Measuring resistance to chestnut blight (*Cryphonectria parasitica*) and American chestnut (*Castanea dentata*) morphology of backcrossed hybrids in Lesesne State Forest

by

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Submitted to Department of Environmental Science and Policy

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Under the supervision of Dr. Ram Oren

For a Bachelor of Science Degree

May 2023

ABSTRACT

The American Chestnut (*Castane dentata*) was a pivotal species in Eastern hardwood forests before populations declined to near extinction across their entire range following the introduction of *Cryphonectria parasitica*, the fungus responsible for the chestnut blight. Backcross breeding is one mechanism used to introduce blight resistance to *C. dentata* specimens following hybridization with resistant Chinese chestnut (*C. mollissima*) specimens. This study focused on measuring blight resistance and *C. dentata* morphology of backcrossed *C. dentata* specimens in Lesesne State Forest, Virginia (LSF). Observations of blight resistance and *C. dentata* morphology were recorded for a subset of trees in LSF from May 2022 to October 2022 to calculate a phenotypic blight resistance index and *C. dentata* morphology index in the field. Analysis compared the phenotypic blight resistance index and expression of *C. dentata* morphology to genetic-based indices and genotyping-by-sequencing data to identify correlation between field and lab-calculated indices used in selecting specimens for further backcrossing. Results showed a strong positive correlation between estimated *C. mollissima* genotypic content and both phenotypic ($R = 0.88$, $p = 0.0044$) and genetic-based blight resistance indices ($R = 0.95$, $p = 2.2e\text{-}16$). Strong positive correlation was found between phenotypic and genetic-based blight resistance indices ($R= 0.66$, $p = 2.2e\text{-}16$). Moderate negative correlation was found between the phenotypic blight resistance index and the *C. dentata* morphological index ($R = -0.41$, $p = 2.9e\text{-}16$). Weak negative correlation was found between the *C. dentata* morphological index and the genetic-based blight resistance index ($R = -0.34$, $p = 1.4e\text{-}11$). The *C. dentata* morphological index was not found to correlate significantly with *C. dentata* genotypic content ($p = 0.25$) in a small sample. These results identify strengths and weaknesses in relying on field-based indices to make selections for backcross breeding, which will have implications for progress and success in restoring the American chestnut.

INTRODUCTION

The American Chestnut (*Castanea dentata*) was once an integral species in Eastern hardwood forests. Encompassing a range of nearly 200 million acres and numbering near four billion at its peak, *C. dentata* filled a role of high ecological and social importance ("History of the American Chestnut," n.d.) and was often considered a "foundation species" in areas characterized by populations of the species large enough to comprise the majority of biomass or forest canopy area (Jacobs et al., 2013).

Ecologically, *C. dentata* filled a niche rivaled by few hardwood species to date, producing significant and consistent annual crops of chestnuts each year (Diamond,et al, 2000). This crop fed communities, their hog or cattle, and a large portion of the rural agricultural economy and forest ecosystems alike. Logging of *C. dentata* was highly profitable due to the quality of its wood and the rate at which this species is able to resprout following its harvest (Jacobs et al., 2009). Its lumber was used in anything from furniture and log cabin construction to telephone poles, fencing material, and railroad ties- many of which are still in use today (Jacobs, 2007). Farmers often practiced silvopasturing with this hardwood species, allowing them to generate income from livestock, timber, and the annual chestnut crop on one plot of land ("History of the American Chestnut," n.d.).

Forest communities comprised primarily of *C. dentata* benefitted from its influence on decomposition, productivity, and nutrient cycling as a broad-leaf species (Jacobs, 2007). Their fast growth rate allows them to sequester carbon faster and in a shorter period of time than most other hardwood species, with their biomass reaching nearly 3 times that of other species at points in their growth cycle (Jacobs et al., 2009). Their rot-resistant wood also slows the rate at which

carbon is returned to ecosystems from their timber, allowing them to act as a carbon sink in Eastern forests (Jacobs et al., 2009). This alone is reason for their reestablishment due to the role they could play in slowing climate change on a global scale.

Cryphonectria parasitica was introduced to Eastern forests following its importation on Asian chestnut specimens to an arboretum in New York in 1904 (Jacobs et al., 2013). By 1950, *C. parasitica* was endemic across the entire *C. dentata* range and populations had declined by nearly 4.2 billion trees (Westbrook et al., 2020). Today, *C. dentata* found in the wild rarely reach the age of flowering before succumbing to chestnut blight, rendering the species functionally extinct across its entire range. Young specimens still exist in the wild, as the blight cannot infect their root systems, but all succumb to the blight before reaching maturity (Lovat, 2019). .

C. parasitica is a necrotrophic, pathogenic fungus that feeds on stem tissue of aboveground portions of trees (Barakat et al., 2009). Asian chestnut species are tolerant to the chestnut blight caused by *C. parasitica*, but European and American chestnut species are highly susceptible to the effects of this fungus (Lovat, 2019). *C. parasitica* infects the cambial zone of *C. dentata* through stem wounds. A canker is produced at the site of infection that often grows to girdle the tree, killing aboveground stems and leaving behind intact root systems (Barakat et al., 2009). This has resulted in a cycle of infection, death, and resprouting of *C. dentata* specimens that rarely reach maturity again due to their susceptibility to infection (Jacobs et al., 2013). *C. parasitica* is now endemic to North America and survives on a number of host trees, including oak and beech (Anagnostakis, 2001). Because of this, complete eradication of the chestnut blight-causing fungus is not likely with any form of biocontrol. Restoration of *C. dentata* is reliant on development of blight resistance in its genome through some form of hybridization, genetic modification, or a combination of several methods (Curry, 2014).

Many individuals and organizations have focused on restoration of this species with some success. The American Chestnut Foundation (TACF) is one of these organizations. TACF has worked for 40+ years on returning *C. dentata* to its original social and ecological niche in Eastern forests. Their programs focuses on introducing chestnut blight resistance to the species through transgenics, backcross breeding, and biocontrol (Westbrook et al., 2020). Backcross breeding is used throughout the agricultural industry to incorporate desirable genes from one specimen to another. It is often used to increase drought tolerance in rice and wheat (Lafitte et al., 2006; Todkar et al., 2020), and has even been used to increase collection of pollen in domestic honeybee populations (Nye & Mackensen, 1967). Likewise, backcrossed breeding of *C. dentata* may allow for the integration of blight resistance into the genome while maintaining the genetic integrity of this species.

The backcross breeding program under TACF was founded using hybrids bred from two *C. dentata* x *C. mollissima* hybrids, "Clapper" and "Graves", bred to provide two distinct sources of blight resistance in backcrossed trees (Westbrook et al., 2020). Hybrid trees were then bred consecutively with *C. dentata* specimens with the intention of producing an offspring that is phenotypically identical to *C. dentata* while retaining the blight resistance introduced in the first hybridization, as shown in Figure 1 (Westbrook et al., 2020). This assures any reestablished *C. dentata* backcrossed populations will be capable of filling the same economic and ecological role they were once cherished for as a perennial fruit-producing species (Jacobs, 2007). To ensure backcross breeding is successful, individuals must be selected from

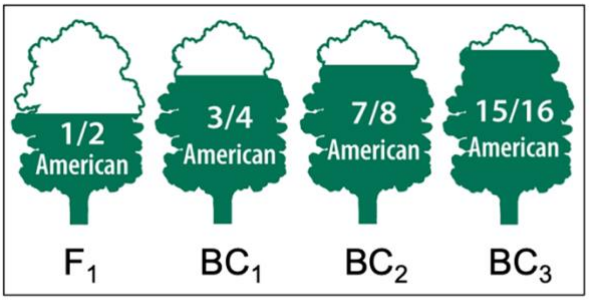

Figure 1: Reestablishment of C. dentata genome through backcross breeding. Credits to PA-TACF, 2016.

each generation that possess the desired amount of blight resistance and similarity to pure *C. dentata* specimens to produce the next generation of backcrossed hybrids.

This work focuses on TACF's backcross breeding program in Lesesne State Forest. Lesesne State Forest (LSF) is unique to the restoration efforts of TACF, as it was originally founded under the Virginia Department of Forestry (VADOF) and is now a collaboration between the two groups to restore this species. In 1955, Ralph Singleton began an experiment exploring whether exposing *C. dentata* nuts to radiation could produce a genetic mutation in the *C. dentata* genome resulting in resistance to chestnut blight (Curry, 2014). Most, if not all, of these specimens eventually succumbed to chestnut blight, leaving behind intact root systems in the state forest (Griffin, 2000). Many of the Largest Surviving Americans (LSA) specimens used in the LSF backcross program come from wild-type *C. dentata* grafted onto irradiated rootstock left from Singleton's experiment (*Lesesne State Forest*, 2009).

Additionally, the VDOF founded their backcross breeding program in Lesesne State Forest using methods that differ from those used by TACF today. They planted these trees with wider spacing to reduce the impact competition between trees may have on disease severity and susceptibility to *C. parasitica* (Jaynes, 1982). Trees in this program were not inoculated by hand but instead were selected for long term survival after naturally contracting chestnut blight from the surrounding environment (Griffin, 2000). Lastly, the program was founded using different *C. mollissima* parents and LSA specimens for breeding. As a result, the trees in this study have a different pedigree than the rest of the TACF backcross breeding program and often express healthier phenotypes than typically observed in the larger TACF program (Westbrook, personal communication, April 2023).

This study serves an exploratory analysis of the methods used to identify backcrossed hybrids that possess the desired amount of blight resistance while resembling their *C. dentata* parent for selection in the backcross breeding program. This can be measured with genetic testing, field-based observations of phenotypic characteristics, or with modeling that combines both genetic and phenotypic data available on a population of backcrossed *C. dentata* specimens. The process will begin with the collection of field-based observations to then calculate a Phenotypic Resistance Index (PRI) and *C. dentata* Morphology Index (CMI). These indices will be compared to genotyping-by-sequencing (GBS) data, outlining *C. mollissima* and *C. dentata* genetic content, in addition to a Genetic Resistance Prediction (GRP). From this, I hope to identify correlation between field-based observational indices and genetic data. This correlation will allow for the assessment and selection of backcrossed specimens without relying on additional resources and access to a lab setting.

MATERIALS AND METHODS

Study Area

Located at approximately 400m in elevation, Lesesne State Forest (LSF) sits in the lower slopes east of Three Ridge Mountain in Nelson County, Virginia. This area receives an average annual precipitation of 1433 mm and sees average annual temperatures ranging from an average winter low of -4 ^{o}C and an average summer high of 31 ^{o}C. This region of central Virginia is characterized by rocky soil composed of brown to sandy brown loam topsoil and clay to sandy clay subsoil, specifically Saunook loam and Edneytown-Peaks complex (Lesesne State Forest – Nelson Scenic Loop). It is well-draining, acidic to very acidic, moderately permeable, and reaches depths of up to 1.52 m (National Cooperative Soil Survey. This soil is common in the

Blue Ridge Mountains throughout Virginia, Georgia, Tennessee, South Carolina, and North Carolina on slopes averaging 15 to 50 percent grade (National Cooperative Soil Survey). Runoff from this soil varies by slope and leaf litter presence but is generally low to moderate in LSF orchard plots.

Lesesne State Forest contains five orchards of *C. dentata* populations: an American Germplasm Conservation Orchard (AGCO) and 4 breeding orchards (F1, BC1, BC2, and BC3). The AGCO orchard in this study was planted between 1962 and 1977, with additional trees added throughout 1980 to 2000. It is now predominantly composed of grafts from LSA and wild-type *C. dentata* specimens. Originally planted with an average spacing of 3 m between rows and columns, spacing between surviving individuals in this orchard has increased significantly due to the approximate 20% survival rate in this orchard. The F1 orchard contains many of the original hybrids bred and planted by the Virginia Department of Forestry between 1969 and 1974 at a spacing of 1.8 m x 1.8 m (Virginia Department of Forestry).

The BC1 orchard, containing predominantly BC_1 specimens with some *C. dentata* x *C. dentata* outcrosses, was planted between throughout 1990 to 1993, with an additional planting in 2001. The BC2 orchard was planted in 2001 and 2009, and the BC3 orchard was planted in 2009 and 2010. These breeding orchards were planted with a 4.57 m x 2.44 m spacing and saw a median survival rate of 60-70% at 10 years of age. Figure 2 shows the BC2 orchard observed in this study.

Figure 2: Lesesne State Forest, BC2 Orchard. Taken by Caragh Heverly.

Data collection for this study occurred between June 2022 to October 2022 and was confined to the BC2 and BC3 breeding orchards. Observations of blight resistance and *C. dentata* morphology were recorded for all 558 living specimens. This population included BC_1, BC_2, and BC_3 crosses. This data was supplemented with genetic data detailing *C. dentata* and *C. mollissima* genetic content from GBS, in addition to genetic blight resistance estimates modeled for all trees in this population. This produced the Genetic Resistance Prediction (GRP) used in the analysis. Methods used for this are described below by Jared Westbrook, Director of Science for TACF.

> "A genetic-based blight resistance index was created from the sum of estimated additive genetic values for this suite of traits. Additive genetic values for blight resistance traits were estimated using a generalized linear mixed model in ASReml-R v. 4.1 (Butler et al. 2018). Genetic liabilities for presence/absence traits were estimated with a binomial model and percent canopy survival was modeled as a continuously distributed gaussian trait. The effect of tree age was treated as a fixed covariate. Genotype effects were assumed to be normally distributed and were estimated with a blend of pedigree and genomic

relationships (VanRaden 2008; Aguilar et al. 2010) using the R package 'AGHmatrix' (Amadeu et al. 2016). To construct the genetic-based blight resistance index, genetic values were scaled 0 to 1 and these scaled values were multiplied by trait heritability (h2 = s2genotype / s2genotype + s2error). Blight resistance index values were then scaled from 0 = mean for susceptible AM chestnut and 100 = mean for resistant CH chestnut controls" (Westbrook, personal communication, February 2023).

Blight Resistance Assessment

Phenotypic blight resistance was measured using visual symptoms indicative of susceptibility to *C. parasitica* infection or strength of resistance to *C. parasitica*. These symptoms include canker formation and appearance, presence of stump sprouts, blight sporulation, canopy loss, blight containment, status of main stem, and presence of exposed. These characteristics are described in detail in Table 1 of Appendix A. These traits were used in calculating the Phenotypic Blight Resistance Index (PRI), for which 1 signifies little to no indication of blight resistance and 5 indicates full resistance as determined by these field-based observations. An example of the appearance of *C. parasitica* infection can be found in Figure 3 and 4.

Figure 3 (left) and 4 (right): Examples of canker formed by *C. parasitica*. Taken by Caragh Heverly.

American Chestnut Morphology

The morphological traits of *C. dentata* specimens on which this study focused were tree form, the presence or absence of an acute petiole angle, leaf shape and sheen, leaves possessing teeth resembling those of pure *C. dentata* specimens, the absence of leaf hairs (pubescence), and presence of sparse and long vein hairs. A detailed description of these traits can be found in Table 2 of Appendix A. Additional morphological differences exist between *C. dentata* and *C. mollissima*, such as bur and catkin characteristics during flowering and fruiting, but these were not included because the period of observation did not coincide with the reproductive season.

Traits were recorded on a binary scale with 1 = *C. dentata* and 0 = *C. mollissima* unless otherwise noted in Table 2. Data collected depended on visual knowledge of the morphological differences between *C. dentata* and *C. mollissima*, with minute traits dealing with leaf pubescence assessed with the use of a hand lens. These traits were then indexed on a scale of 0 to 100, with 100 representing trees expressing all *C. dentata* morphological traits measured. Trees for which not all traits could be measured (e.g., where leaves were out of reach) were indexed

based on the measured traits without penalty. This defines the *C. dentata* Morphology Index (CMI) used in the analysis. Figure 5 and 6 depict morphological differences between *C. dentata* and *C. mollissima* recorded in this study.

Figure 5 (left) and 6 (right): Figure 5, taken by Caragh Heverly, depicts leaf morphology typical of *C. dentata*. Figure 6 (Zhangzhugang, 2018) shows the morphology typical of *C. mollissima*.

Data analysis focused on identifying correlation between genetic data or the genetic-based resistance index and field-based observational indices. Each variable was assessed for normality with the Shapiro-Wilk Test, for which a $p > 0.05$ fails to reject the null hypothesis that the data is normally distributed. Pearson's correlation was used to identify any significant relationship between traits that meet assumptions of normality. Spearman's correlation was used to identify any significant relationship between traits that do not meet assumptions of normality.

Results

I tested for linear covariation between *C. dentata* genotypic content and the *C. dentata* Morphology Index (CMI) using Pearson's Correlation Test. To identify normality in my data, a

Shapiro-Wilk test was applied to both variables, which yielded p = 0.266 and p = 0.111, respectively. This indicates that these variables are normally distributed within Lesesne, as shown in Figure 7 (Appendix B). Applying the Pearson's correlation test here indicated a correlation equal to 0.463 (or between -0.359 and 0.880 with 95% confidence) with a p = 0.248, as shown in Figure 8a. Hence no significant correlation was found within this small sample (n=9).

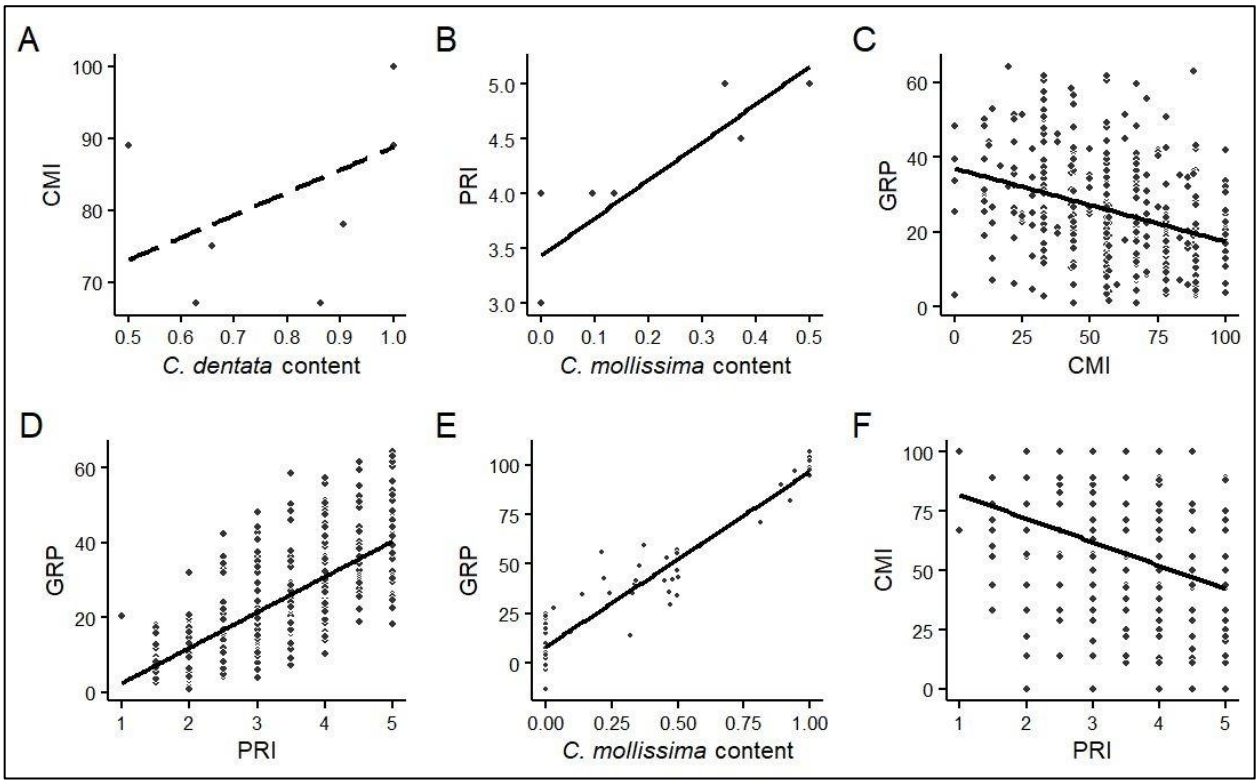

Figure 8: Correlation between field-based indices and genetic data. A) The correlation between the *C. dentata* Morphology Index (CMI) and *C. dentata* genetic content was weak in this sample. B) Phenotypic Resistance Index (PRI) and *C. mollissima* content are strongly correlated with R = 0.88 and a p = 0.004. C) CMI and Genetic Resistance Prediction (GRP) were correlated but left a large amount of unexplained variability, with R = -0.344 and p = $1.41e^{-11}$. D) Correlation between GRP and PRI was fairly strong with R = 0.664 and p = $2.2e^{-16}$. E) GRP and *C. mollissima* content have strong correlation with R = 0.946 and p < $2.2e^{-16}$. F) CMI and PRI were correlated leaving a large amount of unexplained variation as indicated by R = -0.405; p = $2.89e^{-16}$.

The same analysis was applied to test correlation between *C. mollissima* genotypic content and the Phenotypic Resistance Index (PRI) calculated from field observations. Shapiro-Wilk normality testing yielded p = 0.202 and 0.111 for phenotypic resistance and *C. mollissima* content, respectively. This indicates that these variables are normally distributed within my sample, as shown in Figure 9 (Appendix B). The Pearson's correlation test for these variables showed a significant correlation with R= 0.88 (or between 0.446 and 0.977 with 95% confidence) and a p = 0.004, as shown in Figure 8b.

The Shapiro-Wilk test for normality yielding p = 7.45e-7 and p = 3.67e-7 from the Genetic Resistance Prediction (GRP) and CMI, respectively. This indicates that these variables were not normally distributed in this sample, as shown in Figure 10 (Appendix B). The CMI was correlated significantly with GRP based on Spearman's correlation coefficient. This analysis gave an R= -0.375 and p = 1.34e-13, as shown in Figure 8c.

From the Shapiro-Wilk test, I found that PRI (p = 1.19e-9) and GRP (p = 7.45e-7) were not normally distributed in this sample, as shown in Figure 11 (Appendix B). Using Spearman's correlation, PRI was also significantly correlated with GRP, giving an R = 0.688 as shown in Figure 8d. This result was accompanied by a p < 2.2e-16, indicating this correlation is significant.

A Shapiro-Wilk normality test was applied to *C. mollissima* and GRP values, yielding p = 2.36e-8 and p = 7.56e-5, respectively. This test showed that neither variable was distributed normally within Lesesne, as shown in Figure 12 (Appendix B). Using Spearman's correlation, a linear relation was identified between estimated *C. mollissima* genotypic content and GRP for all

trees in Lesesne with GBS data. This correlation is described by $R = 0.900$ and $p < 2.2e\text{-}16$, as shown in Figure 8e.

Finally, the correlation between indices calculated from observations made in the field were calculated. The Shapiro-Wilk normality test yielded $p = 1.74e\text{-}7$ for CMI and $p = 8.01e\text{-}10$ for PRI. This indicates that neither variable was distributed normally in this sample, as shown in Figure 13 (Appendix B). Spearman's correlation test indicated a weak negative correlation with $R = -0.425$ (for $p < 2.2e\text{-}16$), as shown in Figure 8f. This weak correlation indicates a wide range of phenotypic resistance can be found across groups of trees exhibiting a wide range of *dentata* morphology traits.

Discussion

The correlation between the Genetic Resistance Prediction (GRP) and *C. mollissima* genetic content indicates that modeled blight resistance values are representative of measured *C. mollissima* genes in backcrossed hybrids. This allows for inferences to be made regarding *C. mollissima* genetic content regardless of whether genotyping-by-sequencing (GBS) has been performed on all hybrid specimens in the population of interest.

The correlation between the Phenotypic Resistance Index (PRI) and *C. mollissima* genetic content indicates that the PRI is a successful measure of *C. mollissima* genetic content. This relationship is reinforced by the strong correlation found between the PRI and GRP for all individuals sampled in this study. GRP also incorporates data on the heritability of resistance between generations. From this, it is clear that the observed traits used in calculating the PRI successfully represent both estimates of the heritable component of blight resistance and *C. mollissima* genetic content.

The *C. dentata* Morphology Index (CMI) calculated in this study was not significantly correlated with *C. dentata* genetic content for those individuals that had available GBS data. This could be due to the small sample size of GBS individuals (9 of the 558 trees sampled in this study were submitted for GBS). Additionally, this index may not be a successful measure of *C. dentata* genetic content because it does not capture enough of the morphological variability in backcrossed hybrids and their expression of *C. dentata* and *C. mollissima* morphology. Incorporating additional morphological traits may increase the accuracy of this index and its correlation with *C. dentata* genetic content. For example, incorporating morphological traits that vary more significantly by season, such as flowering, bur, and chestnut characteristics, may allow for the calculation of an index that is representative of genetic information for these hybrid trees.

The correlation between the CMI and the GRP indicates a negative relationship between a hybrid's C. *dentata* ancestry and its resistance to *C. parasitica.* In support of this idea, there was a strong positive relationship between *C. mollissima* inheritance and the genetic resistance predictions for blight resistance. The tradeoff between blight resistance and *C. dentata* ancestry implies that many genes control blight resistance. In the traditional breeding program, a key challenge is to select trees for future breeding that balance moderate to high levels of blight resistance on one hand and resemblance to American chestnut on the other hand (Westbrook et al. 2020).

The correlation between the CMI and PRI could be used to optimize selection of hybrids for backcross breeding between each generation if both were correlated with genetic data. Additional GBS of this sample is advised in order to confirm the relationship between *C. dentata* morphology and genetic data. Accounting for additional morphological traits in this index may

also produce the desired significance to confirm that *C. dentata* genetic content can be estimated using field-based observations.

Restoration of the American chestnut has significant social and ecological implications. Their significance in Eastern hardwood forests and surrounding economies is largely attributed to their physiological characteristics (Jacobs et al., 2013). Because of this, restoration of the American chestnut must ensure blight resistant individuals retain the pure *C. dentata* phenotype for reintroduction to Eastern deciduous forest communities or utilization in local economies. This may be challenging on a large scale given the inverse relationship between phenotypic resemblance to *C. dentata* and measures of blight resistance found in this study.

C. mollissima is a productive nut-producing tree that may be capable of replacing *C. dentata* as a cash crop but falls short in its fitness as a forest and timber species. This species cannot compete well in Eastern hardwood forests given their mature height of 12-20 meters (Miller et al., 2014). This is significantly shorter than mature *C. dentata* specimens, which can reach upwards of 30-35m in height (Fei et al., 2012). *C. mollissima* and *C. dentata* differ in tree form as well, with *C. mollissima* often growing with shrub-like characteristics. This poses additional challenges for specimens in Eastern hardwood forests resembling *C. mollissima* more closely. Despite the positive relationship between *C. parasitica* resistance and *C. mollissima* genetic content, establishing blight resistant hybrids in the historical range of *C. dentata* may be challenging given the reduced fitness of the *C. mollissima* phenotype in this habitat (Fei et al., 2012). Alternatively, this does offer some security in ensuring reintroduced *C. dentata* hybrids will not become invasive, as the addition of *C. mollissima* genetics does not enhance their resilience in Eastern forest sites (Miller et al., 2014).

Backcross breeding was utilized in the commercial hazelnut industry in the United States to introduce resistance to Eastern filbert blight (EFB) in European hazelnut trees (Coyne et al., 1998). EFB is a fungal disease caused by *Anisogramma anomala* that occurs naturally on wild American hazelnuts in North America, which tolerate the disease (Molnar & Capik, 2012). Just as was done through crossing Chinese and American chestnuts, resistance to EFB in European hazelnuts was established through an initial cross with their resistant American counterpart. It was important to preserve the phenotype of the European hazelnut during this process as it produces larger nuts that are easier to harvest for commercial production in North America (Lopes et al., 2016). Because of this, producing resistance to EFB in European hazelnuts was met with similar challenges to those faced in restoration of the American chestnut.

This method was successful in producing EFB resistant hybrids, most likely because EFB resistance was found to be controlled by a single, dominant gene in some hybrids produced through backcross breeding (Molnar & Capik, 2012). Resistance to *C. parasitica*, on the other hand, is thought to be inherited polygenically, which complicates the backcross breeding process and its success significantly for *C. dentata* restoration (Westbrook et al., 2020). Backcross breeding alone will likely be insufficient for producing hybrids that both resemble their *C. dentata* parent and possess adequate blight resistance for re-establishment in the wild (Westbrook et al., 2020).

A similar challenge was faced with the American elm and Dutch elm disease (DED). Introduced in the 1940s, the fungus responsible for DED quickly decimated American elms throughout North America. This process may have been accelerated in comparison to the spread of *C. parasitica* due to the role bark beetles play as vectors for the disease, but similarities in the approaches to elm and chestnut restoration still exist (Martín et al., 2019). Though traditional

methods have been used to produce DED resistance in American elms through hybridization, this has not produced the desired outcomes to ensure long-term restoration of the species (Martín et al., 2019). Introducing favorable traits through genetic engineering has allowed for enhancement of agronomic crops and may serve as viable method to introduce resistance in foundational tree species like the American elm and American chestnut (Lafitte et al., 2006).

For both the American chestnut and American elm, backcross breeding alone does not offer substantial guarantee that resistance will be sustained in re-established populations (Merkle et al., 2007). Backcross breeding also limits the genetic diversity in resistant progeny to the few hybrids that successfully passed on resistance, which could limit the success of reestablishing healthy populations of either tree species (Merkle et al., 2007). Transgenesis may offer both these foundational species a fighting chance in recovering from the introduced pathogens that decimated both populations (Merkle et al., 2007). Transgenic trees exhibit resistance introduced by a single gene, which allows for more successful and efficient selection and isolation of resistance in progeny than backcross breeding (Westbrook et al., 2020). Transgenic *C. dentata*, or a combination of transgenesis and backcross breeding, may give this species the greatest chance of being restored to its former ecological and economic glory.

Conclusion

Success of the *C. dentata* backcross breeding program is dependent on selecting individuals from each generation that possess the desired expression of traits from parent genotypes. These individuals can be identified using genetic testing but submitting every individual in a backcrossed generation for genotyping is inaccessible due to the substantial resources that would be required to assess 100+ orchards within the TACF backcross breeding

program. With the creation of field-based observational indices that correlate directly with genetic data, *C. dentata* hybrids can be readily assessed in the field and selected for additional backcrossing. Though enhancing the selection of backcrossed progeny for additional breeding may increase the likelihood of this method producing its desired result, additional sources of resistance should be explored to give this species the chance of reintroduction on a large scale.

Acknowledgments

I would like to thank the following people for their contributions to this study. Dr. Ram Oren, for his guidance and mentorship throughout this process. Sarah Kelso, who was instrumental to the completion of this work. Jared Westbrook, Director of Science at TACF, for sharing his knowledge of genetics and American chestnut restoration. And John Scrivani, for his generational knowledge and constant feedback.

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Appendix A

TRAIT	DESCRIPTION	SCORING
Blight Containment	Blight contained within margins of canker	Yes = 1, No = 0
Canker Size	Canker larger than 15cm in diameter	Yes = 0, No = 1
Canker Appearance	Canker sunken, swollen, or flat	Sunken/swollen/flat
Canker Superficial	Infection on bark surface or in cambial tissue	Superficial / deep
Blight Sporulation	Presence of active sporulation or visible signs of old sporulation	Yes = 0, No = 1
Exposed Wood	Visible wood due to deep canker reaching sapwood	Yes = 0, No = 1
Main Stem	Main stem alive or dead	Yes = 1, No = 0
Stump Sprouts	Presence of stump sprouts (sign of stress)	Yes = 0, No = 1
Percent Canopy Dead	Calculated from main stem alone	0-100

Table 1: Symptoms of blight resistance used in calculation of Blight Resistance Rating for BC_2F_1 hybrids, with 0 = resistance typical of *C. dentata* and 1 = resistance typical of *C. mollissima*

TRAIT	DESCRIPTION	SCORING
Acute @ petiole	Presence of acute angle where leaf blade meets stem	1, 0
Boat Shape Leaf	Leaf is boat shaped, not round	1,0
Dull Leaf	Leaf is dull, not glossy	1, 0
Large Tooth	Large teeth, not small	1, 0
Hooked Tooth	Teeth have hooked end, not wedged	1, 0
Tapered Tooth	Leaf tooth comes to tapered end, not wedged	1, 0
Long Vein Hairs	Hairs on main vein of leaf are long	1, 0
Sparse Vein Hairs	Hairs on vein are sparse	1, 0
No Leaf Hairs	No pubescence on back of leaf anywhere	1, 0
Tree Form	Ranging from straight with acute branch angles and one dominant trunk to numerous trunks with no clear leader and obtuse branch angles.	1-5

Table 2: catalog of categorical variables used in analysis, with 1 = *C. dentata* and 0 (or 5) = *C. mollissima*

Appendix B

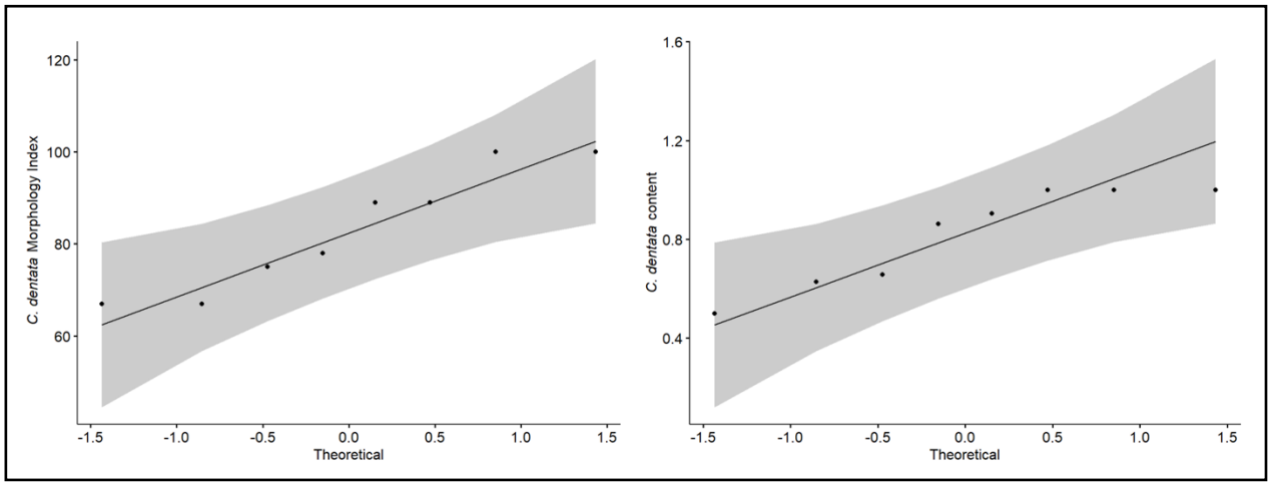

Figure 7: Results of Shapiro-Wilkes test for normality. *C. dentata* Morphology Index (left) is normally distributed based on p = 0.266. *C. dentata* genetic content (right) is normally distributed based on p = 0.111.

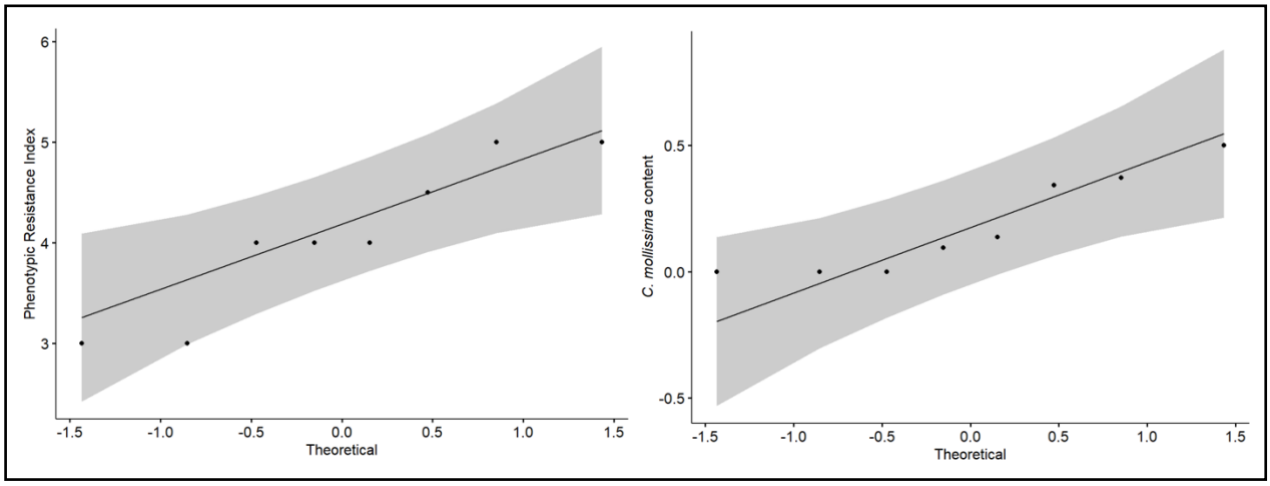

Figure 9: Results of Shapiro-Wilkes test for normality. The Phenotypic Resistance Index (left) is normally distributed in this sampled based on p = 0.202. *C. mollissima* genetic content (right) is also normally distributed in this sample based on p = 0.111.

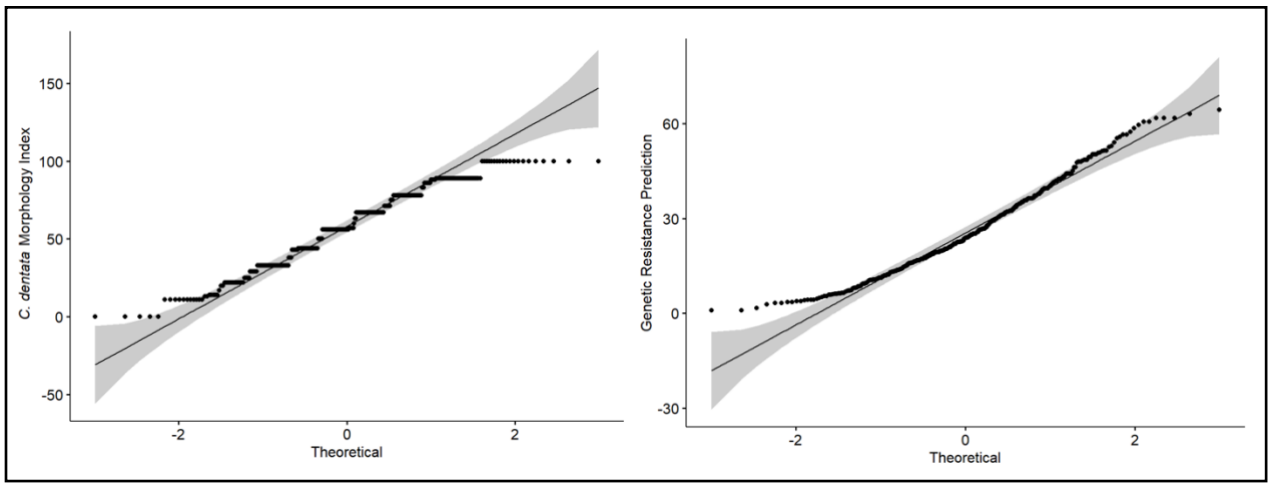

Figure 10: Results of Shapiro-Wilkes test for normality. *C. dentata* Morphology Index (left) and Genetic Resistance Prediction (right) were not normally distributed in this sample given p = 3.67e-7 and p = 7.45e-7, respectively.

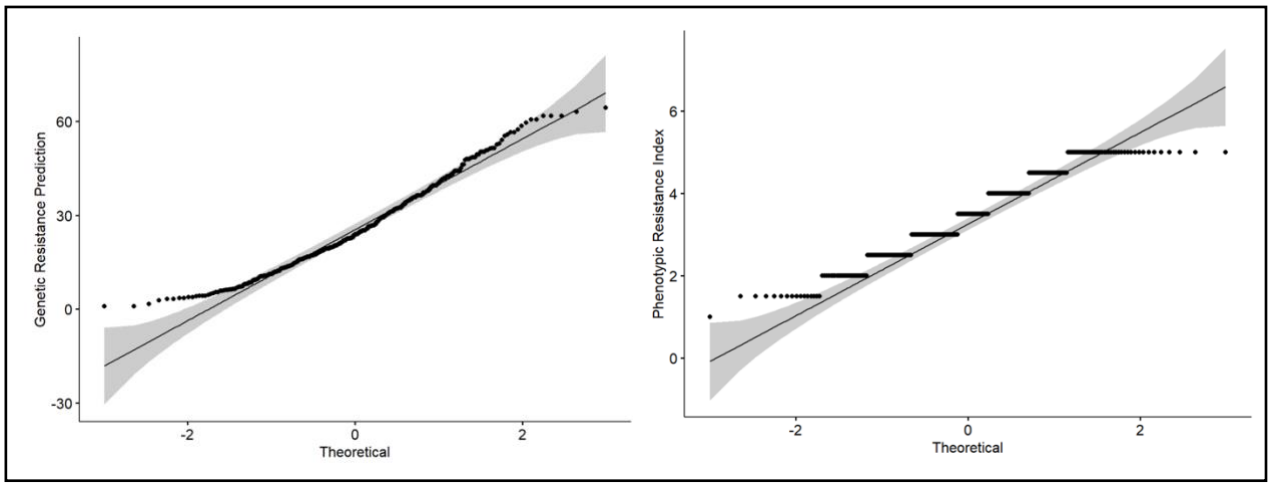

Figure 11: The Genetic Resistance Prediction (left) and Phenotypic Resistance Index (right) were not normally distributed in this sample based on the Shapiro-Wilkes test. This test yielded p = 7.45e-7 and p = 1.19e-9, respectively.

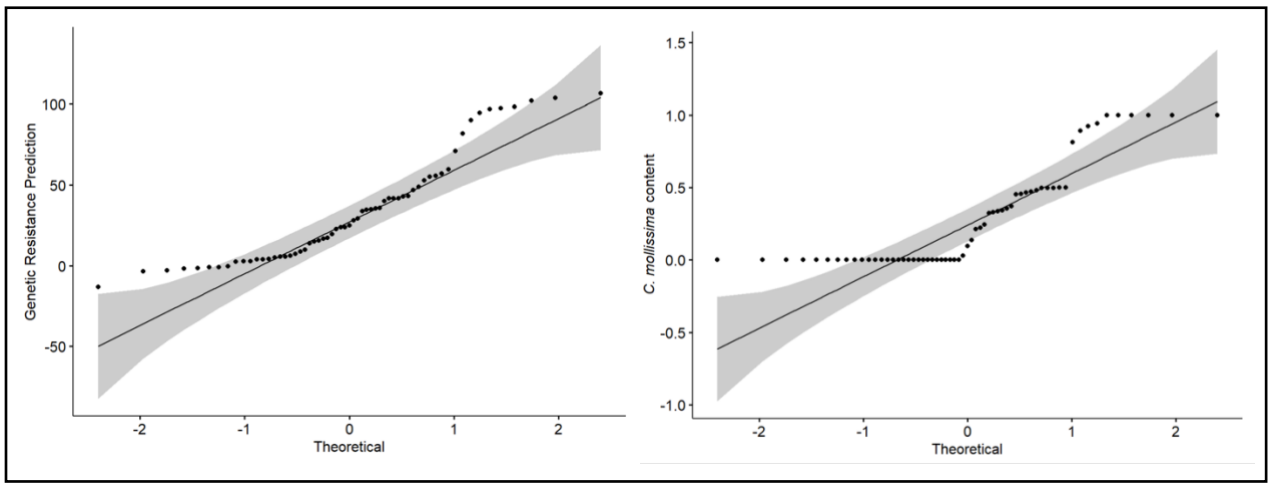

Figure 12: Results of Shapiro-Wilkes test for the Genetic Resistance Prediction and *C. mollissima* genetic content in Lesesne State Forest. P = 7.56e-5 and 2.36e-8, respectively, indicate neither variable is normally distributed in this sample.

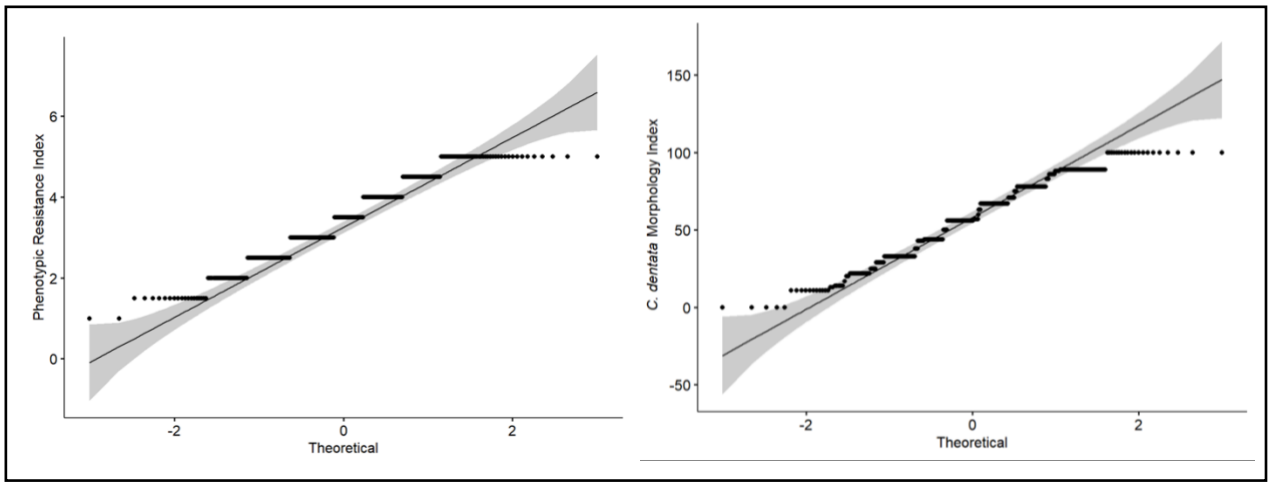

Figure 13: From the Shapiro-Wilkes test, the Phenotypic Resistance Index (left) yielded p = 8.01e-10 and *C. dentata* Morphology Index (right) yielded p = 1.74e-7. These variables were not normally distributed in the sample.