth and competitiveness of *C. dentata* as well as its dominance in pre-blight stands. For example, *C. dentata* trees that were introduced into a site in southwestern Wisconsin rapidly invaded an adjacent woodland and largely outcompeted and replaced associated species, such as oaks and hickories, maintaining themselves over time as the dominant forest canopy trees (McEwan et al., 2006; Paillet and Rutter, 1989). Thus, *C. dentata* exhibits characteristics of both a pioneer (facilitated by aggressive stump sprouting and juvenile competitiveness) and late-successional species (based on its extended stand longevity).

By studying development of *C. dentata* relative to six co-occurring species across a broad range of light and nutrient levels under controlled conditions, Latham (1992) helped to elucidate mechanisms for *C. dentata*'s competitive ability. *Castanea dentata* outranked all other species in traits associated with competitive ability over the wide range of resource level combinations, implicating *C. dentata* as both a broad generalist and strong competitor (Latham, 1992). Furthermore, there is evidence that leachate from *C. dentata* litter may have allelopathic

properties that suppress the development of common competitors (Vandermaast and Van Lear, 2002).

Fire Tolerance

The early forest literature indicates that *C. dentata* is relatively susceptible to fire based upon several life-history characteristics (Buttrick and Holmes, 1913; Mattoon, 1909; Zon, 1904). For example, *C. dentata* has relatively thin bark and shallow root system compared to fire-tolerant species, such as oaks (Buttrick and Holmes, 1913). Although *C. dentata* sprouts prolifically (a trait commonly associated with fire tolerance), its sprouts originate from shallow root collar buds that may be poorly protected from fire (Mattoon, 1909; Zon, 1904). However, seedling growth after two years was highest in experimental sites that had been both thinned and burned (McCament and McCarthy, 2005). While the thinning increased light availability, burning reduced understory competitors. Paillet (2002) observed that light surface fires promote chestnut sprouts by decreasing understory competitors and concluded that the effects of fire on chestnut is likely a complex relationship that depends on both site characteristics and fire conditions such as intensity, frequency, and timing.

Dispersal

Similar to other large hard mast species, nuts of *C. dentata* fall close to the parent tree although blue jays, squirrels, and other rodents were likely significant historical consumers and dispersers (Diamond et al., 2000; Russell, 1987; Steele et al., 2005). A blight-free stand of *C. dentata* in southwestern Wisconsin provided unique insight into dynamics of regeneration and migration (and potential competitive dominance) of the species (Paillet and Rutter, 1989). In 70 years, nine original planted *C. dentata* trees supplied sufficient regeneration to spread the species over 1 km; within about 0.5 km from the original source trees, *C. dentata* comprised at least 25% of total canopy basal area and predominated among advanced saplings entering the canopy. Evidence from this stand suggests that migration of *C. dentata* regeneration involved a multi-step process, including i) establishment of individuals or groups of pioneer trees following seed dissemination in light gaps, ii) development of large pools of advanced regeneration in the understory of these pioneer trees, and iii) persistence of these seedlings and saplings underneath the established canopy until being released by disturbance to assume canopy dominance (Jacobs, 2007; Paillet and Rutter, 1989).

Other Pathogens and Pests

Several pathogens and pests other than chestnut blight fungus pose a threat to *C. dentata*. Principal among these is the introduced soil-borne oomycete pathogen, *P. cinnamomi* Ronds, causing ink disease in which lesions that form on roots inhibit water and nutrient uptake (Maurel et al., 2001a; Maurel et al., 2001b) and lead to reduced tree vigor and eventual mortality (Anagnostakis, 2001; Rhoades et al., 2003). Ink disease development-- growth, reproduction, and dissemination of the pathogen-- are favored under compacted, saturated soils with poor aeration because this promotes sporangia formation and zoospore release (Rhoades et al., 2003; Wilcox and Mircetich, 1985). The impact of ink disease was noted in the southern U.S. prior to introduction of chestnut blight (Anagnostakis, 2001), and current evidence suggests that the pathogen presents another significant obstacle for *C. dentata* reintroduction (Rhoades et al., 2009; Rhoades et al., 2003). Careful site selection, identification of ectomycorrhizae that confer protection to roots, and additional resistance breeding have been suggested as means to help combat the impact of *P. cinnamomi* (Anagnostakis, 2001; Rhoades et al., 2003). In addition, evidence specifically implicates the oriental gall wasp (*Dryocosmus kuriphilus* Yasumatsu), Gypsy moth (*Lymantria dispar* L.), and ambrosia beetles (*Xylosandrus crassiusculus* Mot. and *Xylosandrus saxeseni* Blandford) as pests that may negatively affect *C. dentata* following reintroduction (Anagnostakis, 2001; Oliver and Mannion, 2001; Rieske et al., 2003).

Compared to blight resistance (as discussed above), less is known about the genetics of resistance in *Castanea* spp. to other pathogens and pests. For ink disease resistance, both two and one gene models have been proposed (Bowles, 2006; Guedes-Lafargue and Salesses, 1999). An informative early (i.e., first-year seedlings) screening system is being deployed to progeny test interspecies backcross parents produced in the TACF breeding program (Jeffers et al., 2009; Sisco, 2009). Segregation for resistance is often noted (P. Sisco, personal communication), providing a rich source of genotypic and phenotypic material for genetically mapping the resistance factors. The genetic situation for gall wasp resistance is beginning to emerge (Anagnostakis et al., 2009) with the chestnuts (*C. mollissima*, *C. crenata*, and *C. dentata*) apparently being more susceptible relative to the chinkapins (*C. pumila*, *C. pumila* var. *ozarkensis*) and *C. henryi*. Strong differences among trees within interspecies backcrosses were noted in a field test in North Carolina where gall wasp pressure was high. Segregation for resistance (i.e., no or few galls per tree vs. many galls) was noted in both crosses suggestive of a single, dominant gene controlling resistance (Anagnostakis et al., 2009). Additional crosses will need to be evaluated under high gall wasp pressure to further evaluate the inheritance of gall wasp resistance. In all three cases—blight, ink disease, and gall wasp—resistance is available in the Asian chestnuts for the first two and the chinkapins for the third. Resistance to all pests appears to be at least partially dominant and much of the variation seems to be controlled by one or two genes. Whether these genes are the same in different species or even genotypes within species remains to be seen, but application of emerging genomic technologies (Wheeler and Sederoff, 2009) should help to resolve the situation and provide tools for precisely tracking the genes in breeding programs. This would enable introgression of genes for resistance to ink disease, gall wasp, and blight together.

Optimal Restoration Habitats

Restoration of *C. dentata* to its native range may be initiated through reforestation and afforestation plantings of blight-resistant seedlings. Recent evidence has demonstrated excellent growth and competitiveness of *C. dentata* over a wide range of sites in natural stands (McCament and McCarthy, 2005; McEwan et al., 2006; Rhoades et al., 2009). In addition, mine reclamation sites and marginal agricultural lands would provide abundant planting sites for afforestation of *C. dentata* (Jacobs, 2007; Jacobs and Severeid, 2004). Despite the characteristic competitiveness of juvenile *C. dentata*, effective silvicultural management may be necessary to ensure vigorous establishment of high-value blight-resistant seedlings following planting (McCament and McCarthy, 2005; McNab, 2003; Rhoades et al., 2009). Specific recommendations for underplanting (Wang et al., 2006) or thinning and burning (McCament and McCarthy, 2005) have been proposed to promote competitiveness of *C. dentata* in natural stands. Similarly, recommendations are available for herbicide application to control competing vegetation and promote *C. dentata* development in field plantations (Robertson and Davis, 2011; Selig et al., 2005).

Selecting sites for restoration that optimize growth development and minimize exposure to environmental stresses, such as cold or drought, may help to ensure expression of blight resistance (Griffin, 2000; Jones et al., 1980). The absence of *C. dentata* in high pH, limestone derived soils (Russell, 1987) suggests that these site types should be discriminated against for restoration plantings. Additionally, the susceptibility of *C. dentata* to *P. cinnamomi* indicates that careful site selection may be needed to strategically locate restoration plantings on very well drained sites (Rhoades et al., 2003). Furthermore, public opinion regarding harvesting, fire, and other forms of forest disturbance may restrict the capacity of land managers to employ silvicultural treatments that have been demonstrated to promote *C. dentata* development, particularly on public lands (Jacobs, 2007; McEwan et al., 2006). This implies that target sites for *C. dentata* restoration may shift toward reforestation and afforestation of private lands (Jacobs, 2007). Much of the large-scale hardwood afforestation plantings in the US for carbon

sequestration, conservation, wildlife, and timber occur in the Midwest, which encompasses a limited portion of the original *C. dentata* range. This presents a new challenge, as targeting *C. dentata* plantings in this region is incongruent with the fundamental mission to restore *C. dentata* to the original species range (Jacobs, 2007). Additionally, *C. dentata* has demonstrated its ability to thrive when introduced outside of its native range (Jacobs et al., 2009; Jacobs and Severeid, 2004; McEwan et al., 2006; Paillet and Rutter, 1989), raising ecological considerations regarding its potential to suppress indigenous vegetation (Jacobs, 2007).

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