Merging backcross breeding and transgenic blight resistance to accelerate restoration of the American chestnut

The American Chestnut Foundation's breeding and selection plan
2015 - 2025

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Revised February 12th 2018

Summary

The American Chestnut Foundation's breeding program is approaching major milestones. Selection of the most blight-resistant backcross trees in seed orchards at Meadowview Research Farms is expected to be complete between 2020 and 2025. The offspring from selected seed orchard parents are predicted to have blight resistance that is, on average, intermediate between American chestnut and Chinese chestnut. Once selection in BC_3F_2 seed orchards is complete, BC_3F_3 progeny will be planted in restoration trials to determine if backcross resistance is sufficient for trees to compete and reproduce in eastern forests as canopy trees. Meanwhile, collaborators at the State University of New York have demonstrated that transgenic American chestnut containing the oxalate oxidase (OxO) gene has blight resistance that similar to that of Chinese chestnut in trials on seedlings. Federal regulatory review for the release of transgenic American chestnut is expected to begin soon. If approved, transgenic American chestnut pollen will be available for breeding as early as 2020. First generation progeny of transgenic trees are expected to be available for restoration trials and demonstration plantings by 2021.

Beyond these milestones, this document summarizes plans to increase the speed and accuracy of selection for blight resistance in backcross populations through accelerated progeny testing with small stem assays combined with genomic selection. Blight resistance in backcross populations will be improved through two additional generations intercrossing and selection. In addition, new sources of resistance alleles will be incorporated into the breeding population to minimize vulnerability to the breakdown of blight resistance through evolution of the fungus that causes chestnut blight (*Cryphonectria parasitica*).

Pending regulatory approval, the transgenic founder tree will be outcrossed to pure American chestnut and backcross trees over three generations. The objectives of outcrossing are: 1) Combine blight resistance from backcross trees with OxO to produce progeny with more robust resistance than possible from backcross or transgenic approaches pursued independently. 2) Combine OxO with backcross resistance to phytophthora root rot (PRR), to increase survival in the southern portion of the *Castanea dentata* range, where *Phytophthora cinnamomi* is most prevalent. 3) Create a restoration population with an effective size > 500 to minimize the deleterious effects of inbreeding and to increase adaptability of the restoration population. The conservation of American chestnuts for outcrossing will be augmented by collecting seeds and transplanting wild American chestnuts from regions that are most genetically diverse and underrepresented by TACF's breeding program. A diversified American chestnut population in which transgenic blight resistance is combined with backcross blight and PRR resistance is expected to be available for restoration between 2030 and 2050. While major milestones are within reach, long-term commitment to continued breeding and selection is required for full-scale restoration.

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Objectives for the Meadowview backcross breeding program

The primary objective for Meadowview backcross breeding program in the next decade is to finish planting and selection in Clapper and Graves BC_3F_2 seed orchards and to transition from backcross breeding to recurrent selection for these sources of resistance. A secondary objective is to advance additional sources of resistance through backcross breeding and seed orchard selection. The following sections detail breeding and selection plans that are specific to the Meadowview breeding program. Plans for chapters will be treated in a subsequent section.

I. Objectives for the Clapper and Graves sources of resistance

A. Finish planting Meadowview seed orchards

For the Clapper source of resistance, 24 lines of BC_3F_2 trees have been planted in nine blocks on the Duncan Farm. For the remaining five Clapper lines, 4 – 6 plots have been planted. Considering that planting is complete for more than 20 Clapper lines, no additional BC_3F_2 trees will be planted in the Clapper seed orchard. For the Graves seed orchard on the Wagner Farm, 15 lines have been planted in nine blocks. To complete the planting of nine blocks of 20 Graves lines, 21 additional 150 tree plots (3150 trees) will be planted on the Wagner Farm.

B. Conduct additional phenotypic selection against blight-susceptible trees

An inventory of the Clapper and Graves BC_3F_2 seed orchards at Meadowview was conducted in the winter of 2016. A total of 5110 Clapper BC_3F_2 trees remained on the Duncan farm and 3136 Graves BC_3F_2 trees remained on the Wagner farm. In 2017, a total of 1813 Clapper and 892 Graves BC_3F_2 trees with blight susceptibility phenotypes were removed. A total of 5514 BC_3F_2 trees remain in both seed orchards. Approximately 1800 of the remaining trees were inoculated with *C. parasitica* for the first time in 2017 and another 3150 trees have yet to be planted on the Wagner farm. Assuming 80% of the newly planted and inoculated trees will be removed based on canker assessments after artificial inoculation, a total of 4700 trees will remain from which to make the final selections.

C. Screen progeny from a sufficient number of BC_3F_2 mothers to develop accurate genomic prediction models for disease resistance and finish selection in Meadowview seed orchards with genomic selection

It is not feasible to progeny test all 4700 BC_3F_2 trees that will remain in Meadowview seed orchards to estimate genetic resistance to chestnut blight and/or phytophthora root rot. Final selections of approximately 600 BC_3F_2 trees

that are most resistant to chestnut blight or phytophthora root rot will accelerated with genomic selection. Genomic selection involves the development of a prediction model based on DNA sequencing that will accurately rank trees for disease resistance. The prediction model is developed by estimating correlations between a genome-wide sample of DNA variants and disease resistance in a training population of mother trees that have been progeny tested. The blight and PRR resistance of the mother-tree progeny sets is predicted by genotyping the same DNA variants as were genotyped in the training population and summing the effect of the DNA variants on disease resistance (Meuwissen et al. 2001).

Using the method of Grattipaglia & Resende (2011), the training population size required to create a genomic selection model that is at least as accurate as progeny testing was estimated. Selection accuracy is defined as the strength of correlation between true genetic resistance and genetic resistance estimated from genomic prediction or progeny testing. Assuming a narrow-sense heritability for BC_3F_3 disease severity phenotypes that varies from 0.1 to 0.4, a marker density that is sufficient to explain 75% of the genetic variance in resistance, and resistance that segregates at between 2 - 20 loci, the accuracy of genomic selection is expected be 0.6 to 0.8 with a training population of 250 individuals. This genomic prediction accuracy is comparable to the accuracy of progeny tests comprised of 10 – 30 open pollinated progeny per mother tree (Dekkers et al. 2005).

Genomic prediction models for blight resistance will be developed separately for Clapper and Graves by genotyping training populations of > 250 BC_3F_2 mother trees from each source that have been progeny tested for blight resistance. Genomic prediction for resistance to phytophthora root rot will be developed by genotyping a similar number of BC_3F_2 mothers from the Graves source whose BC_3F_3 progeny have been inoculated with *P. cinnamomi*. There is minimal resistance to PRR among Clapper BC_3F_2 trees (Westbrook et al. 2018), thus no selections for PRR resistance will be made in Clapper. Progeny testing of Meadowview BC_3F_2 mothers will continue until genomic prediction models for blight and root rot resistance have been demonstrated through cross validation to have prediction accuracies similar to progeny tests (i.e., 0.6 to 0.8).

TACF is collaborating with Professor Jason Holliday at Virginia Tech to develop accurate genomic prediction models for blight resistance and PRR resistance. To date, genomic prediction models have been developed by genotyping 195 Clapper BC_3F_2 mothers progeny tested for blight resistance and 166 Graves BC_3F_2 mothers progeny tested for PRR resistance. These training populations represent 26 of 29 Clapper lines and 21 of 25 Graves lines in Meadowview seed orchards. Approximately 80,000 SNPs with minor allele frequencies > 0.01 and < 10% missing data were genotyped with genotyping-by-sequencing in these populations. The SNPs were used to estimate genomic relationships between BC_3F_2 mothers. The genomic relationships, in turn, were used to estimate best

linear unbiased predictions (BLUPs) of average blight canker severity or average PRR mortality among the progeny of each mother tree. A median of 13 and 20 progeny per mother were phenotyped for canker severity and PRR mortality, respectively. The SNP effects were estimated with random regression of SNP genotypes on disease resistance BLUPs (Endelman, 2011). The accuracy of genomic selection was estimated with 10-fold cross validation as the correlation between genomic v. progeny test BLUPs of disease resistance. The accuracy of genomic selection was compared to the accuracy of progeny testing, which was estimated from the narrow sense heritability and numbers of progeny per mother tree following Dekkers et al. (2005).

Genomic selection accuracies were encouragingly high (Figure 1). The average accuracy of genomic selection for blight resistance in the Clapper training population (0.62) was similar to the average accuracy of progeny testing (0.57). While progeny testing for PRR resistance was highly accurate (0.90), the accuracy of genomic selection for PRR resistance is sufficient (0.62) and is expected to increase by progeny testing additional BC_3F_2 mothers.

Currently, blight resistance within the Graves blight resistance training population (N = 83) was not variable enough to develop accurate genomic prediction models. The heritability of canker severity among progeny of the 83 genotyped mother trees was not significantly different than zero. Genomic predictive abilities were correspondingly small (< 0.3). Genotyping has been completed on 194 additional Graves BC3F2 mother trees whose progeny will be screened for blight resistance by 2020. Inclusion of canker severity phenotypes from progeny of these BC3F2 mothers is expected to increase the genetic variation and genomic predictive abilities in the Graves blight resistance training population.

Progeny testing and genotyping of > 250 BC_3F_2 mothers per source of resistance (Clapper and Graves) and disease (blight and PRR) is expected to be complete by 2020. Genotyping of 2000 additional trees that remain in the Meadowview seed orchards but have not yet been progeny tested to predict blight and PRR resistance is ongoing. Culling of the Duncan and Wagner seed orchards based on genomic prediction of blight and PRR resistance is expected to commence in 2021.

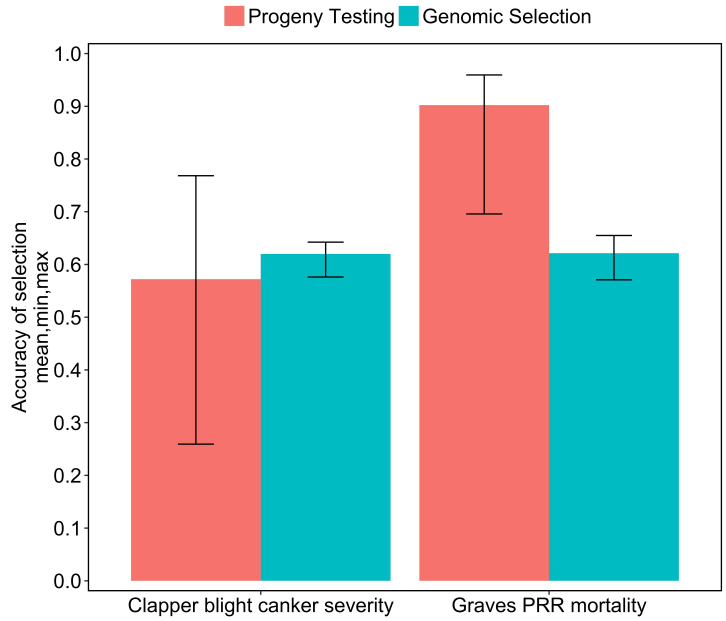


Figure 1. Accuracy of progeny testing compared with the accuracy of genomic selection. Progeny from 195 Clapper BC_3F_2 mothers were phenotyped for blight canker severity 6 months after inoculation with *C. parasitica*. Progeny from 166 Graves BC_3F_2 mothers were phenotyped for mortality 4 – 6 months after inoculation with *P. cinnamomi*. The accuracy of progeny testing for blight resistance was estimated following Dekkers et al. (2005) from a narrow sense heritability for canker severity of 0.16 and 2 and 30 progeny (average 14) evaluated per mother tree. The accuracy of progeny testing for PRR resistance was estimated from narrow sense heritability for mortality of 0.54 and 6 and 74 (average 27) progeny evaluated per mother tree. Progeny tested mothers were genotyped with genotyping-by-sequencing at 79,217 and 77,058 SNPs in the Clapper and Graves populations, respectively. The SNP effects were estimated in rrBLUP (Endelman, 2011) and the accuracy of genomic selection was estimated with 10 fold cross validation over 50 replicates.

D. Incorporate small stem assays to accelerate progeny testing for blight resistance

Small stem assays were evaluated for the purpose of accelerating progeny testing for blight resistance and to increase the number of progeny screened per family. The small stem assay (SSA) consists of inoculating containerized chestnut seedlings with *C. parasitica* and evaluating canker development within their first growing season (Powell *et al.* 2007). By contrast, progeny tests planted in orchards are typically inoculated in their third growing season.

To provide proof-of-concept for the use of small stem assays for progeny testing, 69 BC_3F_3 families from Meadowview + resistant Chinese chestnut and susceptible American chestnut controls were screened for blight resistance with the SSA in a greenhouse at the USFS Resistance Screening Center in 2017.

Forty seeds per family were sowed in a randomized complete block design. One half of the blocks were inoculated with the weakly pathogenic SG2,3 strain and the other half were inoculated with the strongly pathogenic Ep155 strain of *C. parasitica* at age three months. Canker lengths were measured 7, 15, and 24 weeks after inoculation.

The key results were:

- A total of 1812 seedlings were phenotyped for canker length, with a median N of 29 BC_3F_3 seedlings per family. Attrition due to lack of germination and inoculation failure was 36%.
- Canker lengths were significantly greater for American chestnut as compared with Chinese chestnut for seedlings inoculated with 15 weeks after inoculation with Ep155 and 24 weeks after inoculation with SG2,3.
- Variation canker length among individual BC_3F_3 trees was heritable starting at 15 weeks post inoculation with Ep155 ($h^2 = 0.32 \pm 0.08$) and 24 weeks post inoculation with SG2,3 ($h^2 = 0.19 \pm 0.11$).
- Average canker lengths among BC_3F_3 families were genetically correlated when length measurements taken 15 weeks after inoculation with Ep155 were compared to those taken 24 weeks after inoculation with SG2,3 ($r_{genetic} = 0.89 \pm 0.26$).
- For 13 BC_3F_3 families previously included in orchard progeny tests, family blight resistance rankings were positively genetically correlated between orchard and SSA, although the precision of the correlation estimate was low ($r_{genetic} = 0.96 \pm 0.63$). All 68 families included in the SSA were also planted in 2017 in Meadowview orchard progeny tests to increase the precision orchard v. SSA genetic correlation estimate. Results from a larger SSA v. orchard comparison will be available in 2020.

Based on these promising results, progeny testing with small stem assays will continue. In subsequent years, Meadowview BC_3F_3 families will be screened in SSAs and in orchard progeny tests. The canker length data from orchards and SSA will be analyzed together to increase the precision of blight resistance rankings of BC_3F_2 mothers and to increase the accuracy of genomic prediction models for blight resistance. To increase the number of informative canker observations within each family, all seedlings will be inoculated with the strongly pathogenic Ep155 strain and inoculation techniques will be optimized.

F. Conduct controlled pollination among the most blight resistant BC_3F_2 trees and plant progeny in orchards secluded from susceptible trees

Progeny test results to-date have identified BC_3F_2 mothers in Meadowview seed orchards with above-average blight resistance. In 2015 and 2016, 16 controlled pollinations (CPs) were conducted among 19 BC_3F_2 trees with blight resistance

BLUP values > 35 on a 0 = American and 100 = Chinese chestnut scale. In 2017, 328 progeny from these crosses were planted in a randomized complete block design on the Price Farm. Chinese chestnut and BC_3F_3 trees from open pollination of three BC_3F_2 parents used in CPs were also planted as controls. The trees in this orchard will be inoculated with *C. parasitica* in 3 – 5 years to estimate gains in blight resistance at BC_3F_3 once selection is complete among BC_3F_2s in Meadowview seed orchards. Canker severity among the progeny will be compared as follows:

- BC_3F_3 progeny from CPs will be compared to Chinese chestnut controls to predict if BC_3F_3 trees will be as blight resistant as Chinese chestnut after selection at BC_3F_2 is complete.
- The BC_3F_3 progeny from CPs will be compared to the BC_3F_3 progeny from OPs of the same parents to test for the effect of the pollen cloud in BC_3F_2 seed orchards.
- The BC_3F_3 progeny from CPs within sources will be compared to CPs between sources and to Chinese chestnut controls to test if crossing sources of resistance affects blight resistance.

The intention is to use this orchard to generate BC_3F_4 seed with improved blight resistance with open pollination in advance of completing selection in Meadowview BC_3F_2 seed orchards. Thus, the Chinese chestnut controls and trees with the most severe cankers will be culled from the orchard prior to the trees flowering so that only the most blight resistant BC_3F_3 trees will intercross.

G. Transition to recurrent selection for blight resistance

Recurrent selection for blight resistance at BC_3F_3 and beyond is expected to result in additional gains in blight resistance. It is presumed that BC_3F_2 trees inherited different subsets of blight resistance alleles from the Clapper and Graves BC_1 sources of resistance (Steiner et al. 2017). Intercrossing BC_3F_2 trees is therefore expected to result in subsets of BC_3F_3 progeny that inherited greater numbers of resistance alleles - some potentially in a homozygous state - as compared with their parents. Intercrossing Clapper and Graves BC_3F_2 trees may result in progeny with novel combinations of resistance alleles if Clapper and Graves have different alleles for blight resistance.

Simulations were performed to estimate the number of generations of intercrossing and recurrent selection to enhance average blight resistance to 75 on a 0 = American chestnut to 100 = Chinese chestnut scale (see Appendix I for simulation methods). Two selection scenarios were simulated: 1. 99% of the population within each generation is culled based on canker phenotypes, 2. 90% of the population is culled on canker phenotype and 9% is culled based on higher

accuracy progeny testing and genomic selection. Assuming that 1% of trees that were selected within each generation intercrossed randomly, it was assumed that the mean and genetic standard deviation in blight resistance of their progeny was equal to these parameters for the selected parents.

In simulations, average blight resistance approached the target of 75 after higher accuracy selection (scenario 2) within the BC_3F_3 and BC_3F_4 generations or lower accuracy phenotypic selection (scenario 1) within in the BC_3F_3, BC_3F_4, and BC_3F_5 generations (Figure 2). Based on this simulation, recurrent selection will be performed within the BC_3F_3 and BC_3F_4 generations. Higher accuracy selection will be performed among the 10% of the population that remains after 90% is culled based on canker phenotypes.

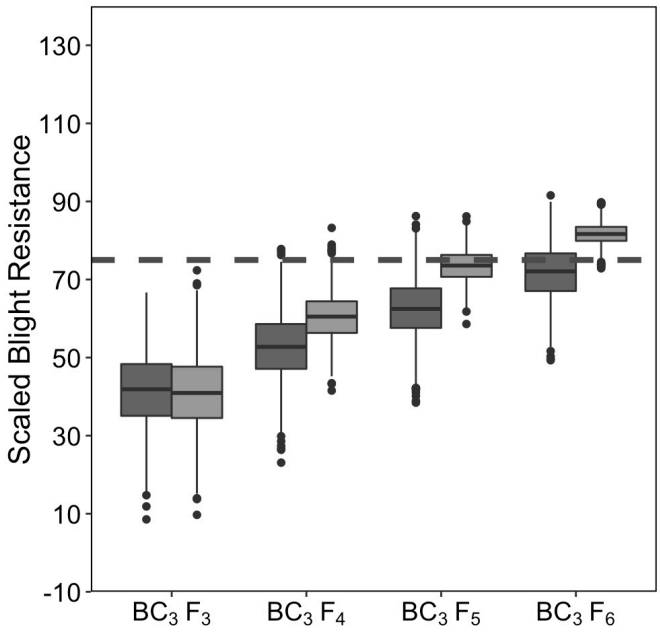


Figure 2. Simulated gains in blight resistance from recurrent selection. Box plots represent genetic variation in scaled blight resistance among unselected progeny after one percent of the parental population was selected for blight resistance. Two selection scenarios were considered: 1. 99% of population is culled based on canker phenotype at 0.45 accuracy (dark grey boxplots), 2.90% of the population is culled based on phenotype at 0.45 accuracy and 9% is culled based on progeny testing and genomic selection at 0.8 selection accuracy (light grey boxplots).

After selection is complete in Clapper and Graves BC_3F_2 seed orchards, BC_3F_3 progeny from these orchards will be planted in a separate orchard where selection for blight resistance will continue. The orchard will be designed as follows:

- BC_3F_3 progeny from each backcross line (i.e., 24 Clapper lines and 20 Graves lines) will be planted in 3 blocks.

- Each block will contain 60 subplots. Approximately 44 of subplots will be composed progeny of open pollination of BC_3F_2 representatives of each line. The remaining 16 subplots will composed of progeny of controlled pollination between Clapper and Graves BC_3F_2s. The purpose of the CPs will be to determine if crossing between Clapper and Graves enhances resistance as compared with crossing within these sources of resistance.
- Each block will contain a family subplot composed of 100 or less BC_3F_3 siblings from each backcross line.
- Each subplot will contain at least one Chinese chestnut and F_1 control.

We will start by planting 100 BC_3F_3 progeny in each block, although fewer trees may be needed to select 5-10 trees per plot with a canker length that is significantly shorter than F_1 control and preferably indistinguishable from Chinese chestnut. Final selection of the most blight resistant tree per plot will be made through a combination of progeny testing and genomic selection.

H. Optimize methods to screen BC_3F_3 chestnut seedlings for blight resistance prior to field planting

Infrared spectroscopy is currently being tested as a non-destructive method to screen large numbers of chestnut backcross seedlings for blight resistance. Infrared spectroscopy measures the absorbance of infrared wavelengths of light as it passes through tissue samples. The absorbance spectrum is correlated with concentration of metabolites in tissues. Infrared spectroscopy has been used to predict disease resistance in other tree species because metabolites (or absorbance at specific wavelengths) is correlated with resistance (Conrad *et al.* 2014). Infrared spectroscopy is non-destructive in that no inoculation of seedlings is required. This selection method could be used to accelerate recurrent selection for blight resistance by enabling us to eliminate most susceptible trees as seedlings prior to planting in the field.

A pilot study of infrared spectroscopy as a method of prescreening was incorporated into small stem assays and screening for Phytophthora root rot BC_3F_3s in at the Resistance Screening Center in 2017. Leaf and stem tissue was collected from six BC_3F_3 families (3 Clapper families & 3 Graves families) prior to inoculation with *C. parasitica*. Tissue from different seedling individuals from the same three Graves families was also sampled prior to inoculation with *P. cinnamomi*. Anna Conrad (Ohio State) will perform infrared spectroscopy on these tissues and develop prediction models of chestnut blight canker length and root rot morality. The accuracy of these prediction models within and among families will be assessed with cross-validation. If infrared spectroscopy is demonstrated to be sufficiently accurate and high throughput as a pre-screening

method then TACF will seek funding to purchase instruments for implementation at Meadowview Research Farms.

II. Objectives for advancing additional sources of resistance

In addition to Meadowview's two major sources of blight resistance (Clapper & Graves), 22 additional sources of resistance have been advanced to F_1 and backcross generations (Steiner *et al.* 2017). Incorporating additional sources of resistance into TACF's breeding program may contribute to more robust blight resistance by pyramiding resistance alleles from different Chinese chestnut sources. It is not feasible for the Meadowview breeding program to advance 20+ additional sources of resistance individually to backcross-F2 seed orchards composed of 9 blocks of 20 to 30 backcross lines. As an alternative, a mixed source breeding population will be created at Meadowview by taking the following steps:

A. Advance backcross lines from each of 10+ Asian sources of resistance to the second backcross generation

Backcross lines from additional sources of resistance will be advanced to BC_2 rather than BC_3 to reduce the probability of breeding out blight resistance alleles from Chinese chestnut. First backcross hybrids that descend from 10 different Chinese chestnut grandparents will be crossed with 20 unrelated American chestnuts that have not previously been used as parents in the breeding program. When possible, progeny of large surviving chestnuts (LSAs) will be used as backcross parents. To test whether BC_2 progeny inherit blight resistance from their LSA parents, BC_1 parents will also be test crossed with susceptible American chestnuts. Between 100 and 200 BC_2 individuals from each backcross line will be planted at TACF's Price Farm along with Chinese, American, and F_1 controls.

B. Select for blight resistance with artificial inoculation and select for American chestnut genome fraction with DNA markers

Selection for blight resistance will be conducted at age 3 to 5 by artificially inoculating stems and culling individuals with canker severity ratings that are significantly greater than the average canker severity of F_1 controls. Average canker severity of BC_2 progeny of LSAs and susceptible Americans will be compared to determine if LSA parents contribute to blight resistance.

The current breeding plan carries backcrossing to the third generation (BC_3) in order to dilute the Chinese fraction of the genome to an average of ~6%. However, it may be possible to achieve the same level of dilution at the BC_2 by selecting against alleles from Chinese chestnut that do not contribute to blight

resistance. Assuming, that genome-wide inheritance of American chestnut alleles is normally distributed among BC_2 progeny with a mean of 87.5% and standard deviation of 3.6% (Visscher *et al.* 2006), then 4% of BC_2 progeny are expected have inherited 94% or more of their genome from American chestnut. If 100 individuals from each backcross line are genotyped, there is a 98% probability of identifying at least one BC_2 individual that inherited 94% of its genome from American chestnut. Currently, Jeanne Romero-Severson (Notre-Dame) is developing Ancestry Informative Markers (AIMs) to estimate the proportion of genomes of backcross trees that is inherited from Chinese chestnut and American chestnut. The BC_2 individuals that remain after selection for blight resistance will be genotyped with these markers. Between 3 and 5 individuals from each line that share the most ancestry with American chestnut will be selected.

C. Intercross BC_2 selections with open pollination and screen BC_2F_2 progeny for blight resistance prior to planting in a seed orchard

It is anticipated that selection among BC_2 trees from additional sources of resistance at Meadowview will be complete by 2025. The selected BC_2 trees may be outcrossed to transgenic trees and/or the selections may be intercrossed and their BC_2F_2 progeny planted in seed orchards. Open pollination among BC_2 trees may be used to generate large populations of BC_2F_2s that are segregating for blight resistance. If proof-of-concept is obtained for early blight resistance screening methods (e.g., small stem assays or infrared spectroscopy), then these methods will be used to eliminate the most susceptible BC_2F_2s prior to planting in a seed orchard.

A mixed source seed orchard may be composed of ~ 20 backcross lines (2 lines x 10 sources). Each line will be planted in family block plots consisting of 150 trees with the aim of selecting one tree per family plot. Each backcross line will be planted in three blocks so that 450 trees will be planted per line in total. We will aim to cull 90% of the trees after artificial inoculation with *C. parasitica*.

As remaining trees in the seed orchard reach reproductive maturity, a subset of ~ 1000 individuals that represent all sources of resistance will be progeny tested for blight resistance. To develop genomic prediction models for blight resistance, progeny tested individuals will also be genotyped and marker effects will be estimated on BLUPs estimated from canker severity of BC_2F_3 progeny. Genotyping will be used to predict the blight and PRR resistance of remaining individuals in the seed orchard that have not been progeny tested. Final selections will be made with genomic selection.

Objectives for chapter backcross programs

A. Finish planting seed orchards for Clapper and Graves sources

To reduce the workload and expense associated with seed orchard planting, maintenance, and selection, it is recommended that chapters reduce the number of intended seed orchard blocks from nine, as at Meadowview, to a minimum of three. Table 1 details the number of seed orchard blocks that each chapter intends to plant and their progress on planting as of 2018.

Table 1. Number of intended blocks and planting progress in chapter Clapper and Graves seed orchards

Source	Clapper			Graves		
Chapter	N lines	N blocks	% Planted	N lines	N blocks	% Planted
Maine	20	9	93%	20	6	38%
VT/NH	--	--	--	20	6	7%
MA/RI	20	7	95%	20	6	6%
Connecticut	17	4	16%	--	--	--
PA/NJ	20	9	50%	20	9	40%
Indiana	24	5	33%	--	--	--
Maryland	24	4	46%	--	--	--
Virginia	--	--	--	25	5	9%
Carolinas	13	1	100%	14	0	0%
Kentucky	--	--	--	20	3	33%
Tennessee	25	4	13%	--	--	--
Alabama	23	3	13%	--	--	--
Georgia	--	--	--	18	4	20%
Total lines	**186**			**157**		

B. Cull a minimum of 90% of trees in seed orchards with phenotypic selection

Simulations were performed to compare projected gains in blight resistance at BC_3F_2 with different proportions of phenotypic selection, progeny testing, and genomic selection assuming that blight resistance segregates at 3 or 10 loci (Table 2). Gains were not substantially different between selection scenarios that involved 75% phenotypic selection or 90% phenotypic selection. Higher accuracy

selection with progeny testing and/or genomic selection to select the 1% most blight resistant trees from 10% remaining after phenotypic selection is predicted to result in 33% greater selection gains relative to 100% phenotypic selection. Incorporating genomic selection resulted in significantly greater gains per unit time relative to selection scenarios that involved progeny testing alone.

Table 2. Simulated gains in blight resistance from selection with varying percentages of phenotypic selection (PS), progeny testing (PT), and genomic selection (GS) assuming blight resistance segregates at 3 or 10 quantitative trait loci (QTL). See Appendix II for simulation methods.

Selection scenario	N QTL	Gain	Gain per time
100% PS	3	0.657	0.026
90% PS, 10% PT	3	0.831	0.024
90% PS, 2% PT, 8% GS	3	0.872	0.044
75% PS, 2% PT, 23% GS	3	0.876	0.044
100% PS	10	0.379	0.015
90% PS, 10% PT	10	0.488	0.014
90% PS, 2% PT, 8% GS	10	0.506	0.025
75% PS, 2% PT, 23% GS	10	0.507	0.025

Based on simulation results, it is recommended that chapters cull 90% of BC_3F_2 trees in seed orchards based on blight severity phenotypes. If artificial inoculation with the weakly pathogenic SG2,3 strain is not sufficiently stringent to cull 90% of the population, then additional inoculation and culling with more pathogenic strains of the blight (e.g., Weekly or Ep155) is recommended.

C. Finish selection for blight resistance in chapter programs with progeny testing and genomic selection

When chapters finish culling 90% of seed orchard trees based on blight canker phenotypes, selection of the 1% most blight-resistant trees will be made with a combination of progeny testing and genomic selection. Progeny testing of a minimum of 50 chapter BC_3F_2 parents from each chapter will be required to estimate the prediction accuracy of genomic models developed from progeny tests of Meadowview BC_3F_2 parents. The progeny test data of chapter material will be incorporated in genomic prediction model development to increase genomic prediction accuracy within that chapter's seed orchards. Progeny testing of chapter material may be expedited with small stem assays.

D. Advance other sources of resistance to the second or third backcross

Some of TACF's chapters have incorporated additional sources of blight resistance into their backcross programs. The Nanking source of resistance has been advanced to the BC_3 generation by the TN, VA, and MA/RI breeding programs. The MD chapter has advanced the Musick source of resistance to

BC_3. The PA/NJ and TN chapters have advanced a multitude of additional sources to BC_1. For the Nanking and Musick sources of resistance, it is recommended that chapters finish selection for blight resistance with the BC_3 generation with artificial inoculation. I recommend that the TN and PA/NJ chapters advance additional sources of resistance to BC_2, cull based on artificial inoculation, and select for American chestnut type with ancestry informative markers. When selection is complete in backcross generations, chapters have the options of 1) outcrossing selected backcross trees with transgenic trees and/or 2) intercrossing backcross trees and planting seed orchards. For the Nanking source, it recommended that BC_3F_2 progeny from Meadowview, TN, and VA be planted together in a regional seed orchard. Additional sources may be combined in existing seed orchards or planted in separate seed orchards depending on the land availability and cooperators within each chapter.

Objectives for integration of transgenic blight resistance into TACF's breeding program

A. Outcross transgenic trees over three or more generations

Currently, two transgenic events (Darling 54 and Darling 58) into one American chestnut tree from New York (Ellis1) are being considered for deregulated status. If deregulated status is obtained, clones of Ellis1 transgenic founders (T_0s) will be outcrossed to American chestnut populations to enhance genetic diversity and adaptability among the progeny. More than one generation of outcrossing to American chestnut will be required to dilute the Ellis1 genome contribution [1], minimize inbreeding, and expand effective population size.

Simulations were performed to determine how the number of outcross generations and number of American parents used in each generation influence effective population size and inbreeding upon intercrossing (Table 3). In simulations the number of American chestnut available for outcrossing was fixed at approximately 1000, while the number of generations and number American parents used in each generation varied. Effective population size (N_e) and inbreeding coefficients (f) were estimated in the T_xF_3 generation where, x = number of outcross generations, and F_3 denotes two generations of random intercrossing after the final outcross generation. Effective population size was expanded and inbreeding was reduced most efficiently by outcrossing over at least three generations and using an equal number of American parents in each generation.

Table 3. Simulations to estimate effective population size (N_e) and inbreeding coefficients (f) in the second intercross generation after outcrossing a single transgenic founder to approximately

[1]Darling 54 and Darling 58 differ only in the location of the transgene within the genome of Ellis1.

1000 unrelated American chestnut trees over varying numbers of generations and varying N of American chestnut parents per generation. Simulations were repeated 10 times to obtain averages of N_e and f. See Appendix III for simulation methods.

N outcross generations	N American parents per generation	Avg. N_e 2nd Intercross	Avg. f 2nd intercross
2	2, 1000	154	0.08
2	500, 500	297	0.03
3	2, 500, 500	190	0.02
3	333, 333, 333	383	0.009
4	250, 250, 250, 250	736	0.004

The N_e estimates should be viewed as comparative rather than absolute because the estimates vary considerably according to the method of calculation (Leroy et al. 2013).

TACF will endeavor to outcross transgenic trees to pure American chestnuts and backcross hybrids over three generations to increase the effective population size (N_e) to at least 500 individuals. A target N_e of 500 is based on the hypothesis that populations of this size have sufficient adaptive variation so as to be at minimal long-term risk of extinction due to genetic drift and inbreeding depression (Jamieson & Allendorf, 2012). A simulation in which transgenic trees are bred with 500 unique sets of unrelated American chestnuts over three generations (1500 American parents total) suggest that the effective population size after two generations of random mating will be 530 with an average inbreeding coefficient of 0.01.

In the first generation of outcrossing, T_0 transgenic pollen will be applied to BC_3 or BC_3F_2 representatives from each of TACF's 200 + Clapper lines and 300 + unique American chestnuts. Selection for the inheritance of OxO, which is expected in 50% of outcross progeny, will be performed with enzymatic assays of oxalate oxidase activity. The T_1 representatives from each line that inherited OxO will then be outcrossed with a diverse collection of 500 American chestnuts. The T_2 progeny will be outcrossed with BC_3F_2 representatives from 150 Graves lines that have been selected for phytophthora root rot resistance, 50 Nanking lines and 300 pure American chestnuts. Transgenic outcrosses to individuals from the extremes of range will only be outcrossed to other individuals from these range extremes to create locally adapted subpopulations. This outcross configuration pyramids resistance alleles from TACF's three major sources of resistance with OxO. It also diversifies transgenic populations beyond what is represented in the backcross program by incorporating American chestnuts that have not previously been used in breeding.

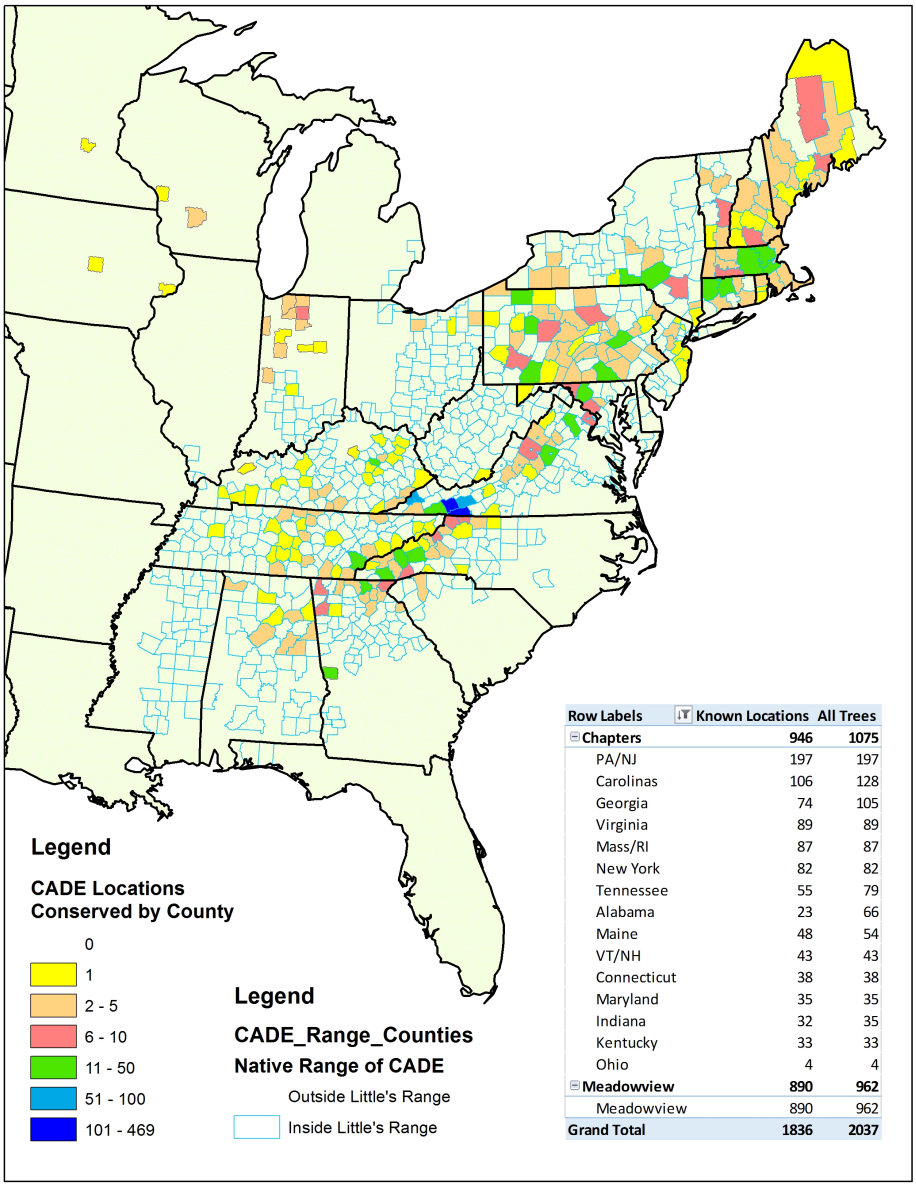
It will take approximately 30 to 40 years to complete three outcross generations and 1 generation of intercrossing to diversify transgenic restoration populations. Assuming it takes 2 - 5 years for transgenic progeny to produce pollen and it takes 5 - 7 years to pollinate 500 backcross and wild Americans per generation, generation time is a minimum of 7-10 years. Up to 150 lines of third outcross

generation progeny may be available in 15 to 20 years by outcrossing transgenic trees to mature backcross trees and accelerating flowering with high light in the greenhouse. It will take another 5 - 10 years for the third generation of outcross trees to reproductive maturity so that they can intercross via open pollination. The purpose of intercrossing via open pollination is to produce sufficient seed for restoration (seed set is lower for controlled pollinations). After the first intercross, TACF may decide to go for full-scale restoration. Alternatively, an additional intercross generation planting only homozygotes for OxO will ensure progeny of these trees are true breeding for OxO resistance. Although a transgenic restoration population with effective size > 500 is the long-term goal, earlier generations of transgenic outcross progeny with lower levels of diversity may be used for demonstration plantings and restoration trials.

B. Establish a genetically diverse core collection of American chestnuts for outcrossing to transgenic trees

A minimum of 1500 genetically diverse and distantly related flowering American chestnut trees will be required for three generations of outcrossing to transgenic trees to expand the effective population size of transgenic trees to 500. Approximately 500 outcrosses may be performed with BC_3 or BC_3F_2 representatives of TACF's backcross lines from the Clapper, Graves, and Nanking sources of resistance. A minimum 1000 additional sources of American chestnut will be required for transgenic outcrossing. Additional sources of American chestnut germplasm will be conserved in TACF's germplasm conservation orchards by collecting seed or transplanting wild trees.

Transplanting in particular will be used to target and accelerate conservation of novel sources of American chestnut germplasm for outcrossing to transgenic trees. A task force will be established to transplant or collect seed from 3000 wild trees from 300 + locations (10 trees per location) into GCOs. The objective is to obtain at least 1000 flowering trees, assuming 33% survival and flowering of transplanted trees (McKenna & Beheler 2016). Regions that have not previously been represented in TACF's backcross program (Figure 3) and that harbor the highest genetic diversity will be targeted for germplasm conservation. A genetic analysis of nine different subpopulations of *C. dentata* indicated that Southern and Western portions of the *C. dentata* range are the most genetically diverse (Gailing *et al.* 2017). Additional germplasm will be collected from across the species range, taking care to represent contrasting aspects, elevations, latitudes and east-west position with respect to the Appalachian chain. Targets for the number trees to transplant or collect seed for each of TACF's chapters are summarized in Table 4. Chapters may transplant or collect seeds from 20 – 50 new sources of American chestnut over the next 5 – 10 years to achieve these targets.



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

Figure 3. Map of American chestnuts sources incorporated into TACF's backcross populations and germplasm conservation orchards as of 2016. Reprinted from Fitzsimmons (2017).

Table 4. Targets for number of new American sources to collect seed from or transplant into germplasm conservation orchards

Chapter	N Sources
AL	250
GA	250
TN	250
KY	250
NC/SC	250
VA	250
WV	250

MD	100
PA/NJ	250
IN	100
OH	100
CT	100
MA/RI	100
VT/NH	100
NY	250
ME	150
Total	**3000**

Survival of American chestnuts within GCO may be increased by treating chestnut blight cankers with super-donor strains of *C. parasitica* capable of transmitting hypoviruses to all (or most) naturalized strains of *C. parasitica* in North America (Zhang & Nuss, 2016). The release of the super-donor strain of *C. parasitica* is currently under regulatory review by the USDA. If regulatory approval is obtained, these super-donor strains may be used TACF's GCOs by obtaining a permit through APHIS (Nuss, pers. comm.).

C. Outcross to phytophthora root rot resistant backcross selections in the final generation of outcrossing to combine blight and root rot resistance

Resistance to chestnut blight and phytophthora root rot will be combined in third and final outcross generation so that selection for the inheritance of OxO and for PRR resistance will only be necessary in the T_3 and T_3F_2 generations. Crossing transgenic progeny with PRR resistant selections will be performed in earlier generations for a small subset of trees for proof-of-concept on the breeding plan to combine blight and PRR resistance. To generate a diverse population of phytophthora-resistant backcross trees for outcrossing to transgenic trees, 200 BC_3F_2 trees from 150 + of TACF's Graves lines will be screened for resistance to *P. cinnamomi* at the USFS Resistance Screening Center (RSC). The RSC has capacity to screen 5000 seedlings per year. At this screening rate, it will take six years to screen BC_3F_2 progeny from 150 Graves lines for root rot resistance. Individuals that survive the screening will be planted at orchard sites where *P. cinnamomi* is present. Based on previous years of screening BC_3F_3s for resistance to *P. cinnamomi*, it is anticipated that survival within BC_3F_2 families will vary between 5% and 80% with an average survival of 25% among families. Families with many survivors may be inoculated with *C. parasitica* to perform additional selection for blight resistance. The final objective is to obtain one flowering BC_3F_2 tree per line with improved resistance to *P. cinnamomi*. Root rot resistant BC_3F_2s will be outcrossed with transgenic trees in the final generation of outcrossing. The progeny of these crosses will be screened for the inheritance of OxO with enzymatic assays. Transgenic outcross progeny will then be screened

for PRR resistance via inoculation with *P. cinnamomi*. Survivors will be planted in seed orchards.

D. Plant outcross progeny in a seed orchard where open pollination will generate large quantities of seed for restoration

After outcrossing is complete, at least three regional seed orchards (Northeast, Mid-Altantic, and Southern) will be established. Seed orchards will be composed of transgenic outcross progeny whose American chestnut parents were autochthonous to each region. In these orchards, outcross progeny will be intercrossed with open pollination.

All transgenic outcross trees in seed orchards will be hemizygous for OxO, meaning they inherited only one copy of the gene. One fourth of progeny from intercrosses among these trees are expected to inherit OxO in a homozygous state, ½ will be hemizygous, and ¼ will be homozygous susceptible. For restoration plantings, TACF and collaborators may decide to plant only the intercross progeny that are homozygous for OxO such that all progeny in the second generation of intercrossing in the forest will also be homozygous for OxO and therefore blight resistant.

The subset of progeny of outcrosses between transgenic trees and PRR resistant backcross selections will be planted at seed orchard sites where *P. cinnamomi* is present. Outcross progeny are expected to be heterozygous at best for alleles that confer resistance to root rot and intercrossing these trees will be used to enhance PRR resistance. Prior to planting progeny of intercrosses among these trees, TACF may decide to pre-screen trees to identify individuals with enhanced (homozygous) resistance to *P. cinnamomi* with genetic markers or artificial inoculation with *P. cinnamomi.*

Conclusions

Restoration of American chestnut depends on producing founder populations that 1) have sufficient resistance to chestnut blight to survive to reproductive maturity and persist in the forest canopy, 2) have combined resistance to chestnut blight and Phytophthora root rot for Southern subpopulations, and 3) have the genetic diversity needed to adapt to local environmental conditions. Blight resistance will be enhanced by accelerating selection in backcross seed orchards with genomic selection and by outcrossing transgenic trees. Resistance to chestnut blight and Phytophthora root rot will be combined by selecting for PRR resistance in backcross populations and then outcrossing these selections to blight-resistant transgenic trees. The genetic diversity remaining in *C. dentata* populations will be conserved by targeting diverse and under-represented regions for germplasm

collection and outcrossing these wild trees to transgenic trees over multiple generations.

Acknowledgements

I thank TACF's Senior Science Advisor, Kim Steiner for extensive review and insightful comments on earlier drafts this plan. Regional TACF staff, Sara Fitzsimmons, Tom Saielli, Kendra Collins, and Ben Jarrett provided updated information and feedback on plans for chapter breeding programs. Meadowview staff Eric Jenkins, Dan McKinnon, Emily Watson, Brandon Yanez, and Jim Tolton have been instrumental in implementing the first stages of this plan at Meadowview Research Farms. Thank you to the members, donors, and volunteers who have made long-term financial and time commitments towards the mission to restore the iconic American chestnut to its native range.

Appendices

Appendix I. Simulating gains in blight resistance from recurrent selection

Gains in blight resistance from recurrent selection were simulated in R. The initial parameters for the simulation were the mean and genetic standard deviation in blight resistance at BC_3F_3 after completing selection at BC_3F_2. These parameters were estimated from 10% of BC_3F_2 mother trees with the largest estimated breeding values (EBVs) for blight resistance. Breeding values were estimated from average canker severity among BC_3F_3 progeny of 544 BC_3F_2 mothers. True breeding values of the selected BC_3F_2 mothers were simulated as a variable that was correlated with the EBVs at an accuracy (i.e. correlation coefficient) of 0.8. The mean and genetic standard deviation in blight resistance at BC_3F_3 was estimated as the mean and standard deviation of the true breeding values of the selected BC_3F_2 mothers.

These initial parameters were used to simulate true breeding values for blight resistance within a BC_3F_3 population composed of 18,000 trees. Two selection scenarios with different selection accuracies were simulated: 1. 99% of the BC_3F_3 population was culled based on phenotypic assessments of canker severity on individual trees, 2. 90% of the population was culled based on canker severity phenotypes and the remaining 9% is culled based on higher accuracy selection based on progeny testing and genomic selection. The accuracy of phenotypic selection is equal to square root of the narrow sense heritability (h^2) of canker severity for individual trees (Dekkers 2005). In previous orchard progeny tests h^2 averaged 0.2, thus the accuracy of phenotypic selection was assumed to be 0.45. The accuracy of progeny tests or genomic selection was assumed to be 0.8. Canker severity phenotypes and estimated breeding values for blight resistance were simulated as variables that were correlated with true breeding values at their respective selection accuracies. Selection of 1% of the population

was simulated 10 times for each selection scenario. The mean and genetic standard deviation of blight resistance among BC_3F_4 progeny of the selected BC_3F_3 parents was estimated as the mean and standard deviation of the true breeding values of the BC_3F_3 selections averaged over 10 simulations. This procedure of selecting 1% of the population with different selection accuracies and estimating the mean and genetic standard deviation of the next generation was repeated for the BC_3F_3 through BC_3F_6 generations.

Appendix II. Simulating gains in blight resistance from selection with different proportions of phenotypic selection, progeny testing and genomic selection

Selection of 180 BC_3F_2 individuals from a total population size of 27,000 (20 lines x 9 seed orchard blocks x 150 individuals per line and block) was simulated in R under the selection scenarios in Table 2. True breeding values (BVs) for resistance were simulated from the number of resistance alleles that a tree inherited. It was assumed that BC_3 parents were fully heterozygous for resistance and that their resistance alleles independently segregate at either 3 or 10 quantitative trait loci (QTL). Estimated breeding values (EBVs) were simulated from the selection method's accuracy, defined by the expected correlation between estimated and true breeding values. It was assumed that the heritability (h^2) of canker severity for an individual tree is 0.4 and the accuracy of phenotypic selection is the square root of h^2. Accuracy of progeny testing was calculated following Dekkers et al. (2005) assuming that 30 half-sib BC_3F_3 progeny would be evaluated per BC_3F_2 mother. Accuracy of genomic selection was simulated assuming a training population size of 500 individuals (~ 2% of the population), an effective population size of 200 individuals (~ N of backcross lines in Clapper and Graves), and a marker density of 25 SNPs per cM (expected marker density using RAD-seq). Gain was estimated as (BV selected – BV unselected)/BV unselected, where BV selected is the average true breeding value of 180 selected individuals, and BV unselected is the mean breeding value prior to selection. Gain per unit time was calculated from the estimated time to complete selection at BC_3F_2 under the various scenarios (25 years - 100% phenotype, 35 years - 90% phenotype/10% progeny tests, 20-years - scenarios involving genomic selection).

Appendix III. Method for simulating effective population size (N_e) and inbreeding (f) after outcrossing a transgenic founder over varying numbers of generations

Pedigrees were simulated in R. The number of transgenic founder trees was 1 while the number of American chestnut parents to outcross with was fixed at approximately 1000. In different simulation scenarios, the American chestnut parents were outcrossed to transgenic trees over 2, 3, or 4 generations and unequal v. equal numbers of American chestnuts were bred in each outcross generation. After the final generation of outcrossing, two generations of

intercrossing were performed with 500 random matings per intercross generation. Each cross resulted in one progeny. Simulations were repeated 10 times for each scenario. Effective population size was estimated after the 2nd intercross using four methods detailed in Leroy *et al.* (2013): 1) inbreeding rate between two successive generations, 2) co-ancestry rate between two successive generations, 3) individual inbreeding rate, and 4) individual co-ancestry rate. The N_e values reported in Table 3 are an average of all four estimates averaged over 10 simulations. Inbreeding coefficients were estimated in the 'pedigree' package for R and averaged over 10 simulations.

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